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# Mitochondrial reactive oxygen species regulate hypoxic signaling

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## Abstract

Physiological hypoxia results in a host of responses which include increased ventilation, constriction of the pulmonary artery, and a cellular transcriptional program which promotes glycolysis, angiogenesis, and erythropoiesis. Mitochondria are the primary consumers of cellular oxygen and have thus been speculated for years to be the site of cellular oxygen sensing. Many of the cellular responses to hypoxia are now known to be mediated by the production of reactive oxygen species at mitochondrial complex III. While the mechanism by which cytosolic oxidant concentration is increased during hypoxia is unknown, the importance of the maintenance of cellular oxygen supply requires further investigation into the role of ROS as hypoxia signaling molecules. The following is a brief overview of the current understanding of the role of mitochondrial-produced ROS in cellular oxygen signaling.

## Introduction

Most eukaryotic cells utilize oxidative phosphorylation for the maintenance of cellular ATP stores. The importance of oxygen as the terminal electron acceptor of the electron transport chain has led to the evolution of multiple mechanisms by which cells and organisms maintain an adequate supply of oxygen. Acute exposure of mammals to hypoxic environments results in the calcium-dependent constriction of pulmonary arteries, allowing for increased blood oxygen perfusion [1]. Prolonged hypoxia stimulates multiple cell types and induces the Hypoxia Inducible Factors (HIFs) which mediate transcription of a large number of hypoxia-sensitive genes, including the production of erythropoietin in the kidney [2]. Although the importance of the hypoxic response cannot be understated, the mechanism by which cells detect oxygen levels to initiate the hypoxic response remains a subject of debate. Mitochondria are the largest consumers of cellular $O_2$ and as such are likely candidates for the location of the cellular oxygen sensor [3]. Significant evidence has accumulated supporting the role of mitochondria as putative oxygen sensors; however, as a mitochondrial signal could involve cellular energy state, cytosolic redox state, or mitochondrial production of reactive oxygen species (ROS), the nature of the hypoxic signal provided by mitochondria is a contentious point. This review focuses on the role of mitochondrial-generated ROS in the propagation of the hypoxic response through calcium-mediated pulmonary artery constriction and through HIF-mediated transcription.



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## The mitochondrial electron transport chain generates reactive oxygen species

During mitochondrial respiration, electrons from NADH and FADH2 are transferred to mitochondrial complex I and complex II respectively. These electrons are then shuttled through mitochondrial complex III, and on to the final electron acceptor, oxygen, which is reduced to water at cytochrome oxidase (complex IV). The actions of mitochondrial complexes I, III, and IV result in a proton gradient, the free energy of which is used to drive ATP synthesis. It is estimated that 2–3% of $O_2$ consumed by mitochondria is incompletely reduced, yielding reactive oxygen species (ROS) [4]. ROS are produced by the electron transport chain at complexes I, II, and III (Figure 1). While complexes I and II produce ROS only into the matrix, complex III is capable of producing ROS on both sides of the mitochondrial inner membrane [4,5]. ROS produced into the intermembrane space theoretically has an easier route to the cytosol to act as signaling molecules than do ROS produced into the matrix [6].

ROS are produced at complex III of the electron transport chain via formation of ubisemiquinone, a radical form of coenzyme Q. Mitochondrial complexes I and II donate two electrons to ubiquinone producing the reduced form, ubiquinol. Ubiquinol is oxidized at the $Q_o$ site of mitochondrial complex III in a two step process termed the Q-cycle (Figure 1). First, one electron from ubiquinol is transferred to the Rieske iron-sulfur protein (RISP). This electron is transferred sequentially from RISP to cytochrome *c*1, cytochrome *c*, and finally to complex IV. This one electron oxidation of ubiquinone results in the transient formation of ubisemiquinone. The free electron of ubisemiquinone is then transferred by RISP to cytochrome *b*, and is subsequently used to reduce another molecule of ubiquinone at the $Q_i$ site. Ubisemiquinone formed at the $Q_o$ site of complex III is capable of donating its free electron directly to oxygen, forming superoxide [7,8]. Superoxide dismutates either spontaneously, or through the action of superoxide dismutase (SOD), to form hydrogen peroxide [4].

While non-charged hydrogen peroxide can diffuse through the outer membrane to access the cytosol, superoxide reaches the cytosol through voltage-dependent anion channels (VDACs) [6]. In the cytosol, oxidation of cysteines remains the best-studied oxidative signaling modification [9]. Oxidation of the cysteine sulfhydryl group has been demonstrated to alter protein-protein interactions, the DNA binding activity of transcription factors, and the catalytic activity of enzymes, including phosphatases involved in signaling cascades [9,10]. Additionally, oxidation of two intra-, or intermolecular cysteines forms disulfide bridges allowing for conformational changes or oligomerization of proteins [11,12].

## Hypoxia induces a cellular transcription program through induction of HIFs

One of the best-characterized physiological responses to sustained hypoxia is the induction of the glycoprotein hormone, erythropoietin (Epo), which binds to receptors on erythroid progenitor cells, enabling proliferation and differentiation into red blood cells [13]. Hypoxic induction of Epo was first demonstrated by Semenza and colleagues to be mediated *in vitro* by activation of the Hypoxia-Inducible transcription Factor HIF-1 [14,15]. HIF-1 is a hetero-dimeric transcription factor consisting of two basic helix-loop-helix/PAS proteins, HIF-1α and HIF-1β [16]. Epo induction *in vivo* has subsequently been demonstrated to be regulated by a second HIF-α subunit (HIF-2α), and to date, three HIF-α subunits have been identified (HIF-3α being the third). The single β-subunit, HIF-1β is constitutively present in the cell while HIF-α subunits are only present during hypoxia due to normoxic protein degradation through ubiquitin-mediated proteosomal degradation [17–19]. In addition to Epo, HIFs regulate the hypoxia-induced expression of glycolytic, angiogenic, cell cycle regulatory, and survival genes [20,21].

The mechanism of normoxic HIF-1 turnover was elucidated when it was demonstrated that hypoxia inhibits the interaction of HIF-1α with the von Hippel-Lindau (VHL) tumor suppressor protein and that VHL mediates the oxygen-dependent instability of HIF-1α (Figure 2) [22, 23]. It was subsequently demonstrated that proline-directed hydroxylation of HIF-1α during normoxia targets HIF-1α for recognition by VHL and subsequent degradation [24,25]. This hydroxylation reaction is carried out by a family of 2-oxoglutarate-dependent dioxygenases termed prolyl hydroxylases 1, 2, and 3 (PHD1–3) [26,27]. In the hydroxylation reaction, PHDs catalyze the splitting of molecular oxygen, donating one atom to HIF prolines and the other to 2-oxoglutarate, forming succinate and $CO_2$. Ferrous iron is used as a cofactor in the reaction and thus, the hydroxylation reaction is inhibited by the iron chelator desferrioxamine (DFO). As the hydroxylation reaction requires oxygen, it was widely speculated that PHDs act as the direct oxygen sensors of the HIF pathway [28].

## Mitochondrial ROS are required for hypoxic activation of HIFs

The first breakthrough suggesting a mitochondrial oxygen sensor came with the discovery that $\rho^0$ Hep3B cells, which contain no mitochondrial DNA and thus no electron transport, are incapable of HIF-1 DNA binding activity and erythropoietin expression following hypoxia [29]. The observation that antioxidants abolish the hypoxic HIF response suggested that ROS generation at mitochondria is responsible for propagation of the hypoxic signal [29]. Correspondingly, treatment of cells with exogenous $H_2O_2$, treatment of cells with growth factors which induce $H_2O_2$ production, or cellular mutations which lead to $H_2O_2$ accumulation, are sufficient to stabilize HIF-1α during normoxia [30–32]. Further use of mitochondrial inhibitors suggested that ROS generation at mitochondrial complex III is critical for hypoxia signaling as complex I inhibitors (rotenone, MPTP) and $Q_o$ site inhibitors (myxothiazol, stigmatellin) abolish hypoxic HIF-1α induction, but the $Q_i$ site inhibitor antimycin A does not [29,30,33]. The use of pharmacological agents does not however prevent HIF stabilization in response to DFO, demonstrating that the HIF-1 pathway downstream of PHDs is intact. The results of these pharmalogical experiments were subsequently corroborated using shRNA or genetic targeting of mitochondrial proteins. Targeting of the Rieske iron-sulfur protein (RISP) using shRNAs prevents electron transfer from ubiquinol, inhibiting ubisemiquinone formation and hypoxic HIF-1 stabilization [34,35]. Similarly, embryonic stem cells lacking cytochrome *c* fail to stabilize HIF-1 during hypoxia as loss of cytochrome *c* leads to a complete reduction of cytochrome c1 and RISP, inhibiting transfer of electrons from ubiquinol at the $Q_o$ site [36].

As mitochondria are the primary cellular oxygen consumers, it was proposed that mitochondrial consumption of oxygen leaves the cytosol of a wild-type cell more hypoxic than that of a $\rho^0$ cell for any given extracellular oxygen tension [37]. To further test the hypothesis that mitochondrial ROS production, but not oxygen consumption is required for hypoxic HIF-stabilization, genetic approaches were taken using cytoplasmic hybrid (cybrid) cell lines which consist of $\rho^0$ cells reconstituted with either wild-type or mutant mitochondria. Cybrids harboring a deletion of the cytochrome *b* gene are respiratory deficient, yet are capable of generating ROS at the $Q_o$ site of complex III and stabilizing HIF-1α during hypoxia [38]. Targeting of RISP in these cytochrome *b*-deficient cybrids with shRNAs abolished ROS generation and hypoxic HIF induction. These experiments demonstrate that (1) it is not the ability of cells to consume oxygen or conduct oxidative phosphorylation that is important for the hypoxic signaling; (2) it is the ability of the cells to produce ROS at mitochondrial complex III which is crucial for hypoxic induction of HIF-α.

## Mitochondrial ROS are required for hypoxic calcium signaling in pulmonary artery

While HIF-induced transcription occurs over a matter of hours, and erythrocytosis occurs within days of exposure to hypoxic environments, other physiological responses to hypoxia occur within seconds of exposure. In response to hypoxia, the arterial pressure of the pulmonary artery increases, allowing for improved gas exchange by diverting blood from poorly oxygenated regions of the lung. This response is termed hypoxic pulmonary vasoconstriction (HPV). Although the pulmonary endothelium can amplify this response, isolated pulmonary arterial smooth muscle cells (PASMCs) contract during hypoxia, indicating that the HPV response is initiated in PASMCs [39]. Hypoxia triggers contraction of PASMCs through an increase in cytosolic calcium from both intracellular and extracellular stores [40]. This calcium response is critical for cellular contraction due to hypoxia; however, the exact mechanism of this calcium elevation is unresolved [41].

Similar to HIF-1α expression in many tested cell lines, $\rho^0$ PASMCs are deficient for contraction in response to hypoxia [42]. Perfusion of isolated lungs with mitochondrial inhibitors which act on or proximal to the $Q_o$ site of complex III inhibit the hypoxia-induced increase in arterial pressure while antimycin A and cyanide do not. Stimulation of cells with exogenous $H_2O_2$ was sufficient to induce PASMC contraction and an increase in arterial pressure of isolated lungs while hypoxia-induced vasoconstriction was inhibited by perfusion with antioxidants [42–44]. Furthermore, treatment of PASMCs with exogenous hydrogen peroxide is sufficient to induce calcium mobilization, while expression of catalase, which converts hydrogen peroxide to oxygen and water, inhibits hypoxia-induced cytosolic calcium accumulation and vasoconstriction [45–47]. Importantly, ρ0 PASMCs or isolated lungs treated with mitochondrial inhibitors were able to respond to the receptor-mediated vasoconstrictor U46619 [42].

ROS required for HPV have also been suggested to result from the activation of NADPH oxidase in hypoxic PASMCs [48]. Recent studies have demonstrated that mitochondrial ROS generation is required for activation of NADPH oxidase in PASMCs during hypoxia [49]. While inhibition of NADPH oxidase activity attenuated the hypoxic increase in cytosolic calcium and contraction of PASMCs, antioxidants and mitochondrial inhibitors prevented hypoxic induction of NADPH oxidase activity. Thus, hypoxia is sensed in the mitochondria of PASMCs and ROS produced at complex III and NADPH oxidase act synergistically to elevate cytosolic calcium, inducing pulmonary vasoconstriction during hypoxia (Figure 3).

## ROS and hypoxia signaling: Looking Forward

A major point of controversy regarding the role of ROS as hypoxic signaling molecules is that it is seemingly paradoxical that a decrease in a required substrate, $O_2$, would result in an increase in ROS production. Although one might expect to observe a decrease in superoxide production as $O_2$ concentrations drop, mitochondrial ROS production varies with both [$O_2$] and [electron donors] and thus an increase in the reduction state of the electron transport chain could theoretically increase ROS production during hypoxia [50]. Ample evidence for both increased and decreased levels of ROS production during hypoxia exists; however, these studies rely on oxidation-sensitive dyes which can lack specificity, accumulate within organelles, exhibit autooxidation, and have limited intracellular access. Furthermore, none of these probes exhibits ratiometric fluorescence and as such are concentration dependent, adding further difficulty to accurate ROS measurements [51]. The use of new ratiometric fluorescent protein ROS probes offers better assessment of cellular redox status in real-time and have been used to demonstrate increased ROS production during hypoxia [45,52]. Furthermore, evidence

of the accumulation of both DNA and lipid oxidation products during hypoxia suggest that cellular oxidant production increases during hypoxia [53,54].

Although the totality of the evidence suggests that increased production of mitochondrial ROS is required for both HIF-1-mediated transcription and for HPV, the direct targets of oxidation have yet to be determined. Some evidence suggests that oxidation of iron within the catalytic site of PHDs may play a role in ROS-mediated HIF-1 signaling; however, the role of post-translational modifications of PHDs has not been addressed [32]. For HPV, it has also been proposed that ROS act on ryanodine receptors, increasing their open probability, although the mechanism of this action is not determined [51]. In addition to the hypoxic responses presented above, evidence suggests that ROS may also mediate the hypoxic regulation of the Na/K ATPase and AMP-activated protein kinase, suggesting that mitochondrial ROS are a central mediator of hypoxic signaling [55,56]. The discovery of the mechanisms leading to hypoxic ROS production and the downstream effectors of ROS signaling is thus critical for understanding the cellular response to hypoxia.

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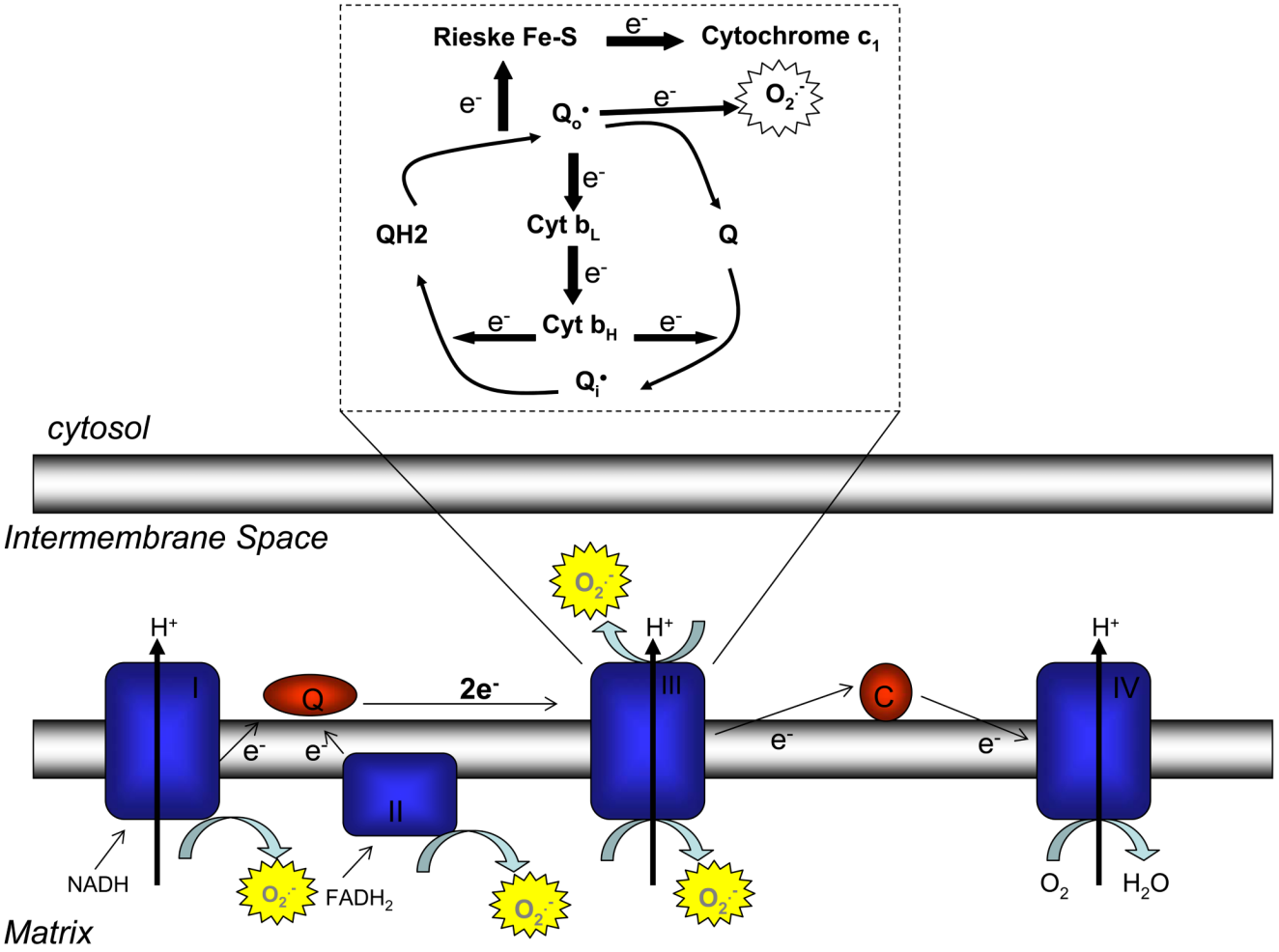


**Figure 1. Mitochondrial generation of reactive oxygen species**

Mitochondrial complexes I, II, and III produce superoxide. While complexes I and II only produce superoxide into the mitochondrial matrix, complex III can produce superoxide on both sides of the mitochondrial inner membrane in a process termed the Q-cycle. Complexes I and II donate two electrons to coenzyme Q, forming ubiquinol. At complex III, the first of these electrons is transferred by the Rieske iron-sulfur protein (RISP) to cytochrome *c*1, leaving the radical ubisemiquinone. Subsequently, ubisemiquinone transfers the second electron to cytochrome *b*. Ubisemiquinone formed at the $Q_o$ site can donate its electron directly to oxygen, producing superoxide.

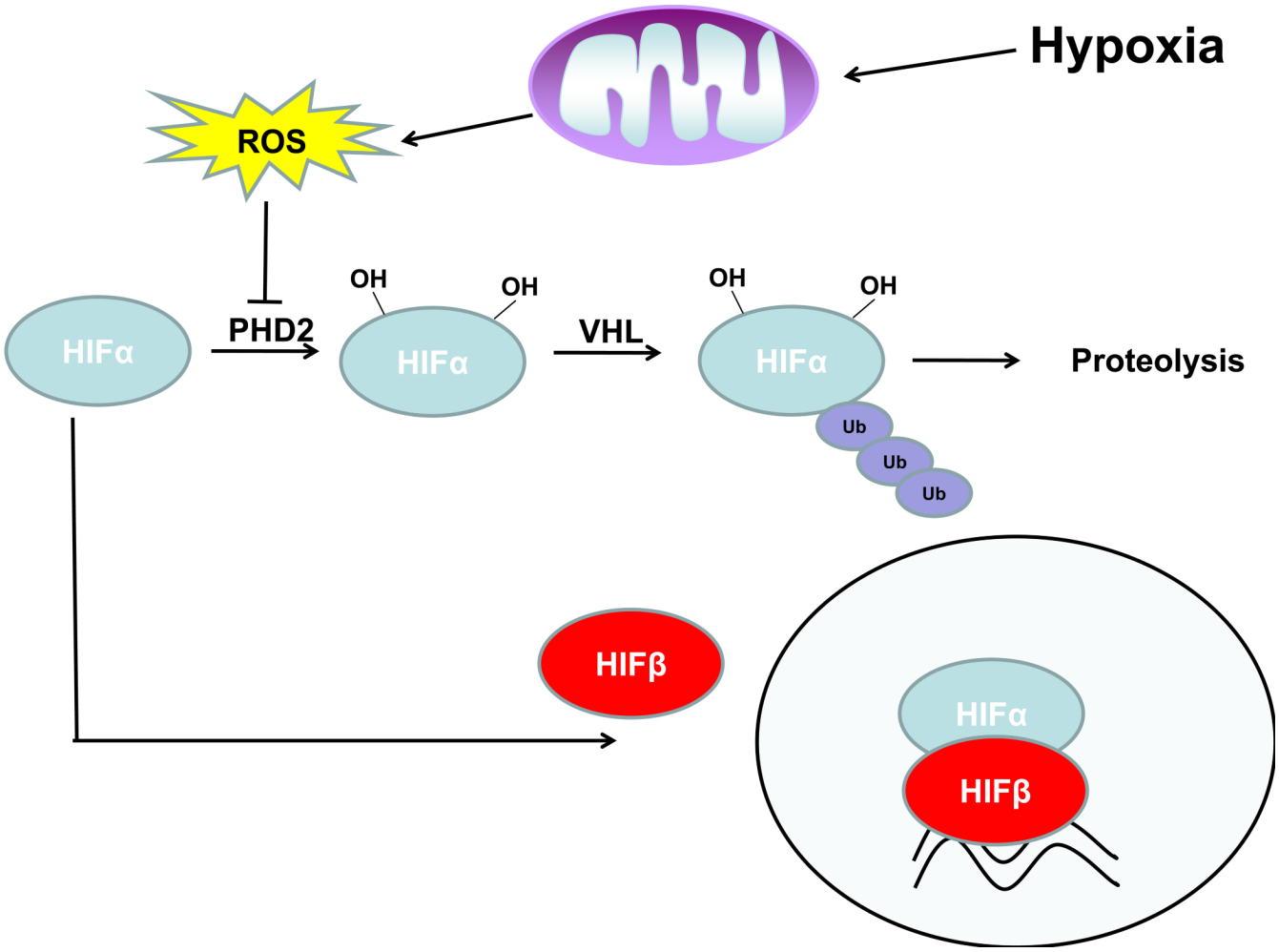


**Figure 2. Hypoxia-induced mitochondrial ROS inhibit HIFα subunit turnover**

Under normoxic conditions, HIFα subunits are hydroxylated on prolines by prolyl hydroxylases (PHDs). Hydroxylation tags HIFα for recognition by the von Hippel Lindau (VHL) tumor suppressor leading to ubiquitination and degradation of HIFα. During hypoxia, mitochondrial production of ROS inhibits the activity of PHDs allowing for stabilization of HIFα subunits and HIF-mediated transcription.

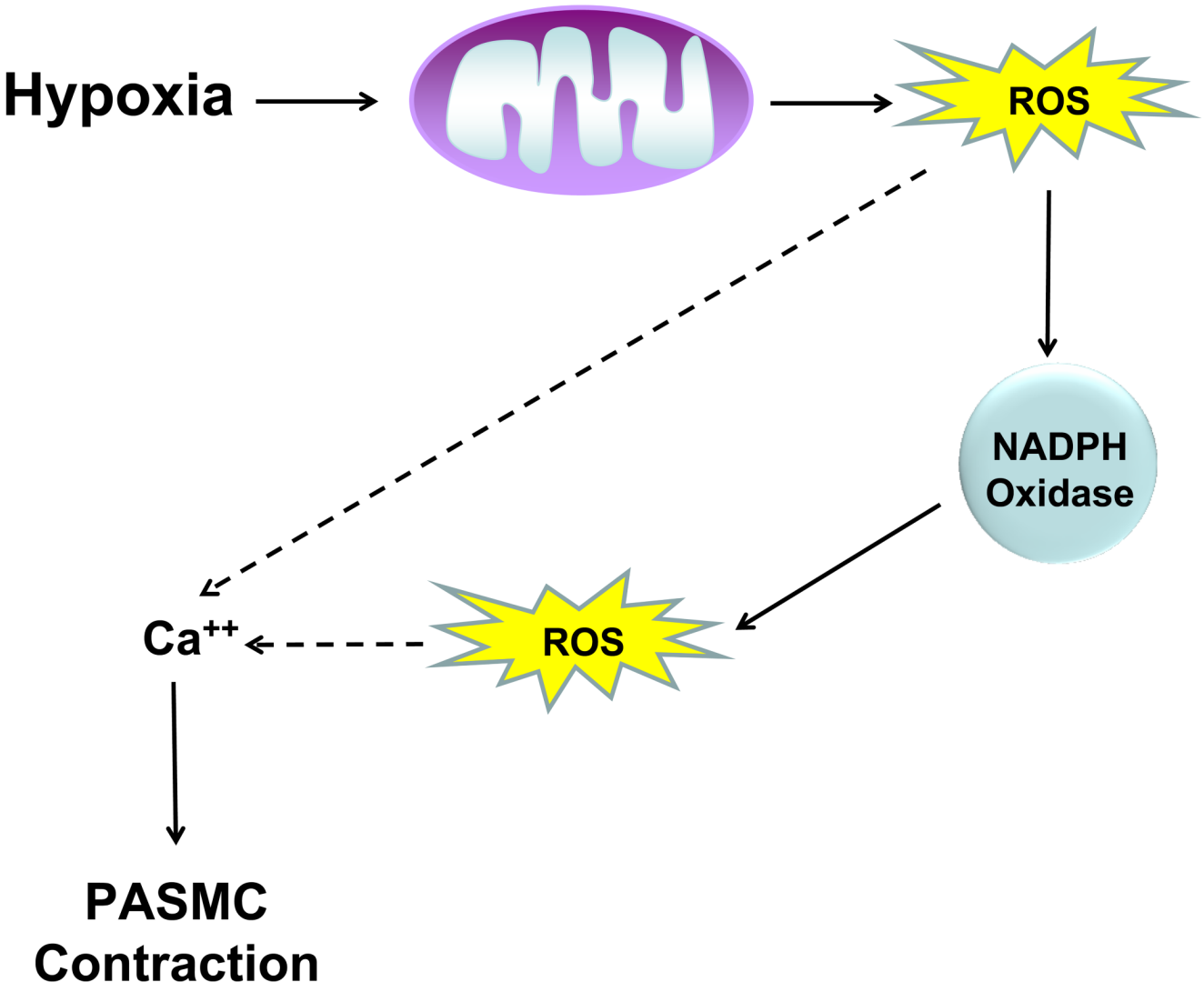


**Figure 3. Hypoxia-induced mitochondrial ROS are required for elevation of cytosolic calcium and contraction of PASMCs**

During hypoxia, mitochondrial production of ROS leads to activation of NADPH oxidases, further amplifying the ROS signal. The combined production of ROS at mitochondria and NADPH oxidase allows for elevation of cytosolic calcium and contraction of PASMCs.