

Review

Mitochondrial complex III regulates hypoxic activation of HIF

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Decreases in oxygen levels are observed in physiological processes, such as development, and pathological situations, such as tumorigenesis and ischemia. In the complete absence of oxygen (anoxia), mammalian cells are unable to generate sufficient energy for survival, so a mechanism for sensing a decrease in the oxygen level (hypoxia) before it reaches a critical point is crucial for the survival of the organism. In response to decreased oxygen levels, cells activate the transcription factors hypoxia-inducible factors (HIFs), which lead to metabolic adaptation to hypoxia, as well as to generate new vasculature to increase oxygen supply. How cells sense decreases in oxygen levels to regulate HIF activation has been hotly debated. Emerging evidence indicates that reactive oxygen species (ROS) generated by mitochondrial complex III are required for hypoxic activation of HIF. This review examines the current knowledge about the role of mitochondrial ROS in HIF activation, as well as implications of ROS-level regulation in pathological processes such as cancer.

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Oxygen Homeostasis and HIF

Maintaining oxygen homeostasis is critical for survival and proper function of cells and organisms.¹ Reduced oxygen levels (hypoxia) initiates proper placental and vascular development. Hypoxia also has a causal role in pathological conditions such as ischemia-related diseases and cancer. A complete absence of oxygen (anoxia) results in cell death.² ATP synthesis is ablated, and most cells undergo apoptosis soon after anoxia exposure.^{3–5} Cells exposed to hypoxia, or reduced oxygen levels, on the other hand, are able to maintain normal ATP synthesis and survive.^{5,6} However, as cells replicate in a hypoxic environment, they reduce the oxygen supply even further and generate levels of local anoxia. Therefore, cells must respond quickly to decreasing oxygen levels before reaching an anoxic state (Figure 1).

Mammalian cells respond to hypoxia by activating broad-action transcription factors named hypoxia-inducible factors, or HIFs, which are expressed by virtually all cells of the body.^{7–9} Three members of the HIF family exist, named HIF1, HIF2 and HIF-3.^{10–13} HIFs bind to hypoxia-responsive elements, consensus sequences in the promoter region of more than one hundred genes, activating the transcription of genes that allow the cell to adapt to and survive in the hypoxic environment.^{14,15} Genes regulated by HIFs include glucose transporter that allow the cells to efficiently import glucose to continue generating ATP despite reduced nutrient availability; and genes that reorganize the microenvironment to bring in oxygen, such as vascular endothelial growth factor, which

stimulates formation of new blood vessels.^{16–18} Importantly for tumorigenesis, HIF also turns on genes such as insulin-like growth factor^{2,19} which induces cellular survival and proliferation, as well as those that promote tumor invasion and migration, such as matrix metalloproteinase-2.²⁰ These factors contribute to tumor growth and invasion and metastasis, implicating HIFs as important protumorigenic factors.

Regulation of HIF Function

HIF is a heterodimer, consisting of a constitutively stable HIF β subunit (also known as aryl hydrocarbon translocator, or ARNT) and an oxygen-sensitive HIF α subunit.^{21–23} HIF α and HIF β are continuously transcribed and translated. However, under normal oxygen conditions (normoxia; defined here as 21% O₂), the HIF α subunit is hydroxylated at conserved proline residues in the oxygen-dependent degradation domain by prolyl hydroxylase enzymes (PHDs) (Figure 2).^{24–25} In addition to oxygen, the PHDs require iron and 2-oxoglutarate as substrates for hydroxylation.²⁶ Indeed, the hydroxylation of the HIF α protein can be inhibited by iron chelators or with dimethylglycylglycine, a competitive inhibitor with respect to 2-oxoglutarate.²⁷ The hydroxylated proline residues of the HIF α protein are then recognized by an E3 ubiquitin ligase, von Hippel–Lindau protein (pVHL), which subsequently targets HIF for rapid degradation by the proteasome.^{28–30} An inherited deficiency in pVHL allows HIF to become constitutively active, leading to renal cell carcinoma, further illustrating the importance of HIF in cancer pathology.^{31,32}

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Abbreviations: COX, cytochrome *c* oxidase; DFO, desferrioxamine; FIH, factor-inhibiting HIF; HIF, hypoxia-inducible factor; NAC, *N*-acetylcysteine; PHDs, prolyl hydroxylases; pVHL, von Hippel–Lindau; Q, ubiquinone; QH2, ubiquinol; ROS, reactive oxygen species

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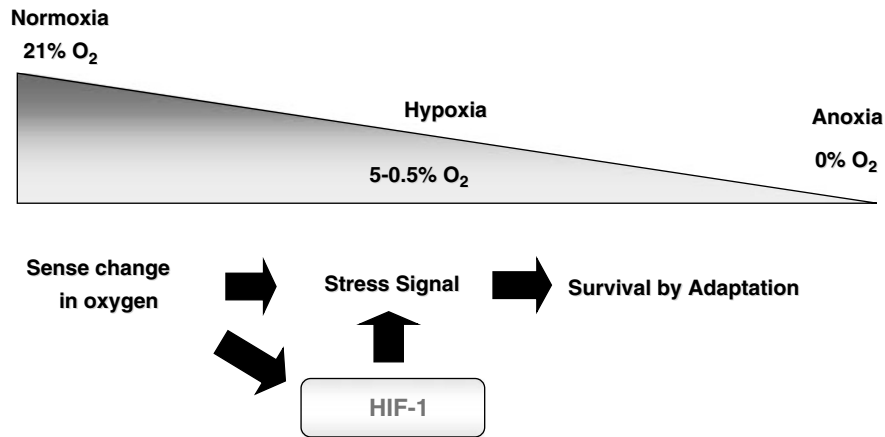


Figure 1 Mammalian cells respond to decreases in oxygen levels by activating a stress signal response, which triggers hypoxia inducible factors 1 and/or 2 for adaptation and subsequent survival

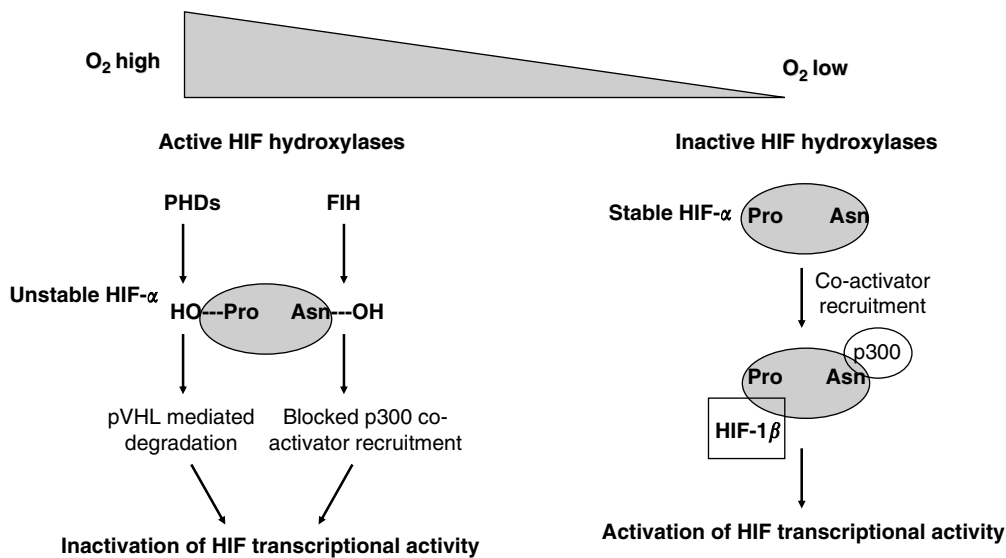


Figure 2 HIF- α protein is hydroxylated at proline and asparagine residues at normal oxygen concentration. This targets the protein for pVHL-dependent degradation and prevents binding to transcriptional coactivators such as p300. As cells become hypoxic, hydroxylation of HIF- α protein is prevented, resulting in stabilization of the protein and binding to p300 to activate its transcriptional activity

HIF is additionally hydroxylated at an asparagine residue by factor-inhibiting HIF (FIH).^{33–35} This prevents HIF from interacting with the transcriptional coactivators p300 and CREB-binding protein (CBP), thereby reducing the transactivating activity of HIF.^{36,37} Under hypoxia, however, HIF α is not hydroxylated by either PHDs or FIH. The lack of prolyl hydroxylation prevents pVHL from recognizing HIF α and targeting it for proteasomal degradation. HIF α can then migrate to the nucleus, dimerize with HIF β , and bind specific hypoxia response elements in the promoter regions of its target genes. The absence of asparagine hydroxylation allows the HIF dimer to interact with transcriptional coactivators and initiate transcription.

PHDs as integral members of oxygen-sensing machinery. The mechanism by which cells sense hypoxia and signal for HIF- α protein stabilization is not fully resolved.

The original and simplest model of oxygen-sensing places PHDs as the direct sensors of oxygen levels. As previously mentioned, PHDs require oxygen for function. A logical extension is that, as oxygen availability decreases to hypoxic levels, PHDs are deprived of this crucial cofactor and become unable to hydroxylate HIF. As a result, the HIF- α protein is not recognized by pVHL and is not degraded. However, although elegantly simple, this model may not by itself explain the oxygen-dependent stabilization of the HIF- α protein. HIF-1 α protein stabilization increases in an exponential fashion as oxygen levels decrease to anoxia.³⁸ By contrast, the PHD activity decreases in a linear fashion as oxygen levels decrease to anoxia.³⁹ Clearly, once cells approach anoxia, the PHDs are the direct sensors for stabilization of the HIF-1 α protein since oxygen becomes a limiting substrate for hydroxylation. Moreover, the rate of

hydroxylation of HIF- α protein is likely dependent on the required cofactors iron and 2-oxoglutarate. Thus an integrated oxygen-sensing model that accounts for the availability of oxygen and other cofactors is likely required to explain HIF-1 α protein stabilization over the hypoxic range.

Mitochondria as integral members of oxygen-sensing machinery.

Although the PHDs are the proximal regulators of HIF- α protein stabilization, much evidence has emerged to suggest that the mitochondrial electron transport chain is involved in oxygen sensing and would therefore need to respond to changes in oxygen levels. This idea has long been proposed, since the electron transport chain consumes most of the cell's oxygen. The electron transport chain, which is embedded in the inner membrane of the mitochondria, consists of five multiprotein complexes. Complexes I and II oxidize the energy-rich molecules NADH and FADH₂, respectively, and transfer the resulting electrons to ubiquinol, which carries it to complex III. Complex III, in turn, shuttles the electrons across the inner mitochondrial membrane to cytochrome *c*, which carries them to complex IV. Complex IV uses the electrons to reduce oxygen to water. Each electron-ferrying step uses the resulting energy to pump hydrogen ions into the intermembrane space. This generates a proton gradient, the dissipation of which is used by complex V, or ATPase, to power the energy-costly phosphorylation of ADP to ATP. Along with carrying electrons, complexes I, II, and III generate reactive oxygen species (ROS), namely superoxide, at low levels.⁴⁰

Initial studies implicating the electron transport chain in HIF-1 α stabilization have demonstrated that certain inhibitors of the electron transport chain, including rotenone (which inhibits complex I distally), myxothiazol, and stigmatellin (which inhibit complex III) block the hypoxic stabilization of HIF-1 α .^{41–43} Genetic deletions of complex I, likewise, blocked HIF-1 α stabilization.⁴⁴ Finally, cells depleted of their mitochondrial DNA by incubation with low levels of ethidium bromide (known as rho-zero cells) fail to stabilize HIF-1 α in response to hypoxia.⁴³ While mitochondrial DNA encodes only 13 genes, these are crucial components of complexes I, III, IV, and V, and cells lacking these components do not have a functional electron transport chain.⁴⁵ These cells, however, are still capable of stabilizing HIF-1 α protein when exposed to hypoxia mimetics, such as the iron chelator DFO, demonstrating that HIF-1 α protein stabilization *per se* is intact.^{41,43} Together, these initial results suggest that hypoxic stabilization of HIF-1 α requires a functional electron transport chain, though they did not show specifically the oxygen-sensing site, nor explained how loss of electron transport may stabilize HIF-1 α .

The findings that a functional electron transport chain is required for hypoxic stabilization of HIF α protein fell under scrutiny, specifically from contradictory reports. In one of these studies, rho-zero cells were shown to be capable of stabilizing HIF-1 α under hypoxia.⁴⁶ However, these studies utilized oxygen levels very close to anoxia (0.1%), rather than true hypoxia (1–2% O₂). Anoxia, like hypoxia, stabilizes the HIF-1 α protein. However, the mechanisms employed by anoxia appear quite different. These differences are explored in a study by Schroedl *et al.*,⁵ which shows that rho-zero cells are, indeed, capable of stabilizing HIF-1 α protein under

anoxia, but not under hypoxia (defined in that study as 1.5% O₂). Anoxic cells, unlike hypoxic cells, experience a significant drop in oxygen consumption and ATP production, and die within 48 h, while hypoxic cells continue to survive and multiply. This study highlighted the differences between anoxia and hypoxia. Since oxygen is entirely limited in anoxia, it is likely that anoxic cells stabilize HIF-1 α protein through impaired PHD function.

An alternative explanation for the contradictory results from rho-zero cells is the use of ethidium bromide, a potential mutagen, to generate these cells. To resolve these discrepancies, three independent groups utilized the power of genetics to conclusively demonstrate that mitochondrial electron transport chain is required for hypoxic stabilization of HIF-1 α protein. The first study by Celeste Simon's group demonstrated that cells lacking cytochrome *c* fail to stabilize the HIF-1 α protein in hypoxia.⁴⁷ The other two studies done by Paul Schumacker's group and ours demonstrated that RNAi of the Rieske-Fe-S protein, a complex III subunit, prevents hypoxic stabilization of HIF-1 α protein.^{48,49} Collectively, these studies provided conclusive genetic evidence that mitochondrial electron transport chain is required for HIF-1 α protein stabilization.

The observation that genetic or pharmacologic inhibition of mitochondrial electron transport chain prevents hypoxic stabilization of HIF- α protein begs the question: which function of the electron transport chain is necessary for HIF- α protein stabilization? Two main theories have arisen to address this question. The first theory suggests that, under hypoxia, mitochondria, with their high oxygen consumption, leave the rest of the cell 'anoxic.' PHDs, then, are deprived of their cofactor, oxygen, and cannot hydroxylate HIF- α protein to target it for degradation.⁵⁰ Another model implicates ROS generation by the electron transport chain as a signaling molecule to HIF-1 α protein stabilization.⁵¹ Support for the ROS theory initially arose from the observation that, paradoxically, hypoxia increases ROS production, and treatment with antioxidants prevents HIF-1 α protein stabilization.⁴¹ Additionally, exogenous H₂O₂ treatment stabilized HIF-1 α protein.⁴¹ Rho-zero cells are unable to generate ROS. Cells genetically depleted of cytochrome *c* or the Rieske-Fe-S protein also fail to increase production of ROS during hypoxia.^{47,48} However, these studies did not conclusively identify the complex within the electron transport chain responsible for hypoxic ROS generation and HIF-1 α protein stabilization.

The Q_o site complex III as part of oxygen sensing machinery.

The pharmacologic and genetic data point to the ubiquinone (Q) cycle of complex III as the source of ROS generation during hypoxia to stabilize HIF-1 α protein.^{41,43,47–49} Unlike complexes I and II, which generate superoxide into the mitochondrial matrix,^{52–55} complex III can also release superoxide into the mitochondrial intermembrane space and subsequently, into the cytosol.^{56–58} The Q cycle is initiated when complex I and complex II transfer electrons to reduce the lipid moiety ubiquinone to ubiquinol (Figure 3). Ubiquinol then enters the Q cycle (Figure 3). The first step in the Q cycle is the transfer of electrons to the Rieske Fe-S protein, which oxidizes ubiquinol (QH₂) to ubisemiquinone (QH•) at

the Q_o site of complex III. This electron is transferred to cytochrome c₁, which in turn reduces cytochrome c. Complex IV oxidizes cytochrome c and then transfers the electron to molecular oxygen, reducing it to water. This pathway only accounts for one of the electrons that reduced ubiquinone to ubiquinol. At this point, the Q cycle is only half complete because the other electron is still maintained by the highly reactive intermediate ubisemiquinone.

Ubisemiquinone quickly reduces the b_L reaction center of cytochrome b at the Q_o site of Complex III. The electron is then passed from the b_L reaction center to the b_H reaction center of cytochrome b. The electron from the b_H reaction center reduces either ubiquinone or ubisemiquinone at the Q_i site of Complex III. When this electron is passed to the Rieske Fe-S protein and consequently cytochrome c₁ the Q cycle is complete. Ubisemiquinone is a highly reactive intermediate that is generated at the Q_o site. As a byproduct of electron transfer through the Q cycle, ubisemiquinone can generate superoxide at the Q_o site. Pharmacologic interventions indicate that Q_o site is responsible for the generation of ROS during hypoxia. Stigmatellin, which inhibits electron flux to Rieske iron-sulfur protein, prevents ROS generation and HIF activation during hypoxia.⁴⁸ In the absence of the Rieske Fe-S protein the Q cycle is not initiated and there is no electron flow through complex III. By contrast, antimycin A does not prevent the hypoxic increase in ROS production or HIF activation.⁴¹ Antimycin A prevents electron flux post cytochrome b. Thus, the Q_o site is maintained while the Q_i site is abolished.

Although the pharmacologic evidence indicates that the Q_o site is a likely site of ROS generation during hypoxia, this needed to be validated with genetic interventions. The best method is to examine ROS production and HIF activation during hypoxia in cells deficient in cytochrome b, which will maintain a Q_o site of ROS generation but not the Q_i site. However, cytochrome b gene is encoded by mtDNA and current siRNA techniques do not target mtDNA transcripts.

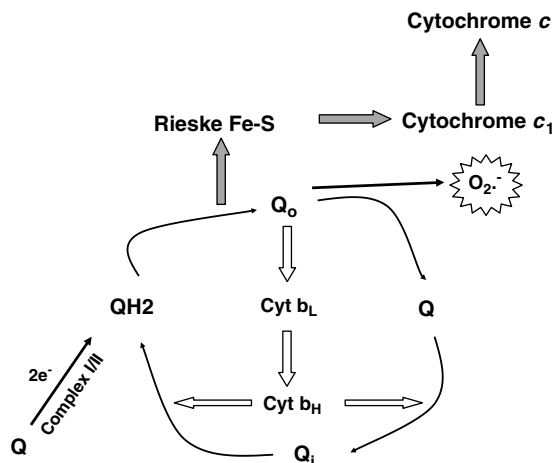


Figure 3 The ubiquinone (Q) cycle is initiated when one electron from ubiquinol (QH₂) is donated to the Rieske-iron sulfur protein and the second electron is donated to cytochrome b. The intermediate moiety is the free radical ubisemiquinone (Q_o), which can donate electrons to molecular oxygen to generate superoxide

Thus, we utilized cytochrome b mutant cybrids, generated by reconstituting 143B rho-zero cells with wild-type mitochondrial DNA or that containing a 4-bp deletion in the cytochrome b gene found in a patient suffering from parkinsonism.⁵⁹ Loss of cytochrome b renders these cells incapable of oxygen consumption and unable to generate ROS at the Q_i site specifically. However, these cells were capable of upregulating hypoxic ROS and stabilizing HIF-1 α protein.⁶⁰ The administration of the mitochondrial antioxidant MITOQ prevented the HIF-1 α protein stabilization. We also utilized RNAi to knock down Rieske Fe-S protein, to abolish ROS generation at the Q_o site in mutant cytochrome b cybrids. This prevented hypoxic ROS generation and HIF-1 α protein stabilization, implicating the Q_o site of complex III as the key site in hypoxic ROS generation and HIF-1 α protein stabilization. These cells, however, retained the ability to stabilize HIF-1 α protein after direct PHD inhibition by dimethylallyl glycine, showing an otherwise intact HIF-signaling pathway. Furthermore, we established a link between hypoxic ROS generation and hydroxylation of HIF-1 α protein. Neutralizing the ROS with antioxidants allowed HIF-1 α protein to remain hydroxylated (and, therefore, primed for degradation) under hypoxic conditions. On the contrary, increasing ROS levels under normoxia by overexpressing glucose oxidase prevented normoxic HIF-1 α protein hydroxylation. Importantly, these data demonstrate that the ROS generated by the mitochondria under hypoxia prevent hydroxylation of HIF-1 α protein (Figure 4).

Our study is not compatible with the oxygen consumption model proposed by Hagen *et al.*,⁵⁰ which states that, as mitochondria consume oxygen under hypoxia, they leave the cytosol with insufficient oxygen for PHD function. Studies using rho-zero cells were unable to distinguish between oxygen consumption and mitochondrial ROS generation, as depleting mitochondrial DNA stops both of these functions. However, the cytochrome b cybrids are incapable of oxygen consumption while still able to generate ROS under hypoxia.

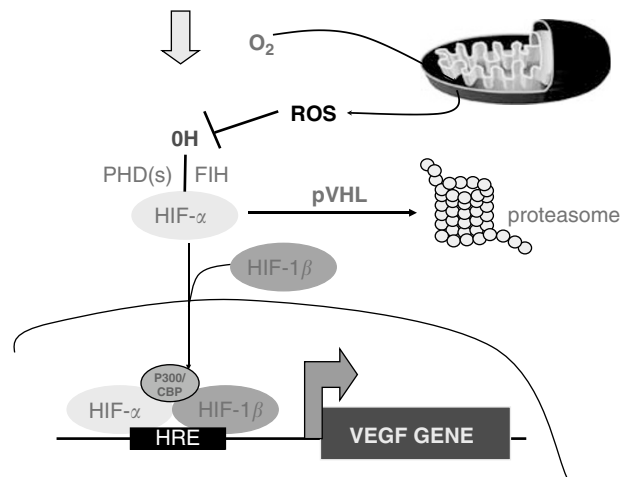


Figure 4 We propose that hypoxia increases the generation of reactive oxygen species that prevent hydroxylation of HIF- α protein, resulting in stabilization and activation of its transcriptional activity

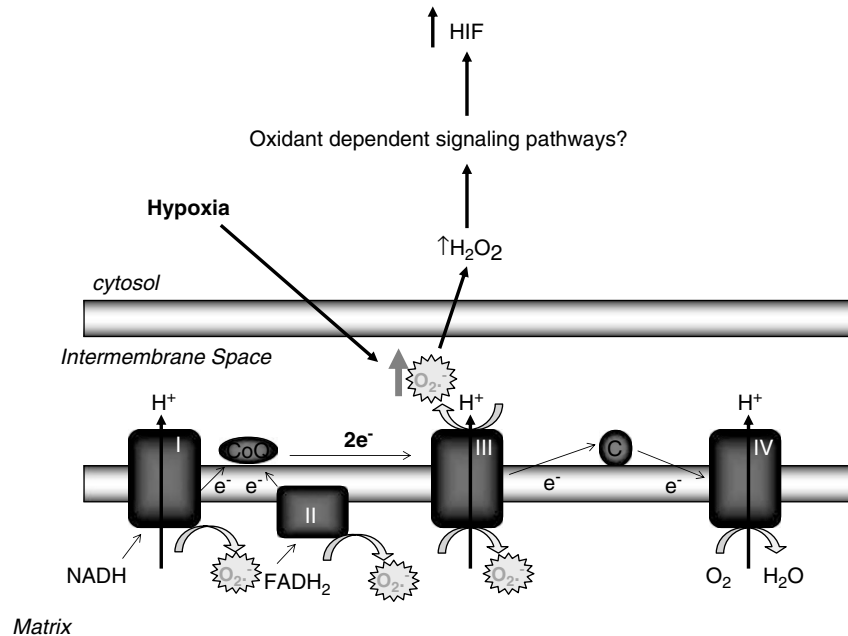


Figure 5 Mitochondrial electron transport chain generates superoxide at complex I, II, and III. Complex I and II generate superoxide within the mitochondrial matrix. Complex III can generate superoxide in both the intermembrane space and the matrix. We propose that complex III is an integral part of the oxygen-sensing machinery in mammalian cells. Hypoxia elicits the release of superoxide from complex III into the cytosol, where it is converted to H₂O₂ to activate oxidant-dependent signaling pathways resulting in the activation of HIF. Currently it is not known how hypoxia increases the release of ROS from complex III, or what oxidant-dependent signaling pathways are required for HIF activation

Unless these ROS are neutralized by antioxidants, HIF-1 α protein is stabilized in these cells under hypoxia. This indicates unequivocally that mitochondrial ROS generation, not oxygen consumption, contributes to HIF-1 α protein stabilization under hypoxia. In certain scenarios, however, a high rate of oxygen consumption by cells or cellular overcrowding under normal oxygen conditions can result in conditions close to anoxia. As the mitochondria utilize most of the oxygen, the cytosol may, indeed, be left anoxic, turning off PHDs ability to hydroxylate HIF-1 α protein. Overall, our recent study clearly implicates the Q_o site of complex III as the source of ROS generation that prevents hydroxylation of the HIF-1 α protein to stabilize it under hypoxia.

Mitochondrial ROS regulate HIF-dependent tumorigenesis. While use of antioxidants has long been known to have anti-tumorigenic effects, the common assumption was that antioxidants prevented oncogenic lesions and genomic instability arising from ROS-induced DNA damage. To test whether antioxidants prevent tumorigenesis through attenuating HIF activation or genomic instability, Dang and colleagues⁶¹ tested efficacy of the antioxidant *N*-acetylcysteine (NAC) in human B cells that overexpress MYC in a xenograft model in SCID mice and transgenic mice that conditionally overexpress MYC in liver cells. Treatment of each kind of mouse with the antioxidant NAC reduced or prevented tumor growth. The human B cells displayed no increase in genomic instability upon MYC induction, indicating that NAC was not preventing tumor growth by decreasing ROS-induced DNA damage. However, NAC treatment of these cells did suppress HIF-1 α

protein stabilization under hypoxia. HIF-1 α protein mutants lacking oxygen-dependent degradation domains rescued tumor growth in the mice treated with NAC. Together, these data suggest that NAC acts on tumorigenesis by preventing HIF-1 α protein stabilization. This study provides a potential mechanistic relationship of why antioxidants might be effective as therapeutic potential for cancer prevention.

Unanswered Questions

Despite recent advances in our knowledge of HIF regulation by mitochondrial ROS, many questions remain unanswered. Particularly, how complex III increases the generation of superoxide to the intermembrane space and how mitochondrial superoxide that is converted to H₂O₂ signals to the PHDs remain unclear (Figure 5). An oxidant-dependent signaling pathway may lead to post-translational modification of PHDs, such as phosphorylation, which would render them inactive. H₂O₂ may also affect PHDs more directly. The PHDs are non-heme iron-dependent oxygenases that require Fe⁺² for their enzymatic activity. H₂O₂ may cause a local Fenton reaction to shift iron ions from Fe⁺² to Fe⁺³ causing inactivation of the enzyme.⁶²

A combination of oxygen, iron, TCA cycle intermediate availability and ROS signaling might optimize the hydroxylation of HIF α proteins.⁶³ Furthermore, recent studies have indicated that HIF-1 activation might prevent excessive ROS production in hypoxic cells by regulating mitochondrial respiration through increased gene expression of PDK1 (PDH (pyruvate dehydrogenase) kinase 1) and switching of cytochrome *c* oxidase subunit IV isoforms.^{64–66} PDK1

phosphorylates and inactivates PDH, the mitochondrial enzyme that converts pyruvate into acetyl-CoA. PDK1 expression would shuttle pyruvate away from the generation of acetyl-CoA to lactate resulting in decreased substrate delivery to the tricarboxylic acid cycle. This would cause reduction in the levels of NADH and FADH₂ delivered to the electron-transport chain, which could potentially decrease ROS generation. The switching of COX composition from increased expression of COX4-2 subunit and decreased in COX4-1 subunit makes the COX enzyme more efficient under hypoxic conditions. It is conceivable that an efficient COX would limit leak of electrons from the electron transport chain thereby diminishing the generation of ROS. Thus, it remains to be tested whether mitochondrial ROS would activate HIF-1, which as a feedback mechanism would decrease excessive ROS production through PDK1 and COX4-2 expression. Future studies are required to answer these questions. However, what is clear is that mitochondrial ROS generation is required for hypoxic stabilization of HIF-1 α protein, and that modulating mitochondrial ROS carries therapeutic implications in cancer and other HIF-dependent diseases.

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