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O_2 sensing, mitochondria and ROS signaling: the fog is lifting

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Abstract

Mitochondria are responsible for the majority of oxygen consumption in cells, and thus represent a conceptually appealing site for cellular oxygen sensing. Over the past 40 years a number of mechanisms to explain how mitochondria participate in oxygen sensing have been proposed. However, no consensus has been reached regarding how mitochondria could regulate transcriptional and post-translational responses to hypoxia. Nevertheless, a growing body of data continues to implicate a role for increased reactive oxygen species (ROS) signals from the electron transport chain (ETC) in triggering responses to hypoxia in diverse cell types. The present article reviews our progress understanding this field and considers recent advances that provide new insight, helping to lift the fog from this complex topic.

Introduction

Eukaryotic cells rely on the availability of nutrients and molecular oxygen (O_2) to meet their metabolic and bioenergetics needs. While single cells or small clusters of cells can sustain adequate cellular O_2 uptake from the environment through the process of diffusion alone, more complex organisms require both convective and diffusive transport mechanisms because diffusion by itself is not sufficient to assure reliable O_2 delivery. Indeed, multicellular organisms have evolved elaborate systems to assure adequate delivery of molecular oxygen and nutrients to each cell, and they regulate that delivery in accordance with local metabolic needs. Moreover, sudden changes in metabolic demand – arising from changes in activity - dictate the need for dynamic systems that respond rapidly to meet the changing needs. Strong evolutionary pressures drive the refinement of these systems, as a failure to respond to a sudden increase in metabolic need or a change in environment can have lethal consequences. Similarly, a prolonged inability to supply adequate O_2 to a respiring cell spells its doom.

Precise regulation of O_2 delivery throughout an organism requires feedback control at the molecular, cellular, tissue and organismal levels. Cells face diverse stresses that challenge oxygen delivery and utilization over different spans of time. Accordingly, organisms have

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acquired complex oxygen sensing systems through evolution, with different time constants that enable them to respond accordingly. Thus, acute decreases in oxygen supply trigger rapid responses, while chronic changes in O_2 availability engage slower mechanisms requiring transcriptional activation that need time to implement. Clearly, a response that requires cell proliferation or tissue remodeling is not likely to protect the organism from a hypoxic stress that develops over a period of seconds. On the other hand, translocation of glucose transporters to the plasma membrane to facilitate anaerobic glycolysis would not, by itself, represent an optimal response to chronic oxygen deprivation. In either case, oxygen sensors must be capable of detecting both acute and chronic changes in oxygen availability, to permit the coordinated engagement of responses with short and long time constants[1]. The present review considers the role of mitochondria as oxygen sensors, it assesses recent advances in this area, and provides a critical analysis of the state of this field with regard to several representative oxygen-sensitive tissues and cells.

Classical theories of mitochondrial O_2 sensing mechanisms

Mitochondria are responsible for the lion's share of oxygen consumption by cells, and thus they represent an appealing site for O_2 sensing. The canonical mechanism of mitochondrial O_2 sensing is based on bioenergetics and ATP supply. According to that idea, mitochondria become oxygen starved as a cell becomes hypoxic; the resulting bioenergetic deficiency then triggers an alarm and activates the response. However, from a biological perspective such a system represents a poor engineering solution. To understand why, consider that as cells consume O_2 an oxygen tension gradient develops between the plasma membrane and the mitochondria. The magnitude of this gradient has been demonstrated to be in the range of 2–4 mmHg based on optical intracellular phosphorescence quenching measurements in whole cells by independent laboratories [2–4]. The gradient increases as the rate of O_2 consumption rises [3], but the difference in O_2 tension between the extracellular space and the mitochondria remains constant as the extracellular O_2 tension varies. In other words, at a given rate of O_2 consumption, changes in the extracellular O_2 tension will produce identical changes at the mitochondria while the difference between the two sites remains constant. For example, if the extracellular O_2 tension is 20 and the gradient is 3 mmHg, mitochondrial O_2 tension will be 17 mmHg. If the extracellular PO_2 increases to 50 mmHg, the mitochondrial O_2 tension will increase to 47 and the gradient will remain at 3. Oxygen is consumed at cytochrome oxidase, the terminal complex in the electron transport chain (ETC). The apparent Km of cytochrome oxidase is less than 2 mmHg, which allows the cell to maintain a normal level of oxygen consumption until near-anoxic conditions have been reached[5]. Hence, mitochondrial ATP production will remain normal until the extracellular O_2 tension is close to zero. By this analysis an oxygen sensor that relies on hypoxia to limit mitochondrial ATP production would be excellent for detecting anoxia, but incapable of detecting physiological hypoxia. Indeed, a system that only detects hypoxia when anoxic conditions have been reached will not be useful in preventing that situation from developing.

To address the limitations described above, some investigators have suggested that specialized oxygen sensing systems, such as the glomus cells of the carotid body, express genetic variants of mitochondrial subunits that produce a low-affinity isoform of cytochrome oxidase[6]. Expression of alternative subunits that shift the Km to a higher level would cause

mitochondrial electron flux and oxygen consumption to become O_2-limited at more physiological levels of hypoxia[7, 8]. Oxidative phosphorylation would then become limited by O_2 availability in response to small decreases in PO_2, producing a signal in the form of decreased ATP concentration. The resulting bioenergetic deficiency would then be transduced by another process that triggers release of neurotransmitters to activate the ventilatory response to hypoxia. In support of that model, it was shown that the rate of fall in carotid body PO_2 - after blood flow to a perfused carotid body was halted - tended to slow as tissue oxygen tension decreased. Those results were taken as evidence that an alternative cytochrome oxidase is expressed in the carotid body[7, 9]. However, other interpretations can also explain those findings. Nevertheless, such a model would dictate that glomus cells in individuals with arterial hypoxemia must exist under continuous bioenergetic stress. If the severity of hypoxia increases, the ATP deficiency would worsen yet the energy demands of the cells for neurotransmitter synthesis, release and reuptake would increase. In any case, although interest in the “low affinity cytochrome oxidase” has persisted [10] no direct evidence of such a system has emerged.

Interestingly, the normal “high affinity” binding of O_2 to cytochrome oxidase reflects an “apparent” rather than a “real” enzymatic Km. In that regard, Wilson and colleagues demonstrated that the ability of mitochondria to sustain O_2 consumption over a wide range of O_2 tensions is achieved not by a low Km, by rather by shifts in the redox status of cytochrome c[11, 12]. Thus at high PO_2, cytochrome c reduction state is low (highly oxidized) and O_2 consumption is normal. As the PO_2 decreases, cytochrome c reduction state increases, allowing O_2 consumption to be preserved even though the abundance of O_2 is decreased. Further increases in cytochrome c reduction continue to develop as the O_2 tension decreases further, allowing O_2 consumption to be sustained until O_2 availability becomes critically limiting. This occurs at PO_2 levels in the range of 1–3 mmHg (at which point the cytochrome c pool is fully reduced)[13–15]. Three corollaries arise from this mechanism. First, there is no need to propose the existence of a low affinity cytochrome, as decreases in PO_2 over a wide range (100–3 mmHg) are transduced into changes in mitochondrial ETC reduction state in diverse cell types. Second, there is no need to postulate the existence of a persistent bioenergetic crisis in energy supply, as changes in ETC reduction state provide a signal indicative of PO_2. Hence, rather than affecting ATP generation, this mechanism transduces changes in PO_2 into changes in redox status of the ETC, which could then be used by the cell to activate hypoxia responses.

Mitochondrial ROS as signaling messengers in hypoxia

If decreases in ATP are not responsible for initiating responses to hypoxia, what signals are? One possibility is that mitochondrial reactive oxygen species (ROS) act in triggering the responses to hypoxia. ROS generation by mitochondria was first identified about 50 years ago[14, 15]. These reactive molecules have long been viewed as damage-inducing agents associated with cell death under pathological conditions such as ischemia-reperfusion injury, UV irradiation, or exposure to hyperoxic conditions[16, 17]. However, under physiological conditions ROS play important roles in regulating a wide range of cellular responses, including proliferation, differentiation, aging, transcription factor regulation, inflammation, and other regulatory functions[18, 19].

The idea that ROS could act as signal transduction messengers in the oxygen sensing response is conceptually attractive. Increases in oxidant generation during hyperoxic ventilation are known to injure the lung through increased ROS generation[20–22], while decreases in the generation of extracellular ROS have been reported to occur during hypoxia[23]. Indeed, studies that began in the 1980's attempted to link the oxygen-sensitive reactivity of pulmonary artery and ductus arteriosus vascular smooth muscle cells to changes in oxygen radical formation[24]. Low oxygen levels *in vivo* are known to cause constriction of pulmonary artery smooth muscle cells (PASMC). High oxygen levels cause constriction of smooth muscle cells in the ductus arteriosus (DA), but cause constriction in systemic artery smooth muscle cells (SASMC). According to the original ROS model developed by Weir, Archer and colleagues, hypoxia should decrease ROS generation in PASMC and in DA[23, 25, 26]. This attenuation in ROS would then cause a chemically reductive modification of membrane voltage-dependent potassium (Kv) channels. In the case of PASMC, decreases in ROS cause the Kv channels to close, resulting in a decrease in membrane potential that triggers the opening of L-type Ca^{2+} channels, entry of extracellular Ca^{2+}, and contraction[27]. In DA cells, increases in oxygen tension would increase ROS generation causing Kv channels to close, thereby leading to membrane depolarization and the associated opening of membrane L-type Ca^{2+} channels[28, 29]. Thus, according to that model the opposite effects of ROS on PASMC and DA constriction would arise from differences in their Kv channels, which close in response to low ROS in PASMC and close in response to high ROS in the DA[30, 31]. Unlike the pulmonary circulation, systemic vascular beds dilate in response to hypoxia. If systemic arterial smooth muscle cells (SASMC) behaved like DA cells, then tissue hypoxia would decrease ROS, causing relaxation via Kv channel opening. However, Michelakis, Archer and coworkers instead proposed that mitochondrial ROS generation occurs in opposite directions in SASMC and PASMC[32]. Thus, hypoxia would augment mitochondrial ROS generation in SASMC, causing Kv channels to open. By contrast, high systemic tissue PO_2 would cause a decrease in mitochondrial ROS generation, which would close Kv channels and cause constriction[33, 34]. Hence, Kv channel sensitivity to ROS would be similar in PASMC and SASMC but opposite to that in DA, whereas mitochondria in PASMC and DA decrease ROS in response to hypoxia whereas those in SASMC increase ROS generation. This complex set of proposed mechanisms has been the topic of previous reviews[33, 35–38], and is summarized in Table 1.

In contrast with the above studies, much recent work reveals that mitochondria in diverse cell types increase, rather than decrease, ROS release to the cytosol during hypoxia[39–45]. This conclusion is based on work from multiple laboratories using a variety of methods for assessing intracellular ROS signaling[44–50]. Why should such a disagreement arise? The most likely explanation is the use of different methods to assess ROS generation. The studies by Archer and coworkers relied heavily on lucigenin – a probe that becomes chemiluminescent upon interaction with superoxide anion[51, 52]. Lucigenin is largely confined to the extracellular space, so their observed decreases in chemiluminescence during hypoxia suggest that extracellular release or generation of superoxide decreases under low oxygen conditions. By contrast, intracellular generation of H_2O_2 increases during hypoxia, and chemical or genetic sensors of intracellular H_2O_2 or superoxide signaling reveal small but significant and reversible increases during hypoxia, as described below[43, 53–55]. So it

seems reasonable to conclude that at least some of the controversy regarding whether ROS generation increases or decreases during hypoxia arises because detection probes or measurement systems located in the extracellular space may be insensitive to reactive oxygen molecules generated within the cell. Indeed, studies reveal that ROS generation within cells is regulated differently among subcellular compartments, such that measurements in one compartment can differ markedly from another. As discussed below, this spatial diversity essentially invalidates the idea that "redox status" for an entire cell can be distilled down to a single number.

Redox chemistry and ROS signaling

ROS generation occurs in mitochondria when a single electron is transferred to molecular oxygen from a site along the electron transport chain or from the tricarboxylic acid (TCA) cycle. The result is superoxide, a radical that can be converted rapidly to hydrogen peroxide (H_2O_2) by the action of superoxide dismutase [56, 57]. Superoxide is a moderately reactive molecule that is highly effective in disrupting iron-sulfur clusters associated with certain proteins. For example, superoxide can inactivate aconitase, an iron-sulfur protein that converts citrate to isocitrate. Superoxide can also react with iron in heme groups, as seen in its ability to donate its extra electron to cytochrome c. H_2O_2 interacts with a different class of cellular targets than superoxide and is perhaps more important as a signaling molecule because it interacts with proteins by oxidizing the thiol groups on cysteine or methionine residues[58]. The oxidation of cysteines can lead to the generation of inter-molecular or intra-molecular dithiol linkages, which can dramatically alter protein structure and function. H_2O_2 levels are kept in check by scavenging peroxidases including glutathione peroxidase, peroxiredoxins, and to some extent glutathione itself. Each of these is expressed or regulated in distinct subcellular compartments, allowing independent regulation of redox signaling in each domain. In each case, these systems depend on NADPH for their reducing ability. Catalase also degrades H_2O_2, but does not require NADPH. Expression of catalase is generally restricted to peroxisomes, its Km for H_2O_2 is relatively high, and its expression level is low in most cells. The oxidation of protein thiol groups by H_2O_2 can be reversed by the action of thioredoxins or glutaredoxins, small proteins that seek out oxidized protein thiols and reduce them, using reducing equivalents ultimately derived from NADPH[18]. Although mitochondrial ROS have the potential to induce cellular damage, for example in states such as ischemia-reperfusion injury [59–64] or other disorders [22, 65], much work indicates that mitochondrial oxidants play important physiological roles in the regulation of a variety of biological processes including the process of O2 sensing[6667, 68].

Mitochondria can generate superoxide at complex I [69–72], II [73] or III [74–80], through the escape of electrons from iron-sulfur groups, flavin-containing proteins, or from the free radical ubisemiquinone in the Q cycle of complex III (Figure 1). When generated at a site located on the matrix side of the inner mitochondrial membrane, superoxide is released into the aqueous matrix environment. However, when generated within the inner membrane itself, superoxide is ejected from the membrane into the intermembrane space. This occurs because an electrical field exists within the inner membrane – a consequence of the large transmembrane electrical gradient (−180 mV) and the narrow dimensions of the membrane (~7 nm). The electrical field occurs in a direction that tends to move anions (such as

superoxide) toward the intermembrane space, and its extraordinary strength (256,000 volts/cm) provides a powerful incentive driving them out into the intermembrane space[18].

Pharmacological inhibitors acting at specific sites along the ETC can significantly alter ROS generation[81, 82]. By blocking electron transfer at a particular site, an inhibitor can increase ROS generation from loci proximal to the blockade, but decrease production distal to the site. For example, rotenone, an inhibitor that acts at the downstream end of complex I, increases superoxide production from complex I when NADH is used as a substrate, but decreases ROS generation from complex III by suppressing electron transfer into that complex[69]. Mitochondrial membrane potential is another important modulator of mitochondrial ROS generation. In cells where oxidative phosphorylation is operating briskly, mitochondrial membrane potential is slightly decreased as a consequence of ATP synthase activity. In that case electron flux along the ETC is increased, which tends to limit the window of time that electrons reside at sites where superoxide could be generated. By contrast, when membrane potential is high the electron flux slows because the free energy necessary for proton extrusion begins to limit electron transfer. Consequently, residence time on flavin moieties and Fe-S groups increases, amplifying the likelihood that electrons will escape to O_2. In isolated mitochondria fed succinate (to supply electrons to complex II), high membrane potential can cause complex I function to reverse as electrons from complex II enter in a retrograde direction. This causes ROS generation at a high rate from complex I, an effect that can be abolished by adding rotenone to inhibit electron flux at the distal end of the complex [70].

In the ETC, electrons from complexes I and II are transferred to complex III via ubiquinone, a membrane-associated electron carrier. Two electrons are transferred to ubiquinone at quinone binding sites on complex I and complex II, generating ubiquinol that migrates to a quinol binding site (Qo) at Complex III, where the two electrons are removed sequentially. The first electron is passed to the Rieske iron-sulfur protein (RISP) subunit in Complex III, which subsequently passes the electron to cytochrome c_1, to cytochrome c, and finally to cytochrome oxidase. Removal of the first electron from ubiquinol by RISP generates the transient free radical, ubisemiquinone, at the Qo site. Normally, the second electron is rapidly removed by the b cytochromes, thereby returning ubiquinone to the membrane pool. However, if the removal of the unpaired electron from ubisemiquinone is delayed, superoxide may be generated if the electron is instead captured by molecular O_2. Thus, the lifetime of the ubisemiquinone at complex III represents a potential mechanism for controlling ROS generation at the Qo site. Interventions that prolong the lifetime of ubisemiquinone lead to a marked increase in superoxide formation at the Qo site. The toxin antimycin A for example prevents the removal of the second electron, thereby causing a large increase in superoxide generation at the Qo site [74].

Superoxide requires a membrane anion channel to cross lipid bilayers, whereas H_2O_2 can travel through aquaporins. Superoxide can react with iron-sulfur groups, heme moieties, and transition metals associated with proteins, but does not directly oxidize cysteine thiol groups [58]. H_2O_2, by contrast, can attack nucleophilic cysteine residues, making it a powerful and reversible modifier of protein structure and function.

While this review focuses on the role of mitochondrial ROS signals in hypoxia, it is important to remember that other oxidant generating systems, such as the NADPH oxidases (Nox systems) may also participate. For example, in the response to hypoxia in the pulmonary vasculature it has been shown that Nox4 is an important contributor, possibly acting as an amplifying ROS generator [83, 84]. Other hypoxia responses may also engage Nox systems [85].

Hypoxia-induced increases in mitochondrial ROS generation

While previous reports had suggested that hypoxia-induced decreases in ROS production were involved in O_2 sensing [52, 86], Marshall et al. suggested the opposite: that hypoxia instead induces increases in ROS production [87]. Their work using pulmonary artery cell homogenates suggested that superoxide generation increased during hypoxia – an effect that was inhibited by diphenylene iodonium, a flavoptrotein inhibitor of NADPH oxidase but not by the mitochondrial inhibitor myxothiazol, which blocks electron entry into complex III. They concluded that an NADPH oxidase system was responsible, although later studies using mice with genetic deficiency of gp91 (NOX2) effectively contradicted that interpretation[88].

The first demonstration that mitochondrial ROS signals control gene transcription in hypoxia came from Chandel et al., who showed that mitochondrial electron transport function is required for stabilization of the HIF-1α transcription factor subunit, through the generation of ROS during hypoxia[41, 67]. These provocative studies were carried out in hepatoma and osteosarcoma cells and utilized mitochondrial inhibitors, ρ^0 cells lacking a functional ETC, and ROS-sensitive fluorescent chemical probes. Those methods led others to question whether the counter-intuitive increases in ROS generation during hypoxia might represent artifacts[89]. To address those concerns, subsequent studies were carried out using genetically encoded ratiometric redox sensors to assess ROS signaling and genetic techniques to disrupt the mitochondrial electron transport chain. Importantly, other laboratories used complementary methods to test and confirm this hypothesis. These later studies were carried out in primary cells from diverse tissue types as well as in transformed cell lines, with remarkable similarity in responses across cell types[55, 90].

Genetic studies implicating complex III in the response to hypoxia were reported by multiple groups, who assessed the role of ROS production from the ETC. Guzy et al. detected increases in cytosolic oxidation during acute hypoxia, using a FRET-based redox sensor, HSP-FRET, and observed a loss of this response in RISP-deficient cells [44]. Similarly, Mansfield et al. measured ROS production in embryonic cells from mice with a genetic deletion of cytochrome c[45]. When cytochrome c is absent, complex III remains fully reduced, which blocks Q cycle operation and prevents ROS generation at the Qo site. As with the RISP-deficient cells, the cytochrome c-null cells lost the ability to generate an oxidant signal in the cytosol during hypoxia, and they also failed to stabilize HIF-1α. However, the same cells retained the ability to stabilize HIF-α in response to prolyl hydroxylase inhibition by DMOG, indicating that the impaired response was specific to the detection of hypoxia. These findings implicate electron flux through complex III as a critical event in the detection of hypoxia in cells.

Evidence for hypoxia-induced increases in ROS generation in the pulmonary circulation came from studies by Waypa et al., showing that hypoxic pulmonary vasoconstriction required electron flux through complex III, and that increases in ROS generation were responsible for eliciting the hypoxic response[49, 55]. Other studies have led to similar conclusions[91–94]. If mitochondria release ROS to the cytosol, an oxidant signal in the mitochondrial intermembrane space should accompany the cytosolic oxidant response. Using the thiol redox sensor roGFP targeted to the cytosol, the intermembrane space or the mitochondrial matrix, Waypa et al. compared the basal and hypoxia-induced changes in pulmonary artery smooth muscle cells (PASMC)[49]. The roGFP sensors are mutants of green fluorescent protein (GFP) that contain adjacent cysteine residues on the outer surface [95]. Oxidation in response to H_2O_2 is mediated by glutaredoxin; the resulting dithiol formation alters the fluorescence properties of the protein and allows it to function as a thiol redox sensor[96]. Oxidation of the sensor is reversible, allowing it to be calibrated so as to yield an absolute measure of the percent oxidation[96, 97]. This property allows comparison of different subcellular regions or cell types.

Using PASMCs under normoxic conditions, Waypa et al. observed that cytosolic roGFP was ~20% oxidized, in the intermembrane space it was ~45% oxidized, and in the matrix compartment it was ~70% oxidized. During acute hypoxia, oxidation increased in the cytosol (to ~35%) as well as in the intermembrane space (to ~65%). However, roGFP oxidation decreased in the matrix during hypoxia. Waypa et al. observed similar results in isolated systemic arterial smooth muscle cells[49]. These findings reveal that hypoxia elicits an oxidative signaling response in the intermembrane space and the cytosol, consistent with the release of superoxide from the inner mitochondrial membrane. Those results also reveal that important differences in redox status can exist among subcellular compartments, with some exhibiting increases and others exhibiting decreases in oxidation during hypoxia. Finally, ROS scavenging in cultured PASMCs during hypoxia abolished the increase in Ca^{2+} induced by hypoxia in the cytosol, indicating that ROS signals are required for the signal transduction linking hypoxia to the contractile response in these cells [43, 53, 98].

In a subsequent study, Waypa et al. extended these findings by studying PASMC and lungs from adult mice with smooth muscle-specific deletion of the RISP gene[55]. In PASMC where RISP was deleted using adenoviral Cre recombinase administration, they observed a loss of hypoxia-induced changes in thiol redox in the cytosol and intermembrane space, again implicating complex III in the response to hypoxia. Using tamoxifen to activate Cre recombinase in smooth muscle cells *in vivo*, they found that the pulmonary vasoconstrictor response to acute hypoxia was lost, as assessed by measurements of right ventricular end-systolic pressure. These findings implicate mitochondrial complex III and ROS signaling in the oxygen sensing response underlying the acute hypoxic pulmonary vasoconstrictor response.

Phylogenetic evidence suggests that post-translational hydroxylation of proteins existed in prokaryotes prior to the evolution of mitochondria. These enzymes are related to modern-day eukaryotic HIF and collagen prolyl hydroxylases, although there is no evidence that the primordial enzymes were involved in hypoxia sensing [99]. It is conceivable that the oxygen sensitivity of today's PHD enzymes represents an evolution of these enzyme systems,

arising from mutations that conferred hypoxic responsiveness to some (e.g. HIF PHDs) but not others (e.g. collagen prolyl hydroxylases).

As multiple antioxidant enzymes in cells function to scavenge and degrade ROS in different subcellular compartments, it is fair to ask why these systems allow oxidant signaling to develop or persist. In addressing this it is useful to note that multiple examples of oxidant signaling have been identified in diverse cell types [100, 101]. Antioxidant systems help to regulate ROS signaling by limiting the extent of protein oxidation both spatially and temporally [102]. In other cases, antioxidant enzymes facilitate ROS signaling by acting as redox relays that transmit oxidant signals from H_2O_2 to selective protein targets [103]. For example, upon oxidation by H_2O_2, the antioxidant enzyme peroxiredoxin-2 oxidizes cysteine residues in the transcription factor STAT3 leading to its activation. A similar example is seen in *S. cerevisiae*, where a glutathione peroxidase (Gpx3) functions as a redox relay that transduces H_2O_2 signals to oxidatively activate the transcription factor Yap1 [104, 105]. Such redox relay systems serve to increase the sensitivity to H_2O_2 signals while conferring greater selectivity toward the intended protein target. Hence, rather than preventing ROS signaling, antioxidant systems both refine and enhance in redox signaling in diverse biological systems.

Further evidence for complex III-derived ROS in O_2 sensing

Studies evaluating the role of mitochondrial ROS generation in cellular responses to hypoxia are hindered by the linkage between mitochondrial electron transport and ROS production, oxidative phosphorylation and NAD(H) redox status in the cytosol and mitochondria. In that regard, studies using mitochondrial inhibitors or genetic deletion of components of the electron transport chain may alter ROS generation, but also affect energy production and biochemical processes coupled to NAD^+ or $NADP^+$. How can the contribution of these independent effects be mechanistically parsed? One possibility is to identify small molecule inhibitors that interact with mitochondrial proteins in a manner that inhibits ROS generation while preserving normal electron transport and energy production. Such an approach was first described by Jung et al., who screened a library of naturally occurring small molecules in search of compounds capable of suppressing the phenotypic response of endothelial cells to hypoxia[106]. They identified terpestacin, a small molecule that inhibited the tubular reorganization of cultured endothelial cells during hypoxia and suppressed angiogenesis in subcutaneous tumor xenografts in mice. Using phage display analysis to identify the cellular target of terpestacin binding, that group identified Uqcrb – a small molecular weight subunit of complex III – as the site of action. Subsequent studies revealed that terpestacin suppresses angiogenesis by inhibiting the generation of ROS by complex III during hypoxia. The loss of the ROS signal in turn limits the hypoxia-induced stabilization of HIF-1α, a transcriptional driver of VEGF expression and angiogenesis[107]. At concentrations that suppress ROS generation, terpestacin did not inhibit cellular respiration or ATP generation. This finding suggests that the loss of O_2 sensing in cells or tissues after deletion of RISP or Ndufs2, or after treatment with mitochondrial inhibitors that block electron entry into complex III, are not the result of ATP deficiency or NADH redox changes. The effects of terpestacin were recapitulated by synthetic small molecules that suppress angiogenesis and ROS generation by binding to Uqcrb and inhibiting hypoxia-induced ROS generation [106]. In

humans, hereditary defects in Uqcrb resulting in early childhood hypoglycemia and lactic acidosis have been reported [108]. Cell lines overexpressing the mutant Uqcrb exhibited increased rates of proliferation, evidence of increased mitochondrial ROS generation, and increased pro-angiogenic activities[109]. Thus, the mutant version of Uqcrb appeared to confer a gain-of-function with respect to ROS generation by the electron transport chain. Treatment of these cells with terpestacin inhibited these effects, indicating that they arose from alterations in complex III function and ROS generation[109].

Collectively these studies suggest that the generation of ROS at complex III is regulated in part by Uqcrb. Exactly how this occurs is not clear, but it is possible that alterations in the structure of this subunit – induced by mutations or by interaction with small molecules like terpestacin – change the conformation of the overall complex in a manner that affects the escape of electrons and the formation of superoxide. Further work detailing the structure of the complex in the presence and the absence of terpestacin may shed light on precisely how alterations in the Uqcrb subunit produce this change.

Studies of terpestacin suggest that complex III controls cellular O_2 sensing responses by generating ROS signals during hypoxia, and rule out the possible contributions caused by altered mitochondrial ATP generation. However, terpestacin treatment does appear to alter mitochondrial membrane potential ($\Delta\Psi$ m)[106]. As $\Delta\Psi$m is critical for the generation of ROS as well as for transport of proteins, ions and small molecules across the inner membrane, it is important to understand if and how that effect could be altering the O_2 sensing response. Important new insight into this question comes from recent studies by Orr et al., who used high throughput chemical screening to identify compounds capable of selectively limiting superoxide production by complex III [110]. The compounds identified in the initial screen were then subjected to further screening to eliminate those that were unselective for the Qo site of complex III, or that produced any impairment of bioenergetic function or $\Delta\Psi$m. This analysis yielded three compounds that selectively limited superoxide and H_2O_2 production at the Qo site of complex III without affecting normal electron flux or bioenergetics capacity. Those investigators then assessed the ability of the three compounds to inhibit the stabilization of HIF-1α in hypoxia. Using HEK-293 cells, they found that the inhibitors significantly but incompletely attenuated HIF-1α induction[110]. The site(s) where these compounds bind is not yet clear and the mechanism by which superoxide generation is inhibited is not known. While it is possible they interact with Uqcrb in a way that mimics the effect of terpestacin on ROS generation, it is also possible that they act at completely different sites. In either case, further studies analyzing the structure of the purified complex in the presence and absence of these interacting molecules is likely to provide important insight into how they alter ROS generation. As pointed out by the authors, it seems likely that the binding of these compounds to complex III leads to a structural shift that limits the ability of ubisemiquinone to transfer its electron to O_2. One way to achieve this would be to shorten the lifetime of the semiquinone at the Qo pocket, by increasing the rate at which the semiquinone's electron is transferred to the b cytochromes. Such an effect would not alter flux of electrons through the complex, but would lessen the probability of electron escape to O_2.

Carotid body O_2 sensors and the role of mitochondria ROS

Although multiple models have been proposed to explain the mechanism of O_2 sensing in the carotid body, no consensus has been reached regarding the underlying mechanisms. The small tissue mass comprising the carotid body has long precluded biochemical analysis, while the diverse mix of cells has complicated the characterization of the transcriptome in the glomus cells. Nevertheless, emerging data points to the role of mitochondria in the O2 sensitivity of those cells, through a mechanism influenced by ROS signaling. Complex I is comprised of approximately 44 subunits, and crystallization studies of a simpler version of this complex in *Thermus thermophiles* reveal a structure containing 64 transmembrane helices and 9 iron-sulfur clusters [111]. One portion of the "L-shaped" complex resides within the inner membrane, while the other region containing the quinone binding site in proximity to the N2 Fe-S cluster extends out into the matrix compartment. Structural characteristics suggest that redox-driven conformational changes involved in electron transfer drive the translocation of 4 protons across the membrane with each cycle. Mutations in genes encoding complex I subunits have been linked to a number of neurodegenerative and cardiomyopathic diseases [112, 113]. Some genetic mutations partially disrupt the function of complex I while essentially preserving its assembly, whereas other deletions prevent assembly of intact complex I. When the structure and function of complex I are disrupted, NADH oxidation and proper electron transfer/proton extrusion may become impaired, thereby inhibiting mitochondrial oxidative phosphorylation. Other genetic alterations of ETC subunits can expose FMN or Fe-S groups, increasing the generation of ROS while preserving the ability to oxidize NADH. Changes in ROS generation by the complex can potentially create oxidant stress, and may thereby disrupt oxidant-mediated signaling in the organelle or the cell.

In a recent study, Fernandez-Aguera and coworkers used conditional deletion of the Ndufs2 subunit of complex I to study the mechanisms of O_2 transduction in catecholaminergic cells, including those of the carotid body[114]. In adult mice, expression of Cre recombinase under the control of the tyrosine hydroxylase promoter led to the cell-specific deletion of Ndufs2, a nuclear-encoded gene. Loss of Ndufs2 abolished the systemic hypoxic ventilatory response without disrupting the ventilatory response to hypercapnia, indicating that complex I function was required for hypoxic sensitivity. Despite loss of ETC function, the morphology of the glomus cells was preserved, as was cellular ATP concentration. This was surprising, because glomus cells have long been considered to have high oxygen consumption rates and to be dependent on oxidative phosphorylation. In cells lacking Ndufs2 the activity of complex I was essentially abolished, indicating that these cells can survive normally on glycolytic ATP generation alone [114]. The authors did show that mitochondria isolated from Ndufs2 null cells can respire normally on succinate, and that succinate levels were increased in the intact cells. However, it is unlikely that intact cells can sustain significant mitochondrial respiration in the absence of complex I function, as the generation of succinate via oxidation of 2-oxoglutarate requires a supply of NAD^+, which cannot be regenerated from NADH in the absence of complex I activity.

Electrophysiological measurements in the Ndufs2-deficient glomus cells revealed that secretory responses to high K+ or zero glucose were normal, whereas the response to

hypoxia was absent. Likewise, increases in cytosolic Ca^{2+} in response to hypoxia were essentially abolished while the responses to K+ or zero glucose were preserved[114]. Perforated patch clamp studies suggested that hypoxia decreases voltage-dependent and background K+ currents in normal glomus cells, and that this response is abrogated in the Ndufs2-deficient cells. To explore the mechanism underlying this effect, they added low concentrations of N-acetyl cysteine (NAC), a thiol reductant, to the recording pipette. In the Ndufs2-deficient cells, this restored the membrane resistance to levels seen in wild type cells, although curiously it did not restore hypoxic sensitivity. By contrast, dialysis of wild type cells with H_2O_2 at low micromolar concentrations produced an increase in input resistance that mimicked the hypoxic response. That finding suggests that hypoxia triggers an increase in oxidative stress that mediates the closure of K^+ channels, leading to Ca^{2+} influx and secretion of neurotransmitters. Based on the high input resistance in the knockout cells during normoxia – a condition reversed by NAC - the data suggested that disruption of complex I function caused by loss of Ndufs2 leads to a constitutive increases in ROS generation that mimics the hypoxic response in normal cells. Collectively these findings implicate ROS generation from mitochondria in triggering the inhibition of membrane K^+ channels of glomus cells during hypoxia.

These interesting findings nevertheless raise new questions. Is complex I normally the source of ROS generation during hypoxia in glomus cells? In the study by Fernandez-Aguera et al. the loss of Ndufs2 blocked complex I activity and appeared to cause an increase in basal normoxic ROS generation that abolished responses to hypoxia. But loss of complex I function also abolishes electron flux into complex III, which in turn will prevent ROS generation from that site. Rotenone, a drug that inhibits the transfer of electrons at the distal end of complex I, has been shown to effectively block hypoxic sensitivity in the carotid body [115]. Therefore, additional studies are needed to establish whether the normal source of ROS generation during hypoxia is complex I or complex III. A second question relates to the biophysics of ROS signaling in response to hypoxia. By virtue of its localization in the mitochondrial inner membrane, superoxide generated at complex III is ejected into the intermembrane space, where dismutation into H_2O_2 would allow it to reach the cytosol to participate in signaling. By contrast, one arm of complex I containing FMN and Fe-S groups extends from the membrane into the mitochondrial matrix. If superoxide is generated at those site(s) during hypoxia, it would be released into the matrix compartment instead. While it is possible that ROS generated in the matrix can leak to the cytosol, the rapid response to hypoxia elicited in the carotid body would suggest that the kinetics of the signaling system must be rapid and concise. Future studies utilizing complementary knockout models that produce different phenotypes in modifying the electron transport chain are likely to help in resolving these issues.

While the above studies provide support for the role of mitochondrial ROS in carotid body hypoxic chemosensitivity, a recent study of the olfactory receptor, Olfr78, challenges that model[116]. In that study, Chang et al. demonstrated that glomus cells express this olfactory receptor, and that homozygous deletion in knockout mice produced a selective loss of the ventilatory response to 10% O_2 without affecting the response to 5% CO_2. While measuring carotid sinus nerve activity, they found no alteration in normoxic spike frequency whereas the increased firing rate during hypoxia was abrogated in the knockouts compared to control

mice. The Olfr78 receptor is sensitive is known to respond to acetate and propionate, but their studies revealed that it also responds to lactate in a dose-dependent manner, and at physiological concentrations. Collectively these findings suggest that hypoxia triggers an increase in ventilation by causing an increase in lactate signaling through the Olfr78 receptor in glomus cells. This model involves mitochondria, which normally metabolize pyruvate through the electron transport chain. As proposed, during hypoxia oxygen availability at cytochrome oxidase would begin to limit cytochrome oxidase activity, shifting the flux of pyruvate toward lactate dehydrogenase and the formation of lactic acid. *In vivo*, the glomus cells would presumably respond to endogenous lactate generated in the carotid body, as well as to lactate produced elsewhere in the body and transported to the glomus cells in the circulation. While intriguing, these provocative findings nevertheless raise important questions. First, why would mitochondrial oxidative phosphorylation become O_2-limited at relatively mild levels of arterial hypoxiemia, given that lung ventilation begins to increase below an arterial PO_2 of ~60 mmHg? Second, the model [116] would lead one to predict that loss of Ndufs2 – reported by Fernandez-Aguera et al. [114]–should have caused a maximal increase in lung ventilation and carotid sinus nerve activity because the loss of complex I activity would redirect the entire pyruvate flux toward lactate. However, that response was not observed in their study. Finally, studies of isolated superfused carotid body preparations continue to show hypoxic responsiveness despite experimental conditions that should limit the ability to accumulate lactate. In any case, the findings of Chang et al. will need to be reconciled with the results of Fernandez-Aguera et al. through future studies.

Mitochondrial O_2 sensing and regulation of Hypoxia-Inducible Factors (HIF)

Gene expression in hypoxia is regulated primarily by Hypoxia-Inducible Factors, HIF-1 and HIF-2. HIFs can potentially regulate many hundreds of genes, with important roles in normal physiology as well as in diseases such as cancer. HIF-1 induces expression of enzymes involved in glycolysis, glucose uptake, vascular mitogens, and genes involved in regulation of vascular tone, and metabolism. HIF-1 is required during embryonic development, and homozygous genetic deletion causes embryonic lethality [117–119].

HIF-1 and HIF-2 are critical for regulation of oxygen homeostasis at all stages of life, in both health and disease.[120–122] HIF functions as a heterodimer comprised of α and β subunits [123]. Under normoxic conditions HIF activity is low, but it becomes increasingly activated as oxygen levels drop below 5% O_2 [124]. The regulation of HIF in accordance with oxygenation arises from the O_2-dependent degradation of the alpha subunit. Both the α and β subunits are continuously transcribed and translated, but under normoxic conditions the α subunit is rapidly degraded. During hypoxia, degradation of the β is progressively inhibited, allowing the protein to accumulate, dimerize, and activate transcription. Proteasomal degradation of the α subunit is regulated by post-translational modification of a region of the protein known as the *oxygen-dependent degradation domain* (ODD) [125]. Degradation is initiated by hydroxylation at proline residues in the ODD by 2-oxoglutarate-dependent hydroxylases[126, 127]. These HIF prolyl hydroxylases (PHDs) incorporate a free Fe^{2+} atom, and require 2-oxoglutarate and O_2 as substrates. HIF prolyl hydroxylases are related to but distinct from the 2-oxoglutarate-dependent hydroxylases involved in collagen processing or demethylation. Although three HIF prolyl hydroxylases have been implicated in HIF

regulation, PHD2 is the only one involved in the O_2-dependent regulation of the α subunit [128]. Genetic loss of PHD2 in mice disrupts the O_2-dependent regulation of HIF-α and causes embryonic lethality [129]. Hydroxylation of HIF-α enables its interaction with von Hippel Lindau protein (VHL). VHL functions as the E3 ubiquitin ligase that targets the protein for proteasomal degradation [130, 130, 131]. Loss of VHL is leads to unregulated activation of HIF [132].

If ROS are released from the mitochondria to regulate HIF, they must cross the intermembrane space to reach the cytosol and the nucleus. To test that idea, Sabharwal et al. expressed peroxiredoxin-5 (Prdx5), a scavenger of H_2O_2, in the intermembrane space (IMS) to intercept and degrade these ROS signals before they reach the cytosol [133]. Prdx5 is normally expressed in the matrix compartment but not in the IMS. To target the protein to the IMS, they used the murine presequence that directs SMAC/Diablo to the IMS. Using viral vectors to express the protein in PASMCs, they confirmed that it attenuated the hypoxia-induced increase in oxidant signaling in the cytosolic and IMS compartments. Moreover, IMS-Prdx5 abrogated the hypoxia-induced increase in cytosolic Ca^{2+} in these cells. Finally, it produced a dose-dependent decrease in HIF-1α stabilization during hypoxia, along with the activation of HIF-dependent gene expression. Importantly, IMS-Prdx5 did not interfere with the mitochondrial ETC or oxygen consumption in the cells [133]. These findings provide alternative evidence that mitochondrial ROS signals are critical for the regulation of HIF-1α stabilization in hypoxia.

How are PHDs regulated by ROS? These hydroxylases require O_2 as a substrate for degradation of HIF-α, so perhaps prolyl hydroxylase itself functions as an O_2 sensor by becoming limited by oxygen availability in hypoxia. If so, then PHD2 should exhibit a Km for O_2 that is compatible with its decreased activity during hypoxia [134]. Studies with recombinant PHD protein demonstrate an inherently low affinity for O_2, consistent with that idea. However, recombinant proteins produced in E. coli lack post-translational modifications that can affect function in mammalian cells. Hence, *in vitro* assays may not recapitulate the function of the enzyme under biologically relevant conditions. However, it is clear that PHDs are inactive during anoxia, so at some level these enzymes must be capable of sensing a lack of O_2.

HIF transcriptional activity is also regulated by the *HIF asparaginyl hydroxylase* (Factor Inhibiting HIF, FIH), a member of the 2-oxoglutarate- and O_2-dependent hydroxylase family. FIH hydroxylates HIF at a conserved asparagine residue near its carboxy terminus [135, 136]. In normoxia, asparagine hydroxylation by FIH disrupts the interaction between HIF and the p300 transcriptional co-activator, preventing transcription of the target gene. By contrast, during hypoxia FIH activity decreases, allowing transcriptional activation of HIF-dependent genes. This mechanism acts largely as a safety switch to prevent accidental activation of hypoxia-regulated genes. FIH has been suggested to act as an oxygen sensor for the same reasons described above for PHDs [137]. However, identical concerns arise regarding the applicability of *in vitro* measurements with recombinant proteins to recapitulate the *in vivo* response.

Nevertheless, hydroxylation of FIH in cells has been reported to be more sensitive to exogenous oxidants than is PHD, whereas PHD is more sensitive to hypoxia [138, 139]. These observations are not inconsistent with the ROS theory of hypoxia sensing per se, as the concentrations and locations of ROS signaling during hypoxia are difficult to replicate when exogenous oxidants are applied to cells. Indeed, different human cell lines exhibit differences in HIF prolyl hydroxylation at a single level of hypoxia, underscoring the complexity of regulation in these systems [139].

Studies utilizing genetic deletion of mitochondrial electron transport complex subunits have been used to demonstrate the role of these complexes in ROS signaling. However, a secondary effect of disabling the ETC is that oxygen consumption is abolished. When this happens, intracellular PO_2 rises because the O_2 gradient between extracellular and intracellular oxygen tension declines. Some investigators have argued that this rise in cellular O_2 tension, rather than the loss of ROS signaling, is responsible for the associated decrease in HIF activation after ETC inhibition [140]. However, based on direct and indirect measurements, the magnitude of the intracellular gradient of oxygen tension is only 2–4 mmHg [2–4, 141]. Hence, inhibition of oxygen consumption would only cause a small rise in intracellular PO_2. Moreover, recent studies have described the ability of site-specific inhibitors of mitochondrial complex III to inhibit ROS generation and prevent hypoxic stabilization of HIF-1α. Those inhibitors do not suppress electron transfer or mitochondrial oxygen consumption, indicating that abrogation of complex III ROS generation is itself sufficient to inhibit hypoxia activation of HIF [110]. Further evidence that mitochondrial ROS, rather than a decrease in oxygen consumption, is responsible for initiating hypoxic responses comes from the studies of terpestacin, and of antioxidants targeted to the intermembrane space of mitochondria [106, 133]. Those studies show an attenuation of hypoxic responsiveness without an effect on O_2 consumption. Collectively these results suggest that loss of oxygen consumption and a corresponding rise in cellular oxygen levels is not responsible for the results observed in the studies of mitochondrial inhibition.

How might mitochondrial ROS signals lead to the inhibition of PHD and FIH? One possibility is that the hydroxylases are post-translationally modified by redox signals. PHD2 is known to interact with other proteins [142], so it is conceivable that ROS signals could modify these protein-protein interactions and thereby affect PHD2 activity. It is also possible that ROS could modify PHD and FIH functions, either by oxidizing cysteine residues or by attacking the coordinated iron atom. [138] In the former case H_2O_2 would be a likely candidate that could act either directly by oxidizing a thiol group or indirectly by oxidizing an antioxidant enzyme in a redox relay system [103]. Oxidation of the iron atom would also inactivate the enzyme, through either H_2O_2 or superoxide attack.

If ROS regulate HIF activation in hypoxia, then mitochondria-targeted antioxidants should abolish that response. MitoQ, a mitochondria-targeted antioxidant compound has such an effect [143], although questions about the effect of this compound on mitochondrial oxygen consumption have arisen.

Regulation of lifespan by hypoxia-induced mitochondrial ROS

HIF has been shown to extend the replicative lifespan in mammalian cells [144, 145]. In *C. elegans*, the HIF homolog EGL-9 has also been shown to extend lifespan [146], although the mechanisms underlying this response are not fully clear. As hypoxia triggers EGL-9 signaling, this suggests that mitochondria-dependent O_2 sensing could contribute to lifespan extending effects that could be independent of HIF. To test this, Schieber and Chandel exposed either larvae or adult *C. elegans* to transient hypoxia and observed a significant increase in lifespan[147]. Using RNAi feeding to alter gene expression in intestinal cells, they suppressed the Target of Rapamycin (TOR) homolog ET-363 and found that the hypoxia-induced lifespan extension was abolished. RHEB-1 is an upstream regulator of TORC1 signaling, and RNAi suppression of TORC1 expression similarly abolished the response to hypoxia, as did suppression of RAPTOR/DAF-15, a scaffold protein partner of TORC1. These findings indicated that HIF-independent TORC1 signaling mediates the increase in lifespan triggered by transient hypoxia in *C. elegans*. In further studies they found that suppression of ELT-2 – an intestine-specific GATA-type erythroid-like-2 transcription factor – also abolished the lifespan response to hypoxia. One gene regulated by ELT-2 is GSTO-1, an intestinal omega-class glutathione-S-transferase and putative antioxidant protein. Expression of GSTO-1 during hypoxia was inhibited by RNAi against TORC1 or RHEB-1, while suppression of GSTO-1 abolished the lifetime extension effect of hypoxia. How do these cells sense a decrease in O_2 and link this to TOR signaling to effect metabolic adaptations and increase lifespan? The answer was suggested from long-lived mitochondrial mutant worms that generate increased basal levels of ROS and activate HIF-1-dependent increases in lifespan [148]. To test whether ROS signals were required for the TORC1-dependent lifespan extension after transient hypoxia, worms were treated with the antioxidant BHA, which attenuated the longevity response to hypoxia. Finally, addition of the ROS-generating compound paraquat was sufficient to confer a lifespan extension that was blocked by RNAi against the TORC1 components RHEB-1 and TOR, or the intestinal transcription factor ELT-2. Collectively these results indicate that hypoxia-induced ROS signaling activates a TOR-dependent upregulation of the ELT-2 transcriptome, leading to upregulated expression of antioxidant proteins including GSTO-1, resulting in lifespan extension.

Summary

Cellular O_2 sensing is an important biological process, and mitochondria have been implicated in the activation of diverse cellular responses to hypoxia (Figure 2). Mitochondria signal the onset of hypoxia through the generation of ROS signals by the ETC. When released to the IMS, these signals can escape to the cytosol where they participate in thiol redox signaling involved in the activation of transcription factors and the initiation of post-translational responses. A wide range of biological processes have been linked to hypoxia-induced release of ROS signals including carotid body O_2 chemotransduction, hypoxic pulmonary vasoconstriction, HIF-1α activation, lifespan regulation, proliferation, differentiation and inflammatory processes. While controversies in the field still exist, emerging work is shedding new light on the mechanisms of mitochondrial O_2 sensing, which is important in both health and disease.

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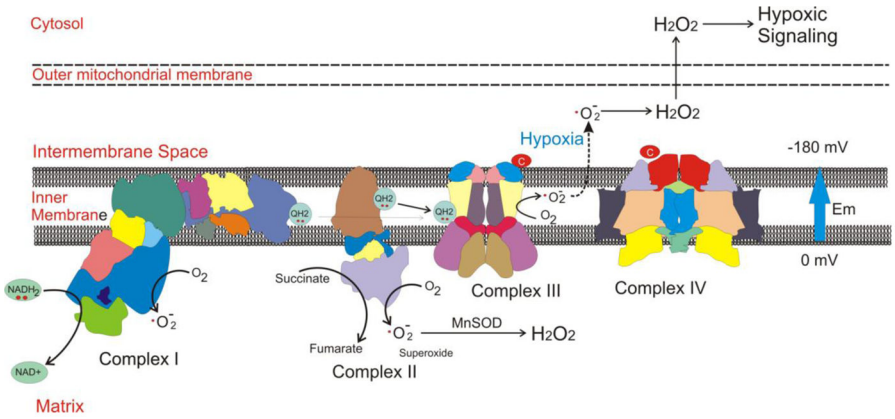

Figure 1.

Mitochondrial ETC complexes can generate superoxide from multiple sites. Complexes I and II release ROS primarily to the matrix compartment, where manganese superoxide dismutatase (MnSOD) converts superoxide to hydrogen peroxide (H_2O_2). Complex III can generate ROS at the Q_0 ubiquinone binding site within the inner membrane. Superoxide generated there is ejected into the intermembrane space by the strong electrical field in the membrane (Em) that results from the electrical gradient (−180 mV).

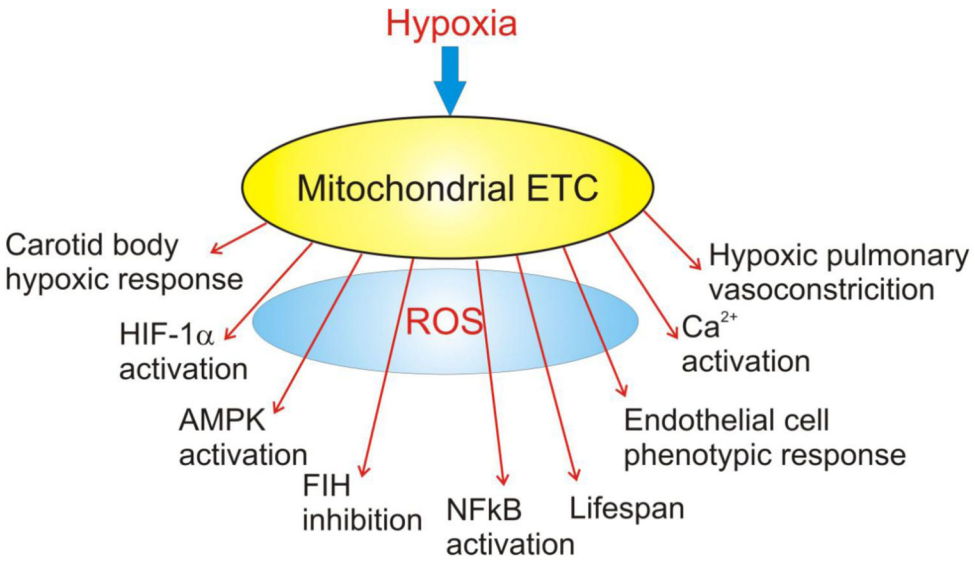

Figure 2.

Mitochondria regulate diverse responses to hypoxia through the release of ROS signals to the cytosol.

TABLE 1

O_2-dependent ROS regulation of Kv and L-type Ca^{2+} channel activity in the regulation of tone in PASMC, Ductus Arteriosus and SASMC, as proposed by Archer, Weir, Michelakis and coworkers.

PASMC		Ductus Arteriosus		SASMC	
Hypoxia	**High PO_2**	**Hypoxia**	**High PO_2**	**Hypoxia**	**High PO_2**
↓	↓	↓	↓	↓	↓
ROS Decrease	ROS Increase	ROS Decrease	ROS Increase	ROS Increase	ROS Decrease
↓	↓	↓	↓	↓	↓
Kv Closure	Kv Open	Kv Open	Kv Closure	Kv Open	Kv Closure
↓	↓	↓	↓	↓	↓
L-type Open	L-type Closure	L-type Closure	L-type Open	L-type Closure	L-type Open
↓	↓	↓	↓	↓	↓
Constriction	Relaxation	Relaxation	Constriction	Relaxation	Constriction

See text for explanation of these responses.