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HYPOXIA-INDUCED CHANGES IN PULMONARY AND SYSTEMIC VASCULAR RESISTANCE: WHERE IS THE O₂ SENSOR?

Gregory B. Waypa and Paul T. Schumacker

Department of Pediatrics, Division of Neonatology Northwestern University Chicago, Illinois, 60611 USA

Abstract

Pulmonary arteries (PA) constrict in response to alveolar hypoxia, whereas systemic arteries (SA) undergo dilation. These physiological responses reflect the need to improve gas exchange in the lung, and to enhance the delivery of blood to hypoxic systemic tissues. An important unresolved question relates to the underlying mechanism by which the vascular cells detect a decrease in oxygen tension and translate that into a signal that triggers the functional response. A growing body of work implicates the mitochondria, which appear to function as O₂ sensors by initiating a redox-signaling pathway that leads to the activation of downstream effectors that regulate vascular tone. However, the direction of this redox signal has been the subject of controversy. Part of the problem has been the lack of appropriate tools to assess redox signaling in live cells. Recent advancements in the development of redox sensors have led to studies that help to clarify the nature of the hypoxia-induced redox signaling by reactive oxygen species (ROS). Moreover, these studies provide valuable insight regarding the basis for discrepancies in earlier studies of the hypoxia-induced mechanism of redox signaling. Based on recent work, it appears that the O₂ sensing mechanism in both the PA and SA are identical, that mitochondria function as the site of O₂ sensing, and that increased ROS release from these organelles leads to the activation of cell-specific, downstream vascular responses.

1. Introduction

Pulmonary arteries (PA) constrict in response to alveolar hypoxia, as part of a response termed hypoxic pulmonary vasoconstriction (HPV). The HPV response was first described in 1946 by von Euler and Liljestrand, who found that PA pressure increased in feline lungs ventilated with low O₂ mixtures, and decreased when ventilated with pure O₂ (von Euler and Liljestrand, 1946). Recognizing that these responses reflected changes in pulmonary vascular resistance, they speculated that a vasoconstrictor response to hypoxia could help to improve gas exchange efficiency by diverting blood flow away from poorly ventilated lung regions and towards areas with better oxygenation. While this response may improve gas exchange when the hypoxia is limited to small regions, widespread hypoxia throughout the lung elicits pulmonary arterial hypertension. This response is beneficial in the fetus, where the diversion of blood away from the lung prevents oxygen loss from pulmonary capillary

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Please address correspondence to: Gregory B. Waypa, Ph.D. or Paul T. Schumacker, Ph.D., Department of Pediatrics, Northwestern University, Morton Building 4-685, 310 East Superior St, Chicago, Illinois 60611 USA, Tel. 312 503-3286, g-waypa@northwestern.edu.

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blood to hypoxic alveolar amniotic fluid. However, in adult lungs the development of global alveolar hypoxia, which can occur at high altitude or in hypoxic lung diseases, leads to chronic pulmonary arterial hypertension that can trigger pulmonary vascular remodeling as well as right ventricular hypertrophy. Unlike pulmonary arteries, systemic arteries relax in response to tissue hypoxia. This response facilitates local arterial vasodilation, thereby enhancing blood flow and oxygen supply in hypoxic tissues (Detar, 1980; Gupte and Wolin, 2008; Guyton et al., 1964; Michelakis et al., 2002; Weir et al., 1997).

A large number of studies have examined the responses to hypoxia in the pulmonary and systemic circulations. In the lung, the HPV response is retained in excised lungs, in lung slices, and in PA rings studied in an organ bath (Archer et al., 1993; Desireddi et al., 2010; Grimminger et al., 1995; Leach et al., 2001; Marshall et al., 1996; McMurtry, 1984; Robertson et al., 2001; Rounds and McMurtry, 1981; Weissmann et al., 1998; Weissmann et al., 1995; Zhao et al., 1996). Isolated PA smooth muscle cells (PASMC) also contract under hypoxia, indicating that these cells are O_2 – sensitive (Lu et al., 2009; Shimoda et al., 2007; Waypa et al., 2001; Waypa et al., 2006; Waypa et al., 2010; Waypa et al., 2002; Zhang et al., 1997). Likewise, the systemic vascular responses to hypoxia are evident in isolated systemic arterial smooth muscle cells (SASMC) (Coburn, 1977; Franco-Obregon et al., 1995; Mehta et al., 2008; Michelakis et al., 2002; Smani et al., 2002; Waypa et al., 2010), indicating that these, too, possess an O_2 sensor. Despite the wealth of information characterizing these responses, the identity of the O_2 sensor(s) is not established. Several previous reviews have dealt with this question, and the reader is directed to them for a broader perspective of this field (Evans, 2010; Evans and Ward, 2009; Gupte and Wolin, 2008; Sommer et al., 2008; Ward and McMurtry, 2009; Waypa and Schumacker, 2008; Weir et al., 2008; Wolin, 2009).

The goal of the present review is to provide an analysis of data examining the acute response to hypoxia in the pulmonary and systemic vasculature. With respect to the HPV response, a growing body of work implicates the mitochondria as the site of O_2 sensing, although the general idea that mitochondria could play such a role is not new. From a parsimonious standpoint it is appealing to predict that a similar mechanism of oxygen sensing could function in both the pulmonary and systemic vascular beds, as the biophysical process of oxygen sensing signal transduction is necessarily complex and the range of possible molecular mechanisms seems limited. However, in view of the heterogeneity of cells in the vascular wall, the diversity of functional responses to hypoxia that have been described in vascular cell types, and the broad range of responses to acute versus chronic hypoxia, it seems unlikely that a single oxygen sensing system is involved. Nevertheless, the definitive identification of even one oxygen sensing mechanism and the mechanisms coupling that sensor to a single functional response would represent an important scientific achievement.

2. Hypoxic pulmonary vasoconstriction

2.1 The HPV response

The HPV response in pulmonary arteries is biphasic (Dipp et al., 2001; Leach et al., 2001). An initial transient constriction (acute phase) is followed by a slow sustained constriction (chronic phase). This response is associated with a biphasic rise in $[Ca^{2+}]_i$ in PASMC (Robertson et al., 1995). The acute phase, which occurs within seconds of a hypoxic challenge, does not require an intact endothelium (Zhang et al., 1997) and likely involves the release of calcium from intracellular stores, closure of voltage-dependent potassium channels (K_V), and opening of both voltage-dependent and voltage-independent calcium channels [also known as non-selective cation channels (NSCC)] (Archer et al., 1998; Lu et al., 2009; Lu et al., 2008; Morio and McMurtry, 2002; Robertson et al., 2000b). The hypoxia-induced increase in cytosolic calcium activates myosin light chain kinase, causing

actin-myosin interaction and PASMC contraction. The chronic phase occurs within minutes after hypoxic challenge and can persist for hours to days, if hypoxia is maintained. This response requires the endothelium, indicating that an endothelial-derived promoting factor may be involved (Aaronson et al., 2002; Dipp et al., 2001; Lazor et al., 1996; Leach et al., 2001; Leach et al., 1994; Liu et al., 2001; Ward and Robertson, 1995). Endothelial denudation does not alter the calcium response to chronic hypoxia; rather, it affects the myofilament sensitivity to $[Ca^{2+}]_i$ in PASMC (Robertson et al., 1995). Calcium sensitivity in PASMC is regulated in part by myosin phosphatase, which is regulated by Rho kinase activity. Thus, inhibition of Rho kinase with Y-27632 attenuates the chronic phase of HPV in isolated PA and in perfused lungs (Robertson et al., 2000a). Rho kinase activity increases in isolated PASMC challenged with hypoxia (Chi et al., 2010), but the effect is amplified in the presence of endothelium (Wang et al., 2001), presumably due to the release of an endothelial-derived factor. Endothelin-1 (ET-1) may contribute to that effect (Liu et al., 2001), as the release of ET-1 is augmented during hypoxia (Bodi et al., 1995; Hu et al., 1998; Yamashita et al., 2001), and the ET-1 receptor subtype A antagonist, BQ-123, attenuates the chronic phase of HPV in intact PA vessels (Liu et al., 2001; Sato et al., 2000). Finally, ET-1 increases Rho kinase activity in PASMC (Barman, 2007; Homma et al., 2007; Weigand et al., 2006). As yet unidentified endothelial-derived factors may act in concert with ET-1 during the chronic phase (Aaronson et al., 2002; Robertson et al., 2003; Robertson et al., 2001). However, this review focuses on the acute phase of HPV, and the O_2 sensor regulating the immediate response to hypoxia.

2.2 Mitochondria as the HPV oxygen sensor

The idea that mitochondria could act as oxygen sensors arose from the expectation that hypoxia should inhibit the mitochondrial electron chain (ETC) at Complex IV. Oxygen limitation of Complex IV could affect the redox state of the upstream electron carriers, which then might signal through an increase in the reduction of mitochondrial electron carriers such as β -NADH, a decrease in [ATP] and energy charge, or changes in cytosolic $[NADH]:[NAD^+]$ redox. If true, then mitochondrial inhibition should mimic hypoxia and trigger HPV. Indeed, Rounds and McMurtry found that certain inhibitors of the ETC and oxidative phosphorylation mimicked the hypoxia response in isolated, blood-perfused lungs, possibly by depleting [ATP] (Rounds and McMurtry, 1981). However, studies in isolated lungs by Buescher et al. and in isolated PA by Leach et al. demonstrated that the energy state does not decline during hypoxia in either of these systems (Buescher et al., 1991; Leach et al., 2000). Those findings suggest that decreases in [ATP] are not required for triggering HPV. Moreover, mitochondrial respiration remains constant until the extracellular PO_2 falls below a critical threshold of 5–7 torr in diverse cell types (Jones and Kennedy, 1982, 1986). It therefore seems unlikely that energy supply in PASMC would become O_2 -limited at oxygen tensions in the range of 10–40 torr where HPV is activated.

2.3 Does hypoxia decrease mitochondrial ROS signaling?

In 1986, Archer and Weir proposed that redox regulates membrane K^+ channels and that a decrease in ROS would induce membrane depolarization by causing closure of those channels (Archer et al., 1986). They later suggested that mitochondria could act as O_2 sensors by slowing their rate of ROS production during hypoxia, and increasing the rate during normoxia. This concept was supported by studies where they measured reciprocal changes in PA pressure and ROS levels during hypoxia or normoxia in perfused lungs, using lucigenin or luminol chemiluminescence to detect ROS generation (Archer et al., 1993). They also found that antimycin A and rotenone, agents that inhibit the ETC, caused chemiluminescence to decrease and PA pressure to increase acutely (Archer et al., 1993). Those findings led them to postulate that the mitochondrial ETC generates constitutive ROS during normoxia, and that the decrease in production during hypoxia leads to a reductive

shift that causes vasoconstriction through the closure of K_V^+ channels (Archer and Michelakis, 2002; Archer et al., 1993). Also, Reeve et al. reported that the Complex I inhibitor rotenone decreased chemiluminescence and increased vascular tone in isolated perfused lungs (Reeve et al., 2001).

They explained the response to antimycin A and rotenone by proposing that these inhibitors decrease mitochondrial ROS generation (Archer and Michelakis, 2002; Archer et al., 1993). In endothelium-denuded rings of distal PA they also observed hypoxia-induced decreases in ROS as assessed by chemiluminescence, Amplex Red (an H_2O_2 -sensitive reagent), and dichlorofluorescein (DCF, a non-specific oxidant-sensitive probe) (Michelakis et al., 2002). They also postulated that ROS could originate from NAD(P)H oxidase, which would presumably decrease its production of superoxide during hypoxia due to a fall in the availability of O_2 (Archer and Michelakis, 2002). However, mice with targeted deletion of the gp91^{phox} subunit (NOX2) of NADPH oxidase still exhibited HPV (Archer et al., 1999), indicating that NOX2 was not required for the hypoxic response.

Archer and Weir then proposed that hypoxia could slow mitochondrial electron transport, possibly decreasing ROS production by Complex I and/or Complex III while increasing cytosolic $[NAD(P)H]/[NAD^+]$ (Archer et al., 1993; Michelakis et al., 2002). This would shift the cytosolic $[NADH]:[NAD^+]$ redox balance to a more reduced state, which they theorized would somehow also shift the glutathione redox pool ($[GSH]/[GSSG]$) toward a reduced state (Weir and Archer, 1995), although those systems are not directly coupled. According to that model, mitochondrial inhibitors would be expected to mimic hypoxia by augmenting NAD(P)H levels and decreasing ROS during hypoxia. A perplexing aspect of that idea is that increases in NAD(P)H might be expected to augment ROS production by enhancing the activity of NAD(P)H-dependent systems other than NOX2. In either case, they claim that a reductive shift in redox during hypoxia results in the inhibition of K^+ current through redox-sensitive 4-aminopyridine (4-AP)-sensitive K_V channels (Reeve et al., 1995). Indeed, Rettig et al. had identified a cysteine residue in the β -subunit of the K_V1 -type channel expressed in rat brain that was oxidized by H_2O_2 (~40 mM) and reduced by glutathione (5 mM) (Rettig et al., 1994). When the cysteine residue is reduced, the NH_2 -terminal inactivating domain of the β -subunit binds to the inner mouth of the channel pore formed by the α -subunits of the K_V channel causing the inactivation of the channel. Extrapolating those findings to pulmonary vascular smooth muscle, severe oxidant stress during normoxia might keep the K_V channel in an activated state, whereas a reductive stress during hypoxia might cause channel closure, leading to membrane depolarization, the opening of L-type, voltage-gated Ca^{2+} channels, and subsequent myocyte contraction. In support of that hypothesis, they found that the powerful oxidants diamide (1 μM) or dithionitrobenzoic acid (1 mM) caused vasodilation in isolated perfused lungs and PA rings (Olschewski et al., 2004; Reeve et al., 1995; Weir et al., 1985), while the equally powerful reducing agent dithiothreitol (DTT; 3 mM) and the antioxidants co-enzyme Q_{10} (700 μM) and duroquinone (700 μM) attenuated K^+ currents in pulmonary artery smooth muscle cells resulting in their contraction (Olschewski et al., 2004; Reeve et al., 1995). Finally, hypoxia was reported to cause inhibition of steady-state whole-cell K^+ currents and membrane depolarization in rat PA smooth muscle cells (Olschewski et al., 2002a; Olschewski et al., 2002b; Olschewski et al., 2004). However, the molecular identities of the K_V channel(s) that participate in those responses have not been fully identified.

That redox theory was based on the expectation that a decrease in oxygen would result in a decrease in ROS signaling. However, a more critical evaluation of that model raises important questions. First, mounting evidence reveals that the cell normally maintains a highly reduced thiol redox state in the cytosol (Gilbert, 1990), making signaling via further reductive stress unlikely. Moreover, signaling events triggered by angiotensin II, growth

factors, and mechanical strain in vascular cells require increases ROS generation (Ali et al., 2004; Cowan et al., 2003; Lassegue et al., 2001; Liu et al., 2003; Marshall et al., 1996; Paddenberget al., 2003; Waypa et al., 2001). Second, chemical inhibitors of K_V channels as well as specific K_V knockout mouse strains have failed to inhibit HPV, suggesting that (a) multiple K_V channel types are involved, and/or (b) that closure of K_V channels represents a downstream amplification step, rather than an initiating step, in HPV (Archer et al., 2001; Hasunuma et al., 1991; Sham et al., 2000). Third, modeling studies suggest that hypoxic inhibition of K^+ currents (IK_V and IK_{Ca}) is insufficient to induce PAMSC contraction unless it occurs in combination with NSCC activation (Cha et al., 2008). In addition, other investigators report that rotenone decreases ROS production while antimycin A augments oxidant generation (Chandel et al., 2000b; Chen et al., 2003; Cowan et al., 2003; Garcia-Ruiz et al., 1997; Mohazzab and Wolin, 1994; Turrens et al., 1985). Therefore, according to the model of Archer et al. (Archer et al., 1993; Michelakis et al., 2002), the former compound should have activated HPV while the latter should have blocked it. Finally, if decreases in intracellular ROS production trigger HPV, then antioxidants that scavenge ROS should trigger HPV during normoxia by lowering the levels of oxidant signaling. Instead, antioxidants block the response to hypoxia in intact lungs, isolated PA vessels and isolated PA smooth muscle cells, without causing a sustained increase in pulmonary vascular tone (Liu et al., 2003; Waypa et al., 2001; Waypa et al., 2002). Collectively, these observations question the idea that hypoxia-induced decreases in ROS trigger HPV.

2.4 Does hypoxia increase ROS signaling?

In 1996, Marshall et al. argued that hypoxia accelerates ROS generation by NADPH oxidase, based on their observation that diphenyleneiodonium (DPI) inhibited the oxidant signal in PASMC and the contractile response to hypoxia (Marshall et al., 1996). This finding was confirmed by other investigators in cultured PASMC and isolated lungs (Grimminger et al., 1995; Thompson et al., 1998; Waypa et al., 2001). However, DPI inhibits all flavoproteins including NADPH oxidase, mitochondrial Complex I, glutathione reductase, nitric oxide synthase, and prostaglandin synthetase (Holland et al., 1973; Majander et al., 1994). Therefore, the site of inhibition responsible for the attenuation of HPV is not known. To clarify the involvement of an NAD(P)H oxidase in HPV, two groups used apocynin, an inhibitor of the neutrophil form of this enzyme (Grimminger et al., 1995; Waypa et al., 2001). As with mice lacking the gp91^{phox} subunit of the NADPH oxidase, apocynin failed to block the HPV response. However, these results do not rule out that possibility that other NAD(P)H oxidase-like systems could still be involved in HPV. In accordance with this reasoning, Weissmann et al. demonstrated a decrease in HPV by the NOX inhibitor [4-(2-aminoethyl) benzenesulfonyl fluoride], while Jones et al. inhibited HPV in isolated rat PA using cadmium sulfate to inhibit the proton translocation channel of the NOX-2 (NADPH oxidase) complex (Jones et al., 2000; Weissmann et al., 2000). More recently, Weissmann et al. studied the effects of hypoxia on lungs from mice deficient in the NADPH oxidase subunit p47^{phox}, which is suggested to be a key subunit for the function of most NADPH oxidase isoforms (Weissmann et al., 2006b). They observed an attenuation of the acute phase of HPV in perfused lungs isolated from NADPH oxidase subunit p47^{phox} deficient mice compared to wild type and NADPH oxidase subunit gp91^{phox} mice (Weissmann et al., 2006b). This supports the concept that a non-phagocytic NADPH oxidase-derived increase in ROS is involved in triggering the acute phase of HPV.

After the report from Marshall et al., other studies also suggested that acute hypoxia triggers an increase in ROS signaling in PASMC, as measured by the intracellular probe 2',7'-dichlorofluorescein-diacetate (DCFH), lucigenin-derived chemiluminescence and by Electron Paramagnetic Resonance (EPR) spectrometry, although the latter results did not achieve statistical significance (Killilea et al., 2000; Liu et al., 2003; Wang et al., 2007; Waypa et al.,

2001). Moreover, antioxidants such as catalase and glutathione peroxidase attenuated the HPV response while others, like superoxide dismutase (SOD), had either no effect or augmented the response (Wang et al., 2007; Waypa et al., 2001; Waypa et al., 2006; Waypa et al., 2010; Waypa et al., 2002). Weissmann et al. observed that SOD or 4,5-dihydroxy-1,3-benzenedisulfonic acid (to accelerate H_2O_2 generation from superoxide) did not affect HPV, suggesting that H_2O_2 , rather than superoxide, is involved (Weissmann et al., 1998). Also, nitrobluetetrazolium, a putative trap for superoxide, prevented H_2O_2 formation and attenuated HPV (Weissmann et al., 1998). The involvement of increased ROS is further indicated by the observation that exogenous H_2O_2 mimics HPV in cultured PASMCM and in isolated lungs (Pourmahram et al., 2008; Sheehan et al., 1993; Waypa et al., 2001; Waypa et al., 2002; Waypa et al., 2000), although it should be noted that H_2O_2 can also release Ca^{2+} from mitochondria (Richter et al., 1995) and may also trigger nonspecific effects (Jin et al., 1991; Rhoades et al., 1990). Nevertheless, these findings support the idea that an increase in a ROS signaling occurs during HPV.

The idea that HPV requires electron transport in the proximal but not the distal region of the mitochondrial ETC comes from the observation that upstream inhibitors such as rotenone or myxothiazol abrogate HPV (Wang et al., 2007; Waypa et al., 2001; Waypa et al., 2006; Waypa et al., 2002; Weissmann et al., 2003), whereas inhibitors acting at more distal sites, such as cyanide or antimycin A, fail to inhibit the response. Leach et al. extended this observation by showing that rotenone blocked hypoxic contraction of PA vessels, and that HPV could subsequently be restored by using succinate to shuttle electrons into Complex III via Complex II, bypassing the site of inhibition (Leach et al., 2001). That inhibitors of the distal ETC do not abolish HPV suggests that a fully functional ETC is not required for the hypoxic response (Leach et al., 2001; Waypa et al., 2001; Waypa et al., 2006; Waypa et al., 2002). These site-specific effects can be explained by the inability of some compounds to prevent hypoxia-induced increases in ROS production from the proximal region of the ETC, notably Complex III (Chandel et al., 1998; Waypa et al., 2006). For example, antimycin A, which blocks the downstream Q_i site in Complex III and thereby augments ROS production from that Complex, triggers vasoconstriction in normoxic lungs (Rounds and McMurtry, 1981). Cyanide, which inhibits cytochrome oxidase and thereby causes reduction of the entire ETC, enhances ROS production throughout the ETC and causes constriction during normoxia (Waypa et al., 2001; Waypa et al., 2006; Waypa et al., 2002). It also triggers $[Ca^{2+}]_i$ increases in PASMCM (Wang et al., 2003; Waypa et al., 2006; Waypa et al., 2002). Interestingly, Archer et al. found that cyanide induced vasoconstriction during normoxia and augmented HPV without affecting the response to angiotensin II or KCl (Archer et al., 1993). This response can be blocked by myxothiazol, which prevents electron flow into Complex III, and by over-expression of catalase (Waypa et al., 2002), which augments the scavenging of H_2O_2 .

While these findings are consistent with a role for increased ROS production in HPV, many of these studies rely on pharmacological inhibitors whose effects can vary depending on concentration. Indeed, some laboratories have reported that mitochondrial inhibitors mimic HPV (Archer and Michelakis, 2002; Archer et al., 1993), while others show that they attenuate the HPV response (Leach et al., 2001; Waypa et al., 2001). To address this, ρ^0 -PASMCM, which lack a functional ETC and are incapable of generating ROS during hypoxia, have been studied. These cells lose their hypoxic response, yet retain the ability to respond to U46619, a thromboxane A_2 analogue (Waypa et al., 2001). Increasingly, genetic deletion of genes involved in redox regulation provides more definitive evidence that the ETC plays a physiologically conserved role in hypoxia-induced ROS signaling and hypoxic responses such as the stabilization of hypoxia-inducible factors (HIFs) (Guzy et al., 2005; Mansfield et al., 2005).

2.5 Critical assessment of ROS signaling during HPV

The finding that hypoxia increases ROS generation is not limited to PASMC, as it has been observed in a wide variety of cell types (Chandel et al., 1998; Chandel et al., 2000a; Duranteau et al., 1998; Killilea et al., 2000; Liu et al., 2001; Waypa et al., 2001). Technical limitations of the tools used to assess oxidant stress have fueled the disagreements regarding ROS signaling during hypoxia. Fluorescent probes such as DCF and dihydroethidium lack specificity (Thannickal and Fanburg, 2000) and can accumulate within organelles, while autooxidation and limited intracellular access can interfere with the ability of lucigenin or luminol to detect intracellular oxidants (Spasojevic et al., 2000). Importantly, none of these probes exhibits ratiometric fluorescence properties, so changes in intracellular dye concentration or fluorescence path length caused by a change in cell volume can affect fluorescence intensity independently from changes in ROS. Investigators are increasingly employing a new generation of ratiometric, redox-sensitive probes to assess ROS signaling during hypoxia. One such probe uses Fluorescence Resonance Energy Transfer (FRET) and consists of enhanced cyan and yellow fluorescent protein motifs (CFP, YFP) linked by the redox-dependent regulatory domain from the bacterial heat shock protein HSP-33 (Janda et al., 2004). The HSP-33 domain contains four highly conserved cysteine residues coordinating a zinc-binding domain. Oxidation of the thiols leads to release of zinc and the formation of two disulfides (Barbicz et al., 2000). Using the redox-dependent regulatory domain from HSP-33, a ratiometric redox reporter was generated (HSP-FRET), in which oxidation causes a change in the optical coupling of CFP and YFP (Guzy et al., 2005). When expressed in cells, HSP-FRET provides a sensitive, real-time assessment of changes in redox in the cytosol in response to hypoxia (Waypa et al., 2006). In cultured PASMC expressing HSP-FRET, superfusion with hypoxic media caused an increase in ROS signaling in the cytosol (Waypa et al., 2006). This response was attenuated by myxothiazol, catalase or glutathione, yet neither cyanide nor SOD altered hypoxia-induced increase in ROS signaling (Waypa et al., 2006). Interestingly, changes in hypoxia-induced ROS signaling were mirrored by changes in $[Ca^{2+}]_i$, and attenuation of the ROS signal abrogated the calcium response (Waypa et al., 2006). However, a characteristic of HSP-FRET protein is that it refolds slowly after being oxidized, hindering the ability to calibrate its redox state. Furthermore, attempts to target its expression to the mitochondria were unsuccessful, impeding its usefulness for assessing sub-cellular redox signaling.

Another redox sensor, roGFP, provides an alternative measure of protein thiol redox status (Cannon and Remington, 2009; Dooley et al., 2004; Hanson et al., 2004; Jiang et al., 2006; Lohman and Remington, 2008). Oxidation resulting in dithiol formation causes reciprocal changes in its emission intensity when excited at two different wavelengths. By exposing cells to strong reducing and oxidizing agents at the conclusion of an experiment, roGFP oxidation state can be calibrated on a cell-by-cell basis.

We generated constructs to target roGFP to the mitochondrial matrix, the inter-membrane space (IMS), and the cytosol (Waypa et al., 2010). Under normoxic conditions the cytosol was highly reduced and the matrix was the most oxidized, while the IMS demonstrated an intermediate level of protein thiol oxidation (Figure 1) (Waypa et al., 2010). During hypoxia, roGFP oxidation in the cytosol and the IMS demonstrated an oxidative shift (Figure 1). However, the oxidant signal was subtle and it produced a relatively small change in protein thiol oxidation state. By contrast, roGFP oxidation in the matrix decreased. The increase in ROS signaling in the IMS and cytosol despite the decrease in O_2 suggests that this is a regulated event, whereas the general O_2 -dependent decrease in oxidation in the matrix suggests that ROS production in that compartment is a non-specific unregulated process. The hypoxia-induced increase in cytosolic ROS signaling was associated with a rise in $[Ca^{2+}]_i$, which was attenuated by over-expression of cytosolic catalase, implicating H_2O_2 involvement. Similar responses to acute hypoxia were measured when roGFP was expressed

in the PASMC of mouse lung slices (Desireddi et al., 2010). Furthermore, it was observed that the hypoxia-induced oxidation of roGFP is maintained when PASMC are incubated under prolonged (24 hrs) hypoxic conditions (Chi et al., 2010).

The compartment-specific redox responses do provide insight into the basis for disagreements among investigators regarding ROS generation in hypoxia. Probes such as lucigenin may not enter the cells, or could accumulate in the mitochondrial matrix. By contrast, probes such as DCF may remain in the extracellular space or can be taken up into the cytosol, mitochondria, endoplasmic reticulum, or other compartments. Once oxidized, many fluorescent probes can migrate between compartments. Thus, depending on where it accumulates, the overall cellular fluorescence/luminescence behavior of a chemical probe could provide a distorted picture of what is occurring in a given region. More importantly, the combined signal would be dominated by large compartments where large shifts occur, and may not detect smaller changes in ROS in specific compartments where they act in a signaling function. To the extent that redox responses differ among subcellular compartments, the unknown and varying distribution of chemical probes among compartments could easily explain why different experiments have yielded inconsistent responses among laboratories. Protein sensors, by contrast, are only expressed in the cells. Immunogold electron microscopy studies confirm the expected targeted expression patterns, and the distribution within the cell does not change over time (Waypa et al., 2010). Importantly, the ability to calibrate the roGFP sensor permits comparisons between cells, and among intracellular compartments. Clearly, more experiments using imprecise tools that provide ambivalent answers are not likely to advance this field.

2.6 Hypoxia-induced ROS signaling triggers the calcium response

While hypoxia-induced increases in $[Ca^{2+}]_i$ could arise from the entry of extracellular Ca^{2+} through voltage-dependent and/or voltage-independent Ca^{2+} channels (Archer et al., 2000; Kang et al., 2002, 2003; Post et al., 1995; Robertson et al., 2000b; Wang et al., 2004), recent studies suggest that an initial intracellular release of Ca^{2+} may then trigger extracellular entry (Dipp and Evans, 2001; Dipp et al., 2001; Morio and McMurtry, 2002; Salvaterra and Goldman, 1993; Weigand et al., 2005). Possible sources of intracellular release include the sarcoplasmic reticulum (SR) and/or mitochondria. Hypoxia-induced release of Ca^{2+} from SR could be mediated by ryanodine receptors (RyR) and/or inositol 1,4,5- trisphosphate (IP_3) receptors. Li et al. demonstrated that the increase in $[Ca^{2+}]_i$ during hypoxia was attenuated in $RyR^{-/-}$ and $RyR^{+/-}$ mice (Li et al., 2009). RyR contain redox-sensitive cysteine thiols (Eu et al., 1999), so ROS released from mitochondria could trigger Ca^{2+} release by activating these channels. In support of this idea, low concentrations of peroxide have been shown to elevate $[Ca^{2+}]_i$ via release from RyR-sensitive stores (Lin et al., 2007; Pourmahram et al., 2008; Rathore et al., 2008; Wang et al., 2007). Alternatively, early calcium increases may reflect SR Ca^{2+} release through stimulation of RyR by cyclic ADP-ribose (cADPR) (Dipp and Evans, 2001; Evans and Dipp, 2002). In that model, hypoxia-induced inhibition of ATP production would increase the AMP/ATP ratio, thereby activating AMPK and increasing production of cADPR (Evans and Ward, 2009). However, ROS and AMPK may also be linked in a system whereby ROS triggers AMPK activation (Quintero et al., 2006). Hence, cADPR-dependent Ca^{2+} release from the SR could occur as a downstream consequence hypoxia-induced increases in ROS. Alternatively, activation of IP_3 receptors could trigger Ca^{2+} release from the SR, based on the ability of ROS to activate phospholipase C (PLC) (Gonzalez-Pacheco et al., 2002). PLC generates diacylglycerol (DAG) and inositol trisphosphate (IP_3) from phosphatidylinositol bisphosphate (PIP_2).

Hypoxia-induced calcium release from the SR may then trigger voltage-dependent and/or voltage-independent calcium entry, thereby amplifying the acute constriction response (Post et al., 1995; Robertson et al., 2000b; Snetkov et al., 2003; Sweeney and Yuan, 2000; Wang

et al., 2004). Voltage-independent calcium entry is one potentially important mechanism contributing to the increase $[Ca^{2+}]_i$ during hypoxia (Robertson et al., 2000b; Snetkov et al., 2003; Wang et al., 2004). Store-operated Ca^{2+} channels (SOCC) are activated by depletion of SR Ca^{2+} stores, which are then replenished by the resulting increase in capacitive calcium entry (CCE) (McDaniel et al., 2001; Wang et al., 2004). SOCC involved in CCE are comprised of mammalian homologs of transient receptor potential (TRP) and transient receptor potential-like (TRPL) proteins, which form light-activated cation channels in photoreceptor cells of *Drosophila melanogaster* (Wang et al., 2004). Wang et al. demonstrated that transient receptor potential channels (TRPC) are expressed in distal intrapulmonary arteries, a major site of vasomotor responses in the pulmonary circulation (Wang et al., 2004). Distal intrapulmonary arteries express mRNA and protein for TRPC1, TRPC4, and TRPC6 (Wang et al., 2004) supporting the possible involvement of CCE in HPV. The endoplasmic reticulum Ca^{2+} sensor, stromal-interacting molecule (STIM1), may interact with most of the TRPC proteins to regulate their function as a SOCC (Yuan et al., 2007). For example, STIM1 directly regulates TRPC1 and TRPC4 as SOCC through this interaction. By contrast, STIM1 does not directly interact with TRPC6, although STIM1 indirectly regulates agonist-induced TRPC6 activation through its interaction with STIM1-regulated TRPC4. In support of a role for TRPC in the acute phase of HPV, hypoxia-induced DAG was shown to activate membrane-bound TRPC6 channels resulting in Na^+ influx, membrane depolarization, and activation of L-type, voltage-gated calcium channels (Weissmann et al., 2006a). Also, the Ca^{2+} release-activated channel (CRAC), formed from the interaction of STIM1 and Orai channel proteins, is highly selective for Ca^{2+} and also represents a likely candidate for HPV responses (Hewavitharana et al., 2007). Finally, a role for voltage-dependent calcium entry was suggested by Post et al. who reported that the release of Ca^{2+} from internal stores caused inhibition of 4-aminopyridine (4-AP)-sensitive K_V channels (Post et al., 1995). The resulting decrease in K^+ current caused membrane depolarization and the opening of voltage-gated L-type Ca^{2+} channels leading to increased $[Ca^{2+}]_i$. However, when voltage-dependent calcium entry was blocked by the L-type inhibitor nifedipine, the acute phase of the HPV response was only partially attenuated (Robertson et al., 2000b). This suggests that voltage-dependent and voltage-independent calcium entry most likely have a synergistic relationship in the acute phase of HPV.

3. Hypoxia-induced systemic vasodilation

While it is well documented that systemic arteries dilate in response to hypoxia, little work has focused on identifying the O_2 sensor responsible for this response. Guyton et al. originally demonstrated that changes in PO_2 could elicit changes in vascular resistance in canine hind limb (Guyton et al., 1964). Decreases in oxygen tension in systemic tissues have been shown to decrease [ATP], increase adenosine release, and increase release of lactic acid and potassium, each of which can affect vascular resistance (Furchgott, 1966; Taggart and Wray, 1998). However, studies suggest that hypoxia-induced SASMC relaxation is independent of energy stores and mitochondrial activity (Coburn et al., 1979). Furthermore, hypoxia-induced SA dilation occurs in a PO_2 range of 20–100 torr, which should be sufficient to maintain mitochondrial oxidative phosphorylation (Detar, 1980).

Hypoxia apparently does not inhibit K^+ currents in SASMC (Post et al., 1992; Yuan et al., 1993). However, decreases in oxygen tension rapidly inhibit calcium currents through voltage-gated Ca^{2+} channels in SASMC, resulting in a decrease in $[Ca^{2+}]_i$ and relaxation (Franco-Obregon et al., 1995; Lopez-Barneo et al., 1999; Saitoh et al., 2006; Smani et al., 2002). Michelakis et al. reported that ROS generation increases during hypoxia in renal PA, as assessed by lucigenin, Amplex Red, and DCF (Michelakis et al., 2002). A similar hypoxia-induced shift in the redox status of SASMC to a more oxidized state was observed by Gupte et al, who measured hypoxia-induced decreases in the $[NADPH]/[NADP^+]$ and

[GSH]/[GSSG] ratios of bovine coronary arteries (Gupte and Wolin, 2006). It was also reported that hypoxia-induced vasodilation was attenuated by the thiol-reducing agent DTT (3 mM) (Gupte and Wolin, 2006). These disparate redox responses of the PASM and SASM to hypoxia lead Michelakis et al. to conclude that tissue-specific differences in mitochondrial function explain the tissue-specific responses to hypoxia (Michelakis et al., 2002). However, Mehta et al. detected decreases in ROS generation in both PASM and coronary artery smooth muscle cells using DCF, dihydroethidium, and Amplex Red (Mehta et al., 2008). We employed the roGFP sensor to examine ROS signaling in SASM. When targeted to the IMS or the cytosol, roGFP detected increases in hypoxia-induced ROS signaling in SASM that were similar to those measured in PASM (Figure 2) (Waypa et al., 2010). Likewise, hypoxia elicited decreases in matrix oxidation during hypoxia. However, hypoxia triggered increases in $[Ca^{2+}]_i$ in PASM, whereas it elicited decreases in SASM (Waypa et al., 2010). These findings suggest that PASM and SASM share a similar O_2 sensor and signaling mechanism, while differences in the downstream signaling pathways lead to opposite functional responses to hypoxia (Figure 3). Possible mechanisms of hypoxia-induced relaxation in SASM include the inhibition of calcium entry, inhibition of intracellular release, and accelerated uptake by the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) pump (Gupte and Wolin, 2006).

4. Mitochondria and extramitochondrial O_2 sensors

A growing body of work indicates that ROS signaling increases during hypoxia in a wide range of cell types, and that this redox response is required for the functional response to hypoxia (Chandel et al., 1998; Duranteau et al., 1998; Gusarova et al., 2009; Guzy et al., 2008; Lavani et al., 2007; Liu et al., 2003; Mansfield et al., 2005; Pastukh et al., 2007; Vanden Hoek et al., 1998; Waypa et al., 2001; Waypa et al., 2006; Waypa et al., 2002). Several pieces of evidence implicate the mitochondria as the site of O_2 sensing. Based on measurements in subcellular compartments, it appears that generation of ROS near the outer surface of the inner membrane is responsible for the cytosolic oxidant shift, rather than from the leakage of ROS from the matrix to the cytosol. Genetic knockdown studies indicate that Complex III function is required for the ROS signaling and for the downstream responses (Guzy et al., 2005), however the molecular details of this process are not yet clear. Recently, a naturally occurring small molecule was found to interact with the cellular oxygen sensor in a highly specific manner. The molecule was identified in a large-scale screen of crude extracts collected from a library of microbial species. Initially, a crude extract that strongly inhibited the functional response to hypoxia in human endothelial cells (HUVEC) *in vitro* was identified from the fungal strain, *Embellisia chlamydospora* (Jung et al., 2010). Fractionation studies identified the active molecule as a bicyclo sesterterpene, terpestacin. Administration of the purified compound to cultured HUVECs recapitulated the effects of the crude extract on the hypoxic response, and administration to mice attenuated tumor growth and tumor angiogenesis in murine xenograft studies. Using a phage-display analysis, the protein target of the compound was identified as UQCRB, a 13.4 kDa subunit component of Complex III. Consistent with that interaction, studies with the fluorescently labeled compound indicated that terpestacin accumulation in the cell colocalized with mitochondria. Subsequent studies revealed that terpestacin inhibits the hypoxia-induced increase in ROS generation from mitochondria, and that it inhibits the hypoxia-induced stabilization of HIF-1 α without abolishing the response to hypoxia mimetic drugs such as deferoximine. Thus, it appears that terpestacin blocks the hypoxic response of cells by binding to Complex III, inhibiting the generation of an ROS signal in hypoxia, and thereby blinding the cell to the presence of hypoxia. This appears to blunt tumor growth by interfering with the hypoxia-induced expression of vascular mitogens through an attenuation of HIF-1 activation. To be sure, the mitochondrial inhibitors stigmatellin and myxothiazol also block the hypoxic ROS signal and HIF-1 α stabilization, but they do so by preventing

electron entry into Complex III. A consequence of that effect is the inhibition of oxidative phosphorylation by the mitochondria, which produces a bioenergetic crisis in many cells. By contrast, terpestacin appears to inhibit the ability of Complex III to function as an O₂ sensor without interfering with the overall electron transport process required for ATP generation below the toxic threshold. In that regard, terpestacin interferes with only one of two biological functions of Complex III. Is UQCRB an oxygen sensor by itself? This possibility seems unlikely, as there is no evidence that this small subunit can interact with molecular O₂ in the absence of the remainder of the Complex. More likely, the binding of terpestacin to UQCRB may alter the function of the intact Complex in a manner that interferes with the ability to increase ROS generation from the Qo site near the outer surface of the IMS, thereby abrogating the generation of a signal that activates the cellular response to hypoxia. However, further work is needed to fully understand the relationship between UQCRB and the oxygen sensing function of Complex III. In any case, the discovery of a naturally occurring molecule that specifically targets the oxygen sensing function of Complex III, identified through an unbiased screen of a microbial library, provides strong independent confirmation that Complex III is a critical component of a mitochondrial oxygen sensing system.

ROS production in the mitochondrial matrix can also occur at Complex II, which consists of four polypeptide subunits A-D. Paddenberget al. suggested the involvement of this Complex in HPV through the reversal of Complex II activity from succinate dehydrogenase to fumarate reductase, thus increasing ROS production during hypoxia (Paddenberget al., 2003). Finally, ROS do not appear to be generated by Complex IV (Fabian and Palmer, 1998), due to the high-affinity trapping of O₂ at the binuclear center (Verkhovsky et al., 1996). Cyanide, which blocks Complex IV, appears to induce smooth muscle contraction during normoxia by causing electrons to backup in Complexes I, II and III, causing those sites to become fully reduced and increasing the generation of superoxide from reduced flavin groups (Archer et al., 1993; Leach et al., 2001; Waypa et al., 2001; Waypa et al., 2006; Waypa et al., 2002).

There is some evidence that hypoxia-induced ROS generation may also originate from non-mitochondrial sources. In that regard it is possible that a NOX system contributes to or amplifies the hypoxia-induced ROS generation, thereby affecting redox conditions in the cytosol and IMS (Weissmann et al., 2006b). While previous research suggests that the mitochondria are the principal source of the hypoxia-induced ROS signal (Chandel et al., 1998; Guzy et al., 2005; Guzy et al., 2007; Leach et al., 2001; Rathore et al., 2008; Wang et al., 2007; Waypa et al., 2001; Waypa et al., 2006; Waypa et al., 2002; Weissmann et al., 2003), Rathore et al. suggested that hypoxia-induced mitochondrial ROS signaling triggers NAD(P)H oxidase activation through PKC_ε activation (Rathore et al., 2008). This would provide a mechanism by which both mitochondria and cytosolic oxidant systems might contribute to the overall increase in ROS signaling during hypoxia.

5. Concluding remarks

Recent advancements in redox sensors have increased our understanding of how mitochondria, functioning as O₂ sensors, can detect a decrease in oxygen tension and translate that into a signal that triggers adaptive responses (Figure 3). The fact that both PASM and SASMC demonstrate similar changes in hypoxia-induced ROS signaling in various sub-cellular compartments while triggering different downstream responses suggests that the mitochondria may coordinate diverse responses to hypoxia in wide range of cell types. The identification of new small molecules that target the mitochondria and that modify the ROS signaling during hypoxia provides further evidence for the role of Complex III in the oxygen-sensing pathway. Additional progress is needed in the development of

genetic tools to moderate and measure ROS signaling, while other studies are needed to identify the downstream targets of ROS signals that mediate their functional responses.

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Figure 1.

Hypoxia increases regulated ROS signaling in the cytosol and IMS of PASM C while decreasing non-specific ROS signaling in the mitochondrial matrix. The redox sensor roGFP was targeted to the cytosol (Cyto-roGFP), the intermembrane space of mitochondria (IMS-roGFP), and the mitochondrial matrix (Mito-roGFP) of PASM C, which were then superfused under controlled O_2 conditions. Hypoxia (1.5% O_2) caused an increase in the oxidation of roGFP in the cytosol and IMS, while it decreased oxidation in the mitochondria matrix. (Data replotted from a previously published report (Waypa et al., 2010)).

Figure 2.

Hypoxia increases regulated ROS signaling in the cytosol and IMS of SASMC while decreasing ROS signaling in the mitochondrial matrix. The redox sensor roGFP was targeted to the cytosol (Cyto-roGFP), the IMS (IMS-roGFP), and the mitochondrial matrix (Mito-roGFP) of SASMC, which were then superfused under controlled O_2 conditions. Hypoxia (1.5% O_2) increased oxidation of roGFP in the cytosol and IMS, and decreased oxidation in the mitochondria matrix. (Data replotted from a previously published report (Waypa et al., 2010)).

Figure 3.

Oxygen sensing model underlying hypoxia-induced responses in pulmonary and systemic vascular cells. PASM, pulmonary arterial smooth muscle cell; SASM, systemic arterial smooth muscle cell; CRAC, Ca²⁺ release activated channel; TRPC6, transient receptor potential channel-6; STIM1, stromal-interacting molecule-1; SR, sarcoplasmic reticulum; DAG, diacylglycerol; RyR, ryanodine receptors; cADPR, cyclic ADP ribose; AMPK, AMP kinase; ROS, reactive oxygen species; SERCA, sarco(endo)plasmic reticulum Ca²⁺-ATPase pump; IMS, intermembrane space.