Chapter 15

# MITOCHONDRIAL REACTIVE OXYGEN SPECIES ARE REQUIRED FOR HYPOXIC HIFα STABILIZATION

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**Abstract:** Multicellular organisms initiate adaptive responses when oxygen ($O_2$) availability decreases. The underlying mechanisms of $O_2$ sensing remain unclear. Mitochondria have been implicated in many hypoxia-inducible factor (HIF) –dependent and –independent hypoxic responses. However, the role of mitochondria in mammalian cellular $O_2$ sensing has remained controversial, particularly regarding the use pharmacologic agents to effect hypoxic HIFα stabilization, which has produced conflicting data in the literature. Using murine embryonic cells lacking cytochrome c, we show that mitochondrial reactive $O_2$ species (ROS) are essential for $O_2$ sensing and subsequent HIFα stabilization at 1.5% $O_2$. In the absence of this signal, HIFα subunits continue to be hydroxylated and degraded via the proteasome. Importantly, exogenous treatment with $H_2O_2$ and severe $O_2$ deprivation is sufficient to stabilize HIFα even in the absence of functional mitochondrial. These results demonstrate that mitochondria function as $O_2$ sensors and signal hypoxic HIFα stabilization by releasing ROS to the cytoplasm. The cytochrome c mutant embryonic cells provide a unique reagent to further dissect the role of mitochondria in $O_2$ mediated-intracellular events.

**Key Words:** transcription, signal transduction, HIF prolyl hydroxylases

## INTRODUCTION

Molecular oxygen is essential for aerobic energy metabolism, whereby the oxidoreduction energy of mitochondrial electron transport is converted into the high-energy phosphate bond of ATP. Oxygen ($O_2$) serves as the final electron acceptor for electron transport at complex IV (cytochrome c oxidase). However, where excess $O_2$ can be toxic, a significant decrease in $O_2$ impairs ATP generation and cell viability. Therefore, most prokaryotic and

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eukaryotic life is restricted to a narrow range of $pO_2$. Most mammalian cells exist at an *in vivo* $pO_2$ of 30 mm Hg to 50 mm Hg (4-7% $O_2$). As $O_2$ levels drop below this range, a number of cellular responses are engaged, including inhibition of ion channel activity, cell division, ribosome biogenesis, and mRNA translation. Cells have also developed transcriptional responses to hypoxia mostly regulated by hypoxia-inducible factors (HIFs) (12). HIFs activate transcription of genes encoding proteins that enhance glycolytic ATP production or increase $O_2$ delivery to affected tissues. This review will focus on models of $O_2$ sensing and their impact on the HIF signaling pathway (Figure 1).

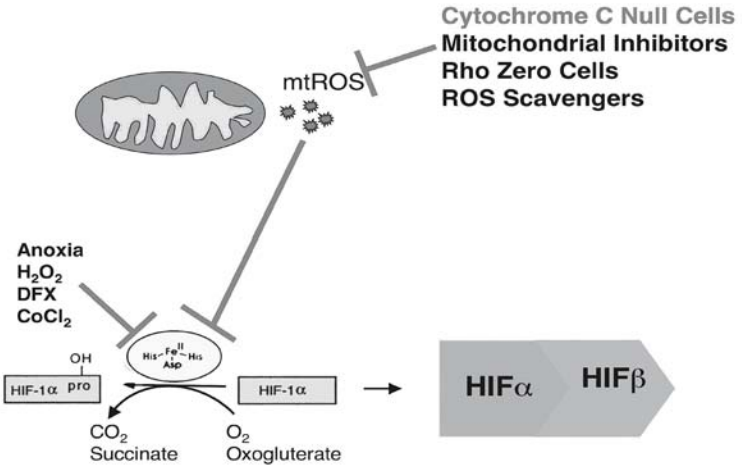


**Figure 1**. Proposed role of mitochondria in cellular oxygen sensing. This figure documents the experimental evidence supporting a role for mitochondrial reactive oxygen species (ROS) in the modulation of HIF stability, including cytochrome c null cells, mitochondrial inhibitors, Rho 0 cells, and ROS scavengers. We believe that mitochondrial ROS inhibit prolyl hydroxylase enzymatic activity in a similar fashion to anoxia, $H_2O_2$, DFX, and cobalt chloride. Inhibition of hydroxylase activity leads to HIFα accumulation, dimerization with HIFα (ARNT), and HIF-mediated gene expression.

## OXYGEN SENSORS AND HYPOXIC SIGNAL TRANSDUCTION

The mechanisms by which eukaryotic cells sense decreased $O_2$ and transduce this signal to the HIF pathway have been debated for at least a decade. HIFs are heterodimeric transcription factors consisting of alpha (HIF-1α, HIF-2α, and HIF-3α), and beta (ARNT, ARNT2) subunits. The beta subunits are constitutively transcribed and translated and reside in the nucleus. In contrast, while the alpha subunits are transcribed and translated at a high rate in normoxic conditions (21% $O_2$), they are rapidly degraded via the proteasome. HIF-1α, HIF-2α, and HIF-3α contain 200 amino acid "oxygen dependent degradation domains" (ODDs) (5). These ODDs include a highly conserved binding domain for the tumor suppressor Von Hippel-Lindau protein (pVHL). pVHL coordinates the assembly of an E3 ubiquitin ligase complex containing Elongin B, Elongin C, Cullin 2, and RBX 1, and this complex ubiquitylates HIFα's, targeting their degradation via the proteasome (6) (7).

Recent data have shown that the interaction between HIFα's and pVHL is regulated via hydroxylation of proline residues in the ODD (6) (7). There are at least three HIF prolyl hydroxylases in mammalian cells (4). In the absence of $O_2$, prolyl hydroxylases are inactive; the unmodified HIFα no longer interacts with pVHL and accumulates. The absolute requirement for $O_2$ by HIF prolyl hydroxylases indicates that these enzymes may function as direct oxygen sensors. However, the precise sensor for the HIF pathway remains contentious. Previous models have included heme-binding proteins, membrane associated NADPH oxidases, and mitochondrial reactive oxygen species (10).

The model based on NADPH oxidase was first proposed in 1996 (8) (15). NADPH oxidases convert $O_2$ into reactive oxygen species (ROS); decreased $pO_2$ would therefore result in *reduced* formation of ROS to stabilize HIFα subunits. However, oxygen sensing and HIFα stabilization during hypoxia are preserved in gp91-*phox*-deficient cells isolated from knockout mice or patients with chronic granulomatous disease (1). These and other observations have shed doubt on a role for NADPH oxidase in oxygen sensing and HIF activation. A third model proposed in 1998 by Chandel et al. (2) suggests that hypoxia results in *increased* production of ROS by the mitochondria. Here, linear decreases in $pO_2$ result in a progressive increase in ROS. Pharmacological inhibitors of different complexes of the mitochondrial respiratory chain support the hypothesis of mitochondria as a key intracellular source of ROS. Inhibition of complex I (via rotenone) suppresses ROS production while inhibition of complex III or IV (antimycin A) increases ROS. Moreover, HIF activation directly correlates with ROS accumulation. Antioxidants such as ebselen and PDTC attenuate the ROS signal and abolish HIF stabilization. Importantly, Rho 0 cells (where mitochondria are deficient in electron transport and oxidative phosphorylation) fail to induce HIF activity. These results were not reproduced using other cell types: Srinivas et al. (13) and Vaux et al. (14) observed that Rho 0 cells display a normal response to hypoxia based on HIF stabilization. Vaux et al. used mutant cell lines with specific genetic defects in the electron transport chain and demonstrated that HIF activation by hypoxia was preserved. Taken together, these results failed to support the model implicating mitochondrial ROS during hypoxia as effectors in HIFα stabilization. However, Schroedl et al. accounted for this discrepancy by comparing HIFα stabilization under hypoxic conditions (1.5% $O_2$) versus anoxic conditions (less than 0.1% $O_2$) (11). Both the Srinivas and Vaux studies observed hypoxic HIFα stabilization using extremely low levels of $O_2$. The Schroedl et al. paper showed that mitochondrial ROS are essential for HIF stabilization at 1% $O_2$, while extremely low levels of $O_2$ (less than 0.01% $O_2$) are independent of mitochondrial electron transport. These results provide a plausible explanation, as the HIF prolyl hydroxylase enzymes are dependent on $O_2$ as a cofactor and at anoxic conditions could become substrate limited.

Both of these models suggest ROS are key players in $O_2$ sensing by the HIF pathway. However, the effector(s) regulated by ROS that transduces the signal to HIF remain unknown. Further investigation is also required to identify these intermediates and determine if they modulate prolyl hydroxylase enzymatic activity.

## CYTOCHROME C IS REQUIRE FOR CELLULAR OXYGEN SENSING AND HYPOXIC HIFA ACTIVATION

While cellular responses to hypoxia have been studied extensively, the precise identity of mammalian cellular $O_2$ sensors remains controversial. Because of their $O_2$ dependence and relatively high kM for $O_2$ *in vitro*, it has been proposed that the HIF prolyl hydroxylases directly sense $O_2$ deprivation to stabilize HIF. However, mitochondrial ROS have been implicated in many cellular processes including HIFα stability and transcriptional activity, myocyte contraction, IL-6 production, glutathione depletion, NA,K-ATPase activity, and adipocyte differentiation (15). Despite this evidence, the role of mitochondria in mammalian cellular oxygen sensing has remained controversial. The use of pharmacological agents to affect HIFα stabilization has produced conflicting data in the literature. Therefore, we investigated this controversy by repeating and extending previous observations.

Mitochondria-deficient Hep3B Rho 0 cells have been shown to be defective in HIFα stabilization (3). Nevertheless, later work suggests this may be an artifact due to their selection with the mitochondrial inhibitor rotenone (14). We initially chose to address this discrepancy by generating Hep3B Rho 0 cell using ethidium bromide and avoiding selection with mitochondrial inhibitors (9). Of note, Hep3B cells exposed to steady doses of ethidium bromide for 3 weeks exhibit no mitochondrial DNA or oxygen consumption. Upon exposure to 1.5% $O_2$, Rho 0 cells failed to stabilized either HIF-1α or HIF-2α. We also generated HEK293 Rho 0 cells and used human osteosarcoma-derived 143B Rho 0 cells and obtained similar results. Hep3B, HEK293, and 143B cells retained responsiveness to desferrioxamine (DFX) with regard to HIF stabilization. These findings indicate that mitochondrial electron transport is required for hypoxic HIFα stabilization at 1.5% $O_2$, indicating that all cells retained active prolyl hydroxylation.

Due to concerns about long-term metabolic adaptation or genetic alteration of Rho 0 cells, we also employed acute mitochondrial inhibition of complex I (rotenone) or complex III (myxothiazol), minimizing non-specific effects by utilizing extremely low nanomolar doses. Treatment of Hep3B cells with rotenone or myxothiazol inhibited respiration and prevented hypoxic HIF-1α and HIF-2α stabilization after 2 hours of treatment. Importantly, myxothiazol treatment also affected HIFα stabilization in response to cobalt chloride, especially at higher doses. These results highlight difficulties inherent to direct pharmacological treatment and have prevented widespread acceptance of mitochondrial ROS during mammalian $O_2$ sensation.

We, therefore, employed a novel genetic model to further investigate the role of mitochondrial ROS in cellular oxygen sensing avoiding the non-specific effects of ethidium bromide or pharmacological inhibition all together. The murine somatic cytochrome c gene has been targeted previously. Loss of cytochrome c prevents oxidation of cytochrome c1, keeping the Reiske's iron sulfur protein reduced. This prevents oxidation of ubiquinol and the formation of the ubisemiquinone radical, eliminating one important source of superoxide anion ($O_2^-$) (and therefore $H_2O_2$) that has been implicated in hypoxic HIFα stabilization. We generated wild type, heterozygous, and null cell lines for cytochrome c using day 8.5 mouse embryos. While wild type and heterozygous cells exhibit similar respiratory rates while null cells are devoid of any measurable mitochondrial $O_2$ assumption or cytochrome c protein.

We next investigated *in vivo* ROS production in the cells using oxidant sensitive dichlorofluorescein (DCFH) and a ROS-sensitive FRET probe. Where wild type and heterozygous cells rapidly and dramatically increase oxidation and FRET activation, cytochrome c null cells exhibit an attenuated response. Importantly, unlike their wild type or heterozygous

counterparts, cytochrome c null cells are unable to stabilize HIF-1α or HIF-2α in response to treatment with 1.5% $O_2$. The null cells, however, retain an appropriate response to DFX. These results argue strongly that the increased ROS production in hypoxic heterozygous cells are mitochondrial, and supports the hypothesis that mitochondrial ROS generated by complex III are responsible for hypoxic HIF-1α and HIF-2α stabilization.

Stable reintroduction of cytochrome c into an independent null cell line restores mitochondrial function and hypoxic mitochondrial ROS accumulation. This correlated with restoration of properly stabilized HIF-1α and HIF-2α levels similar to wild type cells. We concluded that the inhibility of null cells to properly sense $O_2$ is due to the lack of a functional mitochondrial electron transport chain resulting from cytochrome c loss. All cell lines respond similarly to DFX, suggesting that cytochrome c null cells maintain normal HIFα transcription, translation, and degradation even in the absence of functional mitochondrial. However, to test that they continue to hydroxylate and degrade HIF-1α and HIF-2α under hypoxic conditions, cytochrome c wild type and null cells were treated with 1.5% $O_2$ in the presence of the proteasome inhibitor MG132. While the null cells failed to stabilize HIFα in response to hypoxia, inhibition of proteasome degradation by MG132 leads to HIFα accumulation similar to data obtained with wild type cells. This demonstrates that hydroxylation and degradation of HIFα continue to occur in the cytochrome c null cells even at this reduced $O_2$ concentration.

We next determined if HIFα stabilization can be bypassed by severe $O_2$ deprivation (anoxia or approximately 0% $O_2$) where prolyl hydroxylases appear to be substrate limited. Importantly, the null cells fail to stabilize HIF-1α or HIF-2α in response to 1.5% $O_2$. However, exposure to 0% $O_2$ induces both HIF-1α or HIF-2α in a mitochondria-independent manner. These results suggest functional mitochondria are not necessary for cellular responses to severe $O_2$ deprivation. To test whether ROS production is also sufficient to stabilize HIF-1α or HIF-2α, Hep3B cells were treated with exogenous hydrogen peroxide ($H_2O_2$) as previously described (3). This resulted in normoxic stabilization of HIF-1α or HIF-2α similar to that induced by hypoxia in the same period of time. Furthermore, incubation of Hep3B cells with an $H_2O_2$ generating enzyme, glucose oxidase, is sufficient to stabilize both HIFα subunits. This is shown to be $H_2O_2$-dependent as inclusion of catalase abolishes HIFα stabilization. Finally, treatment of cytochrome c null cells with T-butyl hydroperoxide (TBP) a stable $H_2O_2$ analog, also induces HIFα stabilization similar to the hypoxia mimetic cobalt chloride. These results indicate that ROS production is sufficient for HIFα stabilization and likely functions downstream of mitochondria.

In summary, cytochrome c null cells provide novel genetic evidence supporting a role for mitochondrial ROS in the mammalian $O_2$ sensing pathway that leads to HIFα stabilization. How mitochondrial ROS effect HIF prolyl hydroxylase activity is important to understanding cellular $O_2$ sensing. These experiments are ongoing in the laboratory and suggest that ROS may directly inhibit the prolyl hydroxylases perhaps by oxidizing $Fe^{2+}$ at the active site. It is likely that a number of mammalian cellular $O_2$ sensor exist, controlling various hypoxic responses depending on the cell type, $O_2$ tension, and other cellular conditions. The cytochrome c null cells provide valuable tools for investigating the role of the mitochondria and other hypoxic responses such as the inhibition of protein synthesis and cell cycle progression.

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