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INTERACTIONS BETWEEN CALCIUM AND REACTIVE OXYGEN SPECIES IN PULMONARY ARTERIAL SMOOTH MUSCLE RESPONSES TO HYPOXIA

Larissa A. Shimoda and **Clark Undem**
Division of Pulmonary and Critical Care Medicine, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21224

Abstract

In contrast to the systemic vasculature, where hypoxia causes vasodilation, pulmonary arteries constrict in response to hypoxia. The mechanisms underlying this unique response have been the subject of investigation for over 50 years, and still remain a topic of great debate. Over the last 20 years, there has emerged a general consensus that both increases in intracellular calcium concentration and changes in reactive oxygen species (ROS) generation play key roles in the pulmonary vascular response to hypoxia. Controversy exists, however, regarding whether ROS increase or decrease during hypoxia, the source of ROS, and the mechanisms by which changes in ROS might impact intracellular calcium, and vice versa. This review will discuss the mechanisms regulating $[Ca^{2+}]_i$ and ROS in PASMCs, and the interaction between ROS and Ca^{2+} signaling during exposure to acute hypoxia.

1. Introduction

A unique aspect of the pulmonary circulation is the pressor response to hypoxia. While the systemic circulation dilates in response to decreased oxygen (O_2) concentrations in order to increase blood flow and O_2 delivery to tissues, alveolar hypoxia rapidly increases pulmonary vascular resistance beginning within 1–2 minutes after a drop in O_2 levels. Vasoconstriction is maintained for the duration of the hypoxic exposure and rapidly reverses with reoxygenation. This mechanism is thought to divert blood flow from regions of the lung where ventilation is poor in an effort to maintain arterial O_2 tension. However, complications arise when alveolar hypoxia is global and prolonged, as can occur with residence at high altitude or with many chronic lung diseases, resulting in the development of pulmonary hypertension.

The exact mechanism underlying the generation of hypoxia-induced increases in pulmonary vascular tone is unknown. Over the past 50 years, numerous studies have sought to determine whether the hypoxic response is localized to pulmonary arterial smooth muscle cells (PASMCs), or whether signals from the circulation or neighboring cells, (i.e, the endothelium) are required. It is now generally well accepted that while the endothelium can

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Address for Correspondence: Larissa A. Shimoda, PhD, Division of Pulmonary and Critical Care Medicine, Johns Hopkins University, 5501 Hopkins Bayview Circle, JHAAC 4A.52, Baltimore, MD 21224, 410-550-1013, FAX: 410-550-2612, shimodal@welch.jhu.edu.

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influence the magnitude of the response, the PASMCs themselves sense and respond to the hypoxic stimulus.

The smooth muscle cells surrounding the pulmonary arteries provide structural integrity for the vascular wall and are vital for precise regulation of vessel tone, pulmonary arterial pressure and pulmonary vascular resistance. The control of PASMC function is complex, and it has become increasing well recognized that increases in intracellular calcium concentration ($[Ca^{2+}]_i$) and changes in reactive oxygen species (ROS) generation play key roles in hypoxic pulmonary vasoconstriction (HPV). This review will focus on the interaction between ROS and Ca^{2+} signaling in PASMCs during exposure to acute hypoxia.

2. Ca^{2+} signaling in PASMCs

2.1. Mechanisms regulating $[Ca^{2+}]_i$ in PASMCs

$[Ca^{2+}]_i$ is one of the most important regulators of pulmonary vascular function. In PASMCs, changes in $[Ca^{2+}]_i$ modulate contraction(Buckley et al., 1995; Busse and Mulsch, 1990; Emori et al., 1989; Kohno et al., 1992; Martin and Michaelis, 1990; Whorton et al., 1984), cell proliferation and growth by facilitating progression through the cell cycle(Mogami and Kojima, 1993; Rodman et al., 2005; Shukla et al., 2005) and the activation of genes via regulation of Ca^{2+}-sensitive transcription factors(Altura et al., 2003; Ginty et al., 1991; Hardingham et al., 1998; Pribnow et al., 1992; Rothman et al., 1994). That Ca^{2+} signaling plays a crucial role in a wide array of cell functions underscores the importance of understanding the mechanisms regulating Ca^{2+} homeostasis under both physiologic and pathophysiologic conditions.

PASMCs express several Ca^{2+} influx/efflux pathways that may contribute to Ca^{2+} handling and regulation (Figure 1). Plasmalemmal Ca^{2+} channels conduct extracellular Ca^{2+} into the cells, while receptors on the sarcoplasmic/endoplasmic reticulum mediate Ca^{2+} release from internal stores into the cytoplasm. Mechanisms for removing cytoplasmic Ca^{2+} from the cell include the plasma membrane Ca^{2+}-ATPase (PMCA) and Na^+/Ca^{2+} exchange (NCX), the activities of which help to maintain low basal $[Ca^{2+}]_i$.

PASMCs are excitable cells, containing both L-type(Amenta et al., 1998; Fan et al., 2002; Golovina et al., 2001; Resnik et al., 2006; Ricci et al., 2000; Wang et al., 2005; Yuan, 1995) and T-Type(Rodman et al., 2005) voltage-gated Ca^{2+} channels (VGCC). As suggested by their name, VGCC activity is controlled in large part by membrane potential (E_m). L-type, or long-lasting, Ca^{2+} channels exhibit sensitivity to dihydropyridines and are activated by large depolarizations at potentials between −20 and +20 mV. At the normal resting E_m, which in PASMCs appears to be controlled primarily by voltage-gated K^+ (K_V) channels, little activation of these channels occurs, resulting in low basal PASMC $[Ca^{2+}]_i$ and tone(Shimoda et al., 2000). However, when K_v channels are inhibited, the consequent depolarization activates VGCCs and increases $[Ca^{2+}]_i$(Yuan, 1995). T-type, or transient, Ca^{2+} channels have also been identified in PASMCs(Muramatsu et al., 1997; Rodman et al., 2005). T-type channels require small depolarizations in order to activate, are only activated from very negative holding potentials, conduct inward currents at membrane potentials between −70 and 40 mV and typically inactivate rapidly at physiological membrane potentials(Kuga et al., 1990; Mishra and Hermsmeyer, 1994; Wu et al., 2003).

Numerous studies have demonstrated that Ca^{2+} influx into PASMCs could occur via Ca^{2+} permeable channels other than VGCCs. In particular, nonselective cation channels (NSCCs) provide an important Ca^{2+} entry pathway. Based on their proposed modes of activation, these influx pathways were separated into 2 main categories: receptor-operated Ca^{2+} channels (ROCC), which are activated by ligand binding to membrane receptors, and store-

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operated Ca^{2+} channels (SOCCs), which are activated by depletion of intracellular stores(Putney, 1986, 1990). While the activity of VGCCs is primarily driven by depolarization, the activation of NSCCs is relatively voltage-independent. Several labs have demonstrated that Ca^{2+} influx through NSCCs occurs in PASMCs(Golovina et al., 2001; Snetkov et al., 2003; Wang et al., 2004a), and contributes to PASMC contraction and growth(Golovina et al., 2001; McDaniel et al., 2001; Sweeney et al., 2002; Weigand et al., 2006; Yu et al., 2003); however, these channels do not appear to be active under basal conditions(Lin et al., 2004; Wang et al., 2006; Wang et al., 2004b). The exact molecular identity of the proteins encoding Ca^{2+}-permeable NSCCs remains unclear, although most evidence suggests that these channels may be composed of mammalian homologs of transient receptor potential (TRP) proteins, perhaps in combination with the recently identified ORAI1 and stromal interaction molecule (STIM) proteins(Brough et al., 2001; Cioffi et al., 2003; Lin et al., 2004; Lu et al., 2009; Lu et al., 2008; Ng and Gurney, 2001; Sweeney et al., 2002; Wang et al., 2004a; Wang et al., 2006; Yang et al., 2006; Yu et al., 2003).

There are many sites within PASMCs that can store Ca^{2+}, including mitochondria, lysosomes, and the sarcoplasmic (SR). Of these, the SR is the major contributor in regulating cytosolic $[Ca^{2+}]$(Marin et al., 1999; Pozzan et al., 1994) through release of Ca^{2+} from channels on the SR membrane or uptake of Ca^{2+} into the stores via the sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA) pumps(Boittin et al., 1999; Laporte et al., 2004; Wellman and Nelson, 2003). The channels mediating Ca^{2+} release from intracellular stores can be divided into two categories: ryanodine receptors (RyR), which can be blocked by ryanodine and are activated by caffeine, and inositol triphosphate (IP_3) receptors (IP_3R), which can be activated by IP_3 following agonist stimulation. RyR also contain Ca^{2+} binding sites, leading to Ca^{2+}-induced Ca^{2+} release, wgich can be triggered by an increase in cytosolic $[Ca^{2+}]$ due to either IP_3-induced release or Ca^{2+} influx from extracellular sources(Zucchi and Ronca-Testoni, 1997) . Similarly, activation of IP_3R by Ca^{2+} is facilitated by binding of IP_3. Three subtypes of RyR have been identified in PASMCs(Yang et al., 2005; Zhang et al., 2003; Zheng et al., 2005), along with three subtypes of IP_3 receptors(Zheng et al., 2004). Whether RyRs and IP_3Rs access Ca^{2+} stores that are separate and distinct(Flynn et al., 2001) or are the same pools(Pacaud and Loirand, 1995) remains in debate, and may depend on vascular bed(Janiak et al., 2001) or whether cells are freshly isolated or cultured(Ng et al., 2008).

2.2. Mechanisms that control $[Ca^{2+}]_i$ in response to hypoxia

The main site of vasoconstriction in response to hypoxia appears to be the small diameter, resistance arteries, since these vessels exhibit a robust contraction in response to reduced O_2 tensions whereas conduit arteries exhibit relaxation or minimal contraction(Dawson et al., 1978; Fishman, 1976; Madden et al., 1985; Nagasaka et al., 1984; Staub, 1985). In PASMCs isolated from resistance arteries, abundant data has been generated in a variety of species demonstrating that acute exposure to hypoxia (minutes to a few hours) is associated with an increase in $[Ca^{2+}]_i$ that is largely dependent on Ca^{2+} entry from the extracellular compartment(Cornfield et al., 1994; Hong et al., 2004; Kang et al., 2002; Salvaterra and Goldman, 1993; Sham et al., 2000; Urena et al., 1996; Vadula et al., 1993; Wang et al., 2005). Consistent with a role for Ca^{2+} influx, perfusion with Ca^{2+}-free solution reduced HPV to a small transient constriction or abolished it altogether(Dipp et al., 2001; Farrukh and Michael, 1992; Jin et al., 1992; Liu et al., 2001; Robertson et al., 2000; Weigand et al., 2005).

Pharmacologic data indicates that L-type Ca^{2+} channels contribute significantly to the increase in PASMC $[Ca^{2+}]_i$ induced by acute reductions in O_2 tension(Bakhramov et al., 1998; Cornfield et al., 1994; Urena et al., 1996; Wang et al., 2005; Yuan, 1995) and to

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HPV(McMurtry et al., 1976; Redding et al., 1984; Simonneau et al., 1981; Stanbrook et al., 1984; Weigand et al., 2005). However, despite a strong case for VGCC being a main contributor to the Ca^{2+} influx due to hypoxia, recent studies indicate the presence of VGCC antagonists reduced, but did not abolish, hypoxia-induced increases in $[Ca^{2+}]_i$ and Ca^{2+} entry in PASMCs(Ng et al., 2005; Wang et al., 2005). While experiments examining the effect of specific inhibitors of T-type Ca^{2+} channels, such as mibefradil, will need to be performed to definitively determine their role in the maintenance of $[Ca^{2+}]_i$ during hypoxia, mounting evidence indicates that hypoxia-induced increases in $[Ca^{2+}]_i$ can be abolished by antagonists of NSCCs(Ng et al., 2005; Wang et al., 2005; Weissmann et al., 2006). Since release from intracellular stores appears to be a component of the hypoxia-induced increase in $[Ca^{2+}]_i$(Dipp et al., 2001; Jabr et al., 1997; Kang et al., 2002; Zheng et al., 2004; Zheng et al., 2005), a likely scenario is that Ca^{2+} entry via NSCCs is triggered by store-depletion. Store release in PASMCs in response to hypoxia appears to occur, at least in part, via activation of RyRs(Dipp et al., 2001; Zheng et al., 2005). Blockade of RyRs or depletion of the SR with thapsigargin or cyclopiazonic acid inhibited hypoxia-induced increases in PASMCs $[Ca^{2+}]_i$(Vadula et al., 1993; Zheng et al., 2005) and HPV(Dipp et al., 2001; Du et al., 2005; Leach et al., 1994; Liu et al., 2001; Morio and McMurtry, 2002; Robertson et al., 2000; Zheng et al., 2005). These results indicate that hypoxia induces an initial increase in $[Ca^{2+}]_i$ due to Ca^{2+} release from intracellular stores, with a sustained increase in $[Ca^{2+}]_i$ due to Ca^{2+} influx, likely through both NSCCs and VGCCs. Interestingly, blockade of NSCCs completely prevents the increase in $[Ca^{2+}]_i$ induced by hypoxia, whereas blockade of VGCCs only partially inhibited the response(Wang et al., 2005), suggesting the possibility that activation of NSCCs not only leads to Ca^{2+} influx, but also may provide a depolarizing stimulus that can subsequently contribute to the activation of VGCCs.

3. ROS signaling in PASMCs

3.1. Types of ROS and mechanisms of ROS production

It is now recognized that another major component of PASMC signaling is the generation of ROS; O_2-derived small molecules, including the O_2 radicals superoxide and hydroxyl, and nonradicals that are either oxidizing agents or are easily converted into radicals, such as ozone (O_3), singlet O_2, and hydrogen peroxide (H_2O_2). ROS avidly interact with a variety of molecules and have been identified as major contributors to cellular damage, aging and host defense. It has also become clear that ROS play an important role in reversible regulatory processes. For example, H_2O_2 influences pulmonary vasomotor tone(Jin and Rhoades, 1997; Rhoades et al., 1990) and modifies the function of various proteins including transcription factors, kinases, and phosphatases(Droge, 2002; Franklin et al., 2006; Ichiki et al., 2003; Jin et al., 2000; McCubrey et al., 2006; Rao, 2000; Schmidt et al., 1995).

ROS are produced from a cascade of reactions that starts with the production of superoxide, generated by mitochondrial respiration, xanthine oxidase, uncoupled NO synthase, or via reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase (Nox). Superoxide is highly reactive and rapidly dismutates to H_2O_2, either spontaneously or catalyzed by superoxide dismutase. Superoxide can also react with nitric oxide to form peroxynitrite, or the iron-catalyzed Fenton reaction leading to the generation of hydroxyl radicals. Of the mechanisms known to produce ROS, evidence suggests that during hypoxia, the mitochondria and Nox appear to be the predominate sources in PASMCs.

3.1.a. Mitochondria—In mitochondria, a series of redox reactions occurs during respiration along which electrons are transferred from a donor molecule (NADH or QH_2) to O_2, concluding at complex IV (cytochrome oxidase), where molecular O_2 is reduced to water (Figure 2). Formation of superoxide occurs upstream of complex IV, primarily by auto-oxidation of flavins in complex I, where superoxide can enter the matrix, and at

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complex III where superoxide is formed via the Q-cycle. From complex I, two electrons are carried by ubiquinol (QH_2) to complex III (coenzyme Q: cytochrome *c*-oxidoreductase). Another pair of electrons is donated to ubiquinone (Q) at complex II, and also carried by ubiquinol to complex III, where one electron is transferred at the Qo site to the Rieske iron–sulphur protein (Fe-S) and subsequently cytochrome c (Cyt c), while the remaining electron is donated to cytochrome b (bl and bh), followed by transfer to ubiquinone at the Qo site. Cytochrome c then donates the electrons to molecular O_2 in complex IV, producing water. Electron leakage can occur at both the Qo and Qi sites of complex III, resulting in production of superoxide that enters either the mitochondrial intermembrane space or the matrix, respectively(Boveris, 1977;Turrens, 2003;Turrens et al., 1985). Once in the matrix, superoxide is converted first to H_2O_2, by mitochondrial superoxide dismutase, and then to water by glutathione peroxidase (Gpx1). In the inter-membrane space, superoxide can undergo multiple fates; it can be degraded by CuZnSOD, can be scavenged by cytochrome c or can enter the cytosol via voltage-dependent anion channels(Han et al., 2003;Waypa et al., 2001). The relative contribution of complex I and III to superoxide generation appears to be cell and/or tissue specific and dependent on the respiratory status. In PASMCs, rotenone, a complex I inhibitor, and myxothiazol and antimycin A, which prevent production of radicals by inhibiting complex III at the pre- and post-ubisemiquinone sites, respectively, decreased basal ROS production(Archer et al., 1993;Michelakis et al., 2002b;Paky et al., 1993;Waypa et al., 2001), suggesting that constitutive superoxide generation is occurring at both sites.

3.1.b. NADPH oxidase—A second important source of ROS is via Nox. Originally described in phagocytes, five Nox isoforms (Nox1-5) have now been identified. Nox produces superoxide or H_2O_2 via electron transfer from cytosolic NADPH to FAD, and subsequently to molecular O_2 to form superoxide, which is then dismutated to H_2O_2. In vascular smooth muscle, Nox 1, 2, 4 and 5 are expressed and contribute to generation of ROS, although the exact distribution of isoforms varies with vascular bed and species(Fulton, 2009; Lassegue and Griendling, 2010). Nox1 and Nox2, or gp91 phox, are membrane bound and require the assembly of additional membrane associated (p22phox) or cytosolic (Rac 1 and 2, p47phox, and p67phox) subunits for activation(Lambeth et al., 2007) (Table 1), and have been implicated in a variety of cardiovascular diseases(Lassegue and Griendling, 2010). Nox4, also abundant in pulmonary vascular smooth muscle(Ismail et al., 2009; Mittal et al., 2007; Rathore et al., 2008), differs from Nox1 and Nox2 in that it exhibits constitutive activity, does not appear to require additional subunits other than p22phox(Lassegue and Griendling, 2010) and appears to produce primarily H_2O_2 rather than superoxide(Serrander et al., 2007a), although the mechanism by which this occurs is unclear. In contrast to the other Nox isoforms, Nox5 is not expressed in rodents, does not require p22phox or the cytosolic subunits and is the only isoform that is directly activated by Ca^{2+} due to four canonical EF-hands in the N-terminus(Jagnandan et al., 2007; Serrander et al., 2007b). In addition, the NADPH-binding domain of Nox5 contains a calmodulin binding site(Tirone and Cox, 2007), and PKC-mediated phosphorylation of Ser/Thr residues in the FAD-binding domain may increase Ca^{2+}-sensitivity(Jagnandan et al., 2007).

3.2. ROS generation in response to hypoxia

Numerous investigators have demonstrated changes in ROS production in PASMCs during hypoxia, suggesting that ROS may be key mediators in HPV; however, controversy exists concerning whether ROS increase or decrease during hypoxia. Since O_2 is the major substrate for ROS formation and hyperoxia increased ROS generation(Brueckl et al., 2006; Chandel and Budinger, 2007; Freeman and Crapo, 1981), it was reasonable to hypothesize that, due to substrate limitation, hypoxia would decrease ROS. Indeed, several reports indicated that this was the case, contending that either mitochondrial production of ROS was impaired(Archer and Michelakis, 2002; Archer et al., 1993; Michelakis et al., 2002a), or that

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there was a decrease in ROS generation by microsomal NADH oxidoreductase(Mohazzab et al., 1995; Mohazzab and Wolin, 1994a, b). Reports of hypoxia-induced decreases in ROS production were countered, however, by numerous labs which consistently showed increased ROS generation in response to hypoxia(Jernigan et al., 2004; Killilea et al., 2000; Leach et al., 2001; Liu et al., 2003; Marshall et al., 1996; Paddenberg et al., 2003; Rathore et al., 2006; Wang et al., 2007; Waypa et al., 2001; Waypa et al., 2006; Waypa and Schumacker, 2002, 2005; Weissmann et al., 2003). The mechanism by which hypoxia might induce an increase in mitochondrial ROS production remains unresolved; however, it has been hypothesized that decreased interaction between O_2 and protein or lipids at complex III could prolong the lifetime of ubisemiquinone or that hypoxia might increase the access of O_2 to the semiquinone radical at complex III. In both cases, O_2 levels cause alterations in the lipid–protein structure such that electron transfer from ubisemiquinone to O_2 increases, despite decreased availability of oxygen.

The reasons underlying discrepant reports regarding the effects of hypoxia on ROS remain incompletely understood. Some investigators have attempted to use pharmacological inhibitors of mitochondrial-derived ROS to resolve the controversy, with varying results. Most agree that rotenone blocks the hypoxia-induced changes in ROS, regardless of whether the response was an increase(Rathore et al., 2006; Wang et al., 2007; Waypa et al., 2001; Waypa et al., 2006; Waypa and Schumacker, 2002; Weissmann et al., 2003) or decrease(Archer et al., 1993; Michelakis et al., 2002a). Myxothiazol also blocked the acute hypoxic responses in PASMCs(Archer et al., 1993; Michelakis et al., 2002a; Wang et al., 2007; Waypa et al., 2001; Waypa et al., 2006; Waypa and Schumacker, 2002; Weissmann et al., 2003), although more distal electron transport chain inhibitors, such as antimycin-A, had no effect. These data suggest that the mitochondrial subunits prior to the ubisemiquinone site of complex III act to increase generation of ROS in PASMCs during hypoxia.

In addition to mitochondria, studies have also indicated a role for Nox mediated generation of ROS during hypoxia. Acute hypoxia increased Nox activity and translocation of p47phox to the plasma membrane in pulmonary arteries(Rathore et al., 2008). Consistent with these findings, the putative Nox inhibitors, apocynin and diphenyleneiodonium, blocked the hypoxic increase in ROS in PASMCs(Marshall et al., 1996; Rathore et al., 2008); however, a caveat is that both of these inhibitors can have nonspecific effects, as diphenyleneiodonium is a broad spectrum inhibitor of electron transporters(Bedard and Krause, 2007) and apocynin is able to act as an antioxidant independent of its effects on Nox(Heumuller et al., 2008). Nonetheless, if activation of Nox did occur in these studies, it is likely this was secondary to hypoxic activation of PKC, since inhibition of PKCε blocked the hypoxia-induced Nox activity and increase in ROS. Interestingly, rotenone and myxothiazol both attenuated the hypoxia-induced activation of PKCε activity(Rathore et al., 2006), suggesting a link between mitochondrial and Nox generated ROS.

To complement pharmacologic studies, several investigators have used genetic alterations to further explore the role of mitochondrial proteins in the hypoxic ROS response. Overexpression of mitochondrial catalase and Gpx1, which would be expected to augment ROS scavenging, attenuated hypoxia-induced increases in ROS(Wang et al., 2007; Waypa et al., 2006), whereas Gpx1 gene deletion to prevent ROS removal has the opposite effect(Wang et al., 2007). Furthermore, in nonvascular cells, silencing subunits of the mitochondrial electron transport chain reduced hypoxia-induced ROS signaling(Guzy et al., 2005; Mansfield et al., 2005), suggesting that the mitochondria are a primary source for elevating ROS levels. Interestingly, lungs from $p47^{phox}$ knockout mice increased ROS in response to hypoxia, whereas a reduction in ROS was observed in wild-type mice, suggesting that reduced production of extracellular ROS derived from Nox might mask an increase in intracellular mitochondrial-derived ROS under some experimental

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conditions(Weissmann et al., 2006). It is also possible that within the cell, ROS generation is a localized event and that compartmentalization of either ROS or the indicator could produce differential results. With respect to the indicator, experiments using recently developed ratiometric, redox-sensitive fluorescence resonance energy transfer (FRET) probes indicated that hypoxia-induced an increase in ROS which was inhibited by catalase and antioxidants(Waypa et al., 2006; Waypa et al., 2010). Consistent with the possibility of compartmentalized ROS production, recent reports using indicators targeted to the mitochondrial matrix, intermembrane space and cytoplasm show that ROS levels vary within these compartments, with hypoxia increasing ROS in the cytosol and intermembrane space but decreasing ROS in the mitochondrial matrix(Waypa et al., 2010). These results suggest the possibility that the differences reported regarding the effects of hypoxia on ROS formation in PASMCs could reflect a lack of differentiation between extracellular and intracellular sources of ROS or measurements of intracellular ROS within different sub-compartments of the cell. Overall, however, it would appear that the predominance of evidence argues for increased cytosolic ROS during hypoxia, with a fall in ROS occurring extracellularly and within the mitochondrial matrix.

4. Interplay between ROS and $[Ca^{2+}]_i$

4.1. ROS modulate $[Ca^{2+}]_i$

Substantial evidence has accumulated demonstrating that ROS can influence vascular smooth muscle $[Ca^{2+}]_i$. With respect to hypoxia and PASMCs, in experiments where a ratiometric ROS probe was used in conjunction with a FRET-based ratiometric Ca^{2+} sensitive probe, the increase ROS and $[Ca^{2+}]_i$ appeared to occur virtually simultaneously(Waypa et al., 2006). Moreover, catalase, antioxidants and pharmacological inhibitors of the potential sources of ROS, including rotenone and myxothiazol, reduced or prevented the hypoxia-induced elevation in $[Ca^{2+}]_i$ in PASMC(Leach et al., 2001; Rathore et al., 2006; Wang et al., 2007; Waypa et al., 2006). In contrast, antimycin A or cyanide, which inhibits Complex IV, had no effect on or enhanced the $[Ca^{2+}]_i$ response to hypoxia(Leach et al., 2001; Rathore et al., 2006; Wang et al., 2007; Waypa et al., 2006; Waypa and Schumacker, 2002). Consistent with these results, HPV was prevented in the presence of rotenone or myxothiazol(Archer et al., 1993; Leach et al., 2001; Rounds and McMurtry, 1981; Waypa et al., 2001; Weissmann et al., 2003), but not by cyanide(Archer et al., 1993; Leach et al., 2001; Waypa et al., 2001; Weissmann et al., 2003).

In the case of studies where ROS was found to decrease during hypoxia, it has been proposed that reduced ROS leads to inhibition of K^+ channels and depolarization, with subsequent activation of VGCCs and increased $[Ca^{2+}]_i$(Archer and Michelakis, 2002; Archer et al., 2008; Archer et al., 1993; Michelakis et al., 2002a; Michelakis et al., 2002b; Michelakis et al., 2004; Reeve et al., 1995). On the other hand, ROS have been shown to induce Ca^{2+} release from ryanodine-sensitive stores(Lin et al., 2007; Pourmahram et al., 2008; Suzuki et al., 1998) either via action on redox-sensitive cysteine thiols in RyRs(Eu et al., 1999) or stimulation of RyRs via generation of the β-NAD^+ metabolite, cyclic ADP-ribose(Dipp and Evans, 2001; Evans and Dipp, 2002). Release of SR Ca^{2+} could then serve to increase Ca^{2+} entry via store-dependent NSCCs(Wang et al., 2005) and/or activation of VGCCs, secondary to inhibition of K^+ channels and depolarization(Post et al., 1995). Elevated H_2O_2 levels could also initiate an increase in $[Ca^{2+}]_i$ through the activation of phospholipase C(Gonzalez-Pacheco et al., 2002), resulting in the production of both IP_3 and diacylglycerol. While IP3 generation would lead to Ca^{2+} release from internal stores, diacylglycerol could serve to activate NSCCs, resulting in Ca^{2+} influx, as well as activation of VGCCs secondary to depolarization(Weissmann et al., 2006). Superoxide and peroxynitrite have also been shown to inhibit SERCA in systemic vascular smooth muscle(Grover et al., 2003; Suzuki and Ford, 1991), which would serve to elevate cytosolic

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[Ca^{2+}] by decreasing Ca^{2+} uptake into the SR; however, whether this occurs in PASMCs has not been examined.

4.2. $[Ca^{2+}]_i$ influences ROS generation

While several studies provide evidence that ROS alter $[Ca^{2+}]_i$, as described in the preceding section, several reports also indicate that changes in $[Ca^{2+}]_i$ may impact ROS formation. With respect to Nox isoforms, only Nox5 is directly Ca^{2+}-sensitive by virtue of four canonical N-terminal EF-hands(Fulton, 2009). Nox5 also contains a binding site for calmodulin in the NADPH-binding domain and PKC-mediated phosphorylation of Ser/Thr residues in the FAD-binding domain may increase the Ca^{2+}-sensitivity of Nox5(Fulton, 2009). In contrast, $[Ca^{2+}]_i$ may control the activity of other Nox isoforms by regulating the cytosolic subunits. For example, under certain circumstances, Nox-dependent generation of ROS requires serine phosphorylation of p47phox, which occurs in response to activation of protein kinase C(Lassegue and Griendling, 2010). Although the specific PKC isozyme(s) responsible for mediating phosphorylation of p47phox has not been identified, it is possible that conventional, or Ca^{2+}-sensitive, PKC isozymes might be involved. On the other hand, recent evidence suggests that Nox activation in PASMCs during hypoxia is mediated by PKCs(Rathore et al., 2008), which is not Ca^{2+}-sensitive; however, in this case ROS production was only measured during brief hypoxic challenge, and it is not clear whether longer duration of hypoxia might lead to Ca^{2+}-dependent activation of Nox and generation of ROS.

5. Conclusions

Over the last two decades, extensive work has been performed aimed at identifying the role of ROS in the pulmonary vascular response to hypoxia. While abundant evidence now indicates that ROS are an essential factor in HPV, there continues to be some debate regarding the direction of the change in ROS with hypoxia, and how ROS might interact with other signaling pathways, namely $[Ca^{2+}]_i$, to produce HPV. The preponderance of evidence appears to indicate that hypoxia induces an increase in ROS formation, likely mitochondrial in origin, which can trigger increases in $[Ca^{2+}]_i$ and activation of PKC, perhaps further enhancing ROS production through activation of Nox. It also seems increasingly likely that ROS signaling, like that for Ca^{2+}, is highly compartmentalized within the cell, perhaps within microdomains where mitochondria, the SR and/or plasma membrane channels are in close approximation. The consequence of this type of compartmentalization on PASMC function during acute hypoxia remains to be fully resolved.

Acknowledgments

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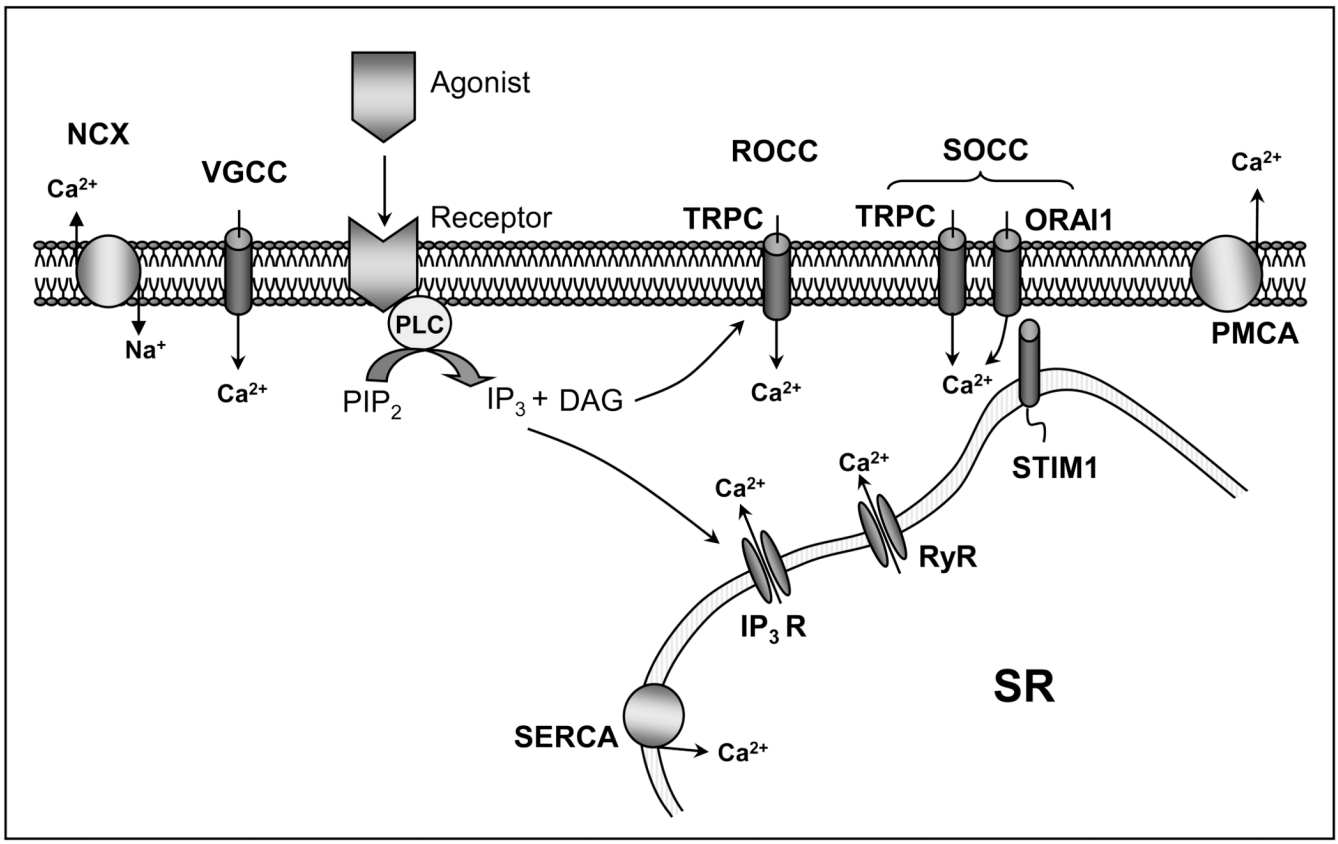

Figure 1.
Schematic illustrating calcium handling pathways in pulmonary arterial smooth muscle cells. PMCA= plasma membrane Ca^{2+}-ATPase; NCX= Na^{+}/Ca^{2+} exchange

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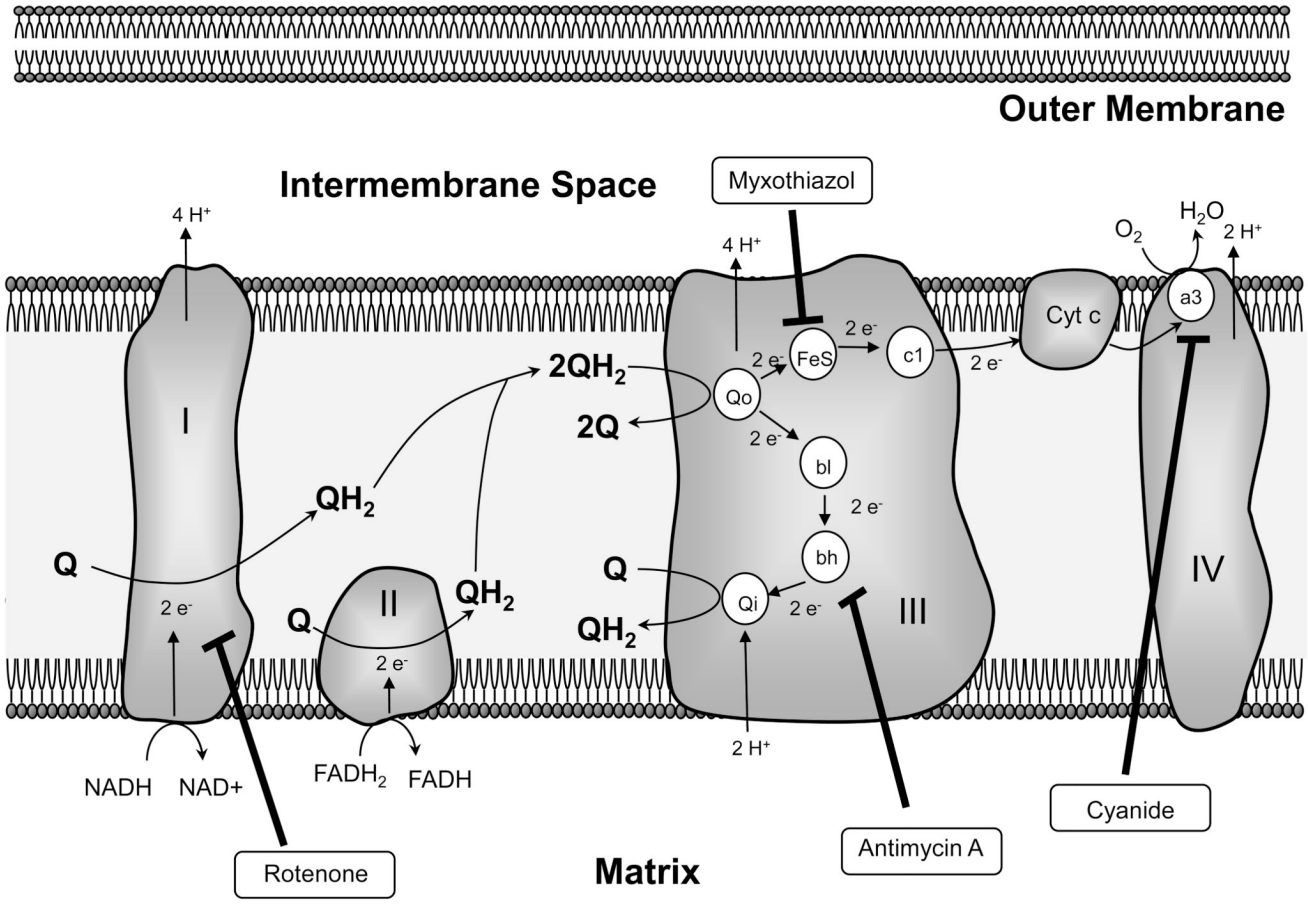

Figure 2.
Depiction of the mitochondrial electron transport chain.

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Table 1

Localization, function and activation of NADPH oxidase (Nox) isoforms.

Isoforms	Tissue expression	Subunits	Activation	Functions	Refs
Nox1	colon epithelium, vascular smooth muscle, endothelial cells, kidney	NoxO1 NoxA1 p22phox Rac	PKC, PLD, growth factors	proliferation, migration, apoptosis	(Clempus and Griendling, 2006; Hoidal et al., 2003; Mittal et al., 2007)
Nox2	phagocytes, endothelial cells, pulmonary arteries, heart	p47phox p67phox p22phox Rac	PKC	immunity, HPV	(Clempus and Griendling, 2006; Lassegue and Griendling, 2010; Liu et al., 2006; Mittal et al., 2007)
Nox3	inner ear non vascular	p22phox NoxO1	constitutive		(Hoidal et al., 2003; Lassegue and Griendling, 2010)
Nox4	vascular and airway smooth muscle, kidney	p22phox	constitutive, Ang II, TGFβ, hypoxia,	proliferation, migration	(Clempus and Griendling, 2006; Liu et al., 2006; Mittal et al., 2007)
Nox5	spleen, kidney, testes, lung, cancers not found in rodents	p22phox	Ca^{2+}	contraction, cell growth	(Fulton, 2009)