HIGHLIGHTED TOPIC | Pulmonary Circulation and Hypoxia

Hypoxic pulmonary vasoconstriction: redox events in oxygen sensing

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Waypa, Gregory B., and Paul T. Schumacker. Hypoxic pulmonary vasoconstriction: redox events in oxygen sensing. J Appl Physiol 98: 404–414, 2005; doi:10.1152/japplphysiol.00722.2004.—Recently, the mitochondria have become the focus of attention as the site of O2 sensing underlying hypoxic pulmonary vasoconstriction (HPV). However, two disparate models have emerged to explain how mitochondria react to a decrease in P02. One model proposes that a drop in P02 decreases the rate of mitochondrial reactive oxygen species (ROS) generation, resulting in a decrease in oxidant stress and an accumulation of reducing equivalents. The resulting shift of the cytosol to a reduced state causes the inhibition of voltage-dependent potassium channels, membrane depolarization, and the influx of calcium through voltage-gated (L-type) calcium channels. A second and opposing model suggests that hypoxia triggers a paradoxical increase in a mitochondrial-induced ROS signal. The resulting shift of the cytosol to an oxidized state triggers the release of intracellular calcium stores, recruitment of calcium channels in the plasma membrane, and activation of contraction. This article summarizes the potential involvement of a mitochondria-induced ROS signal in these two very different models.

reactive oxygen species; oxidants; mitochondria; redox signaling

DURING DEVELOPMENT, fetal oxygenation is achieved by placental gas exchange and the O2 tension in the lung remains low. In response to this alveolar hypoxia, the pulmonary arteries (PA) remain tonically constricted, a response termed hypoxic pulmonary vasoconstriction (HPV). Hypoxic conditions also promote dilation of the ductus arteriosus (DA), allowing it to shunt blood flow from the pulmonary artery to the systemic circulation, thereby bypassing the pulmonary microcirculation (35). At birth, inflation of the lungs with air produces a rapid increase in alveolar O2 tension. These events trigger a relaxation of tone and a decrease in pulmonary vascular resistance, whereas the increase in O2 tension causes the DA to constrict. The decrease in pulmonary vascular resistance and the closure of the DA cause a redistribution of blood flow through alveolar capillaries, thus initiating pulmonary gas exchange. As an individual progresses through life, the pulmonary resistance arteries retain their ability to constrict in response to alveolar hypoxia (104). This physiological response was first described in 1946 by von Euler and Liljestrand, who found that PA pressure increased when feline lungs were ventilated with low O2 mixtures and decreased when ventilated with pure O2 (104). They recognized that these responses reflected changes in pulmonary vascular resistance, and 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 toward areas with better oxygenation. Arterial constriction is unique to the pulmonary circulation; in the systemic circulation, tissue hypoxia causes arterioles to dilate, resulting in a restoration of blood flow and oxygen supply to the affected region (61, 117).

Since the discovery of HPV, many investigators have characterized the pulmonary vascular response to hypoxia, and the importance of this response in health and disease has become apparent. Several excellent review articles dealing with HPV have appeared recently and the reader is directed to those for a more comprehensive analysis of the field (1, 26, 33, 52, 62, 92, 113, 116, 120, 127). However, an important and still unresolved question relates to the underlying mechanism by which vascular cells detect a decrease in O2 tension and convert this into a biological signal. Finding an answer to this question will increase our understanding of the physiology and pathophysiology of the pulmonary circulation and may open the door to our understanding of O2 sensing in other tissues. Although much progress has been made toward identifying the O2 sensor underlying HPV, no consensus of opinion has emerged behind any single mechanism.

Recent studies of HPV suggest that the source of the signal responsible for triggering constriction occurs at the mitochondria. From a teleological standpoint, it is reasonable that the organelle where most of the cellular O2 is consumed would also be the site of O2 sensing. However, two opposing views have emerged regarding mitochondria-dependent O2 sensing during hypoxia. They are similar in that both implicate changes in cytosolic oxidation-reduction (redox) status through the production of reactive oxygen species (ROS). In other respects these models differ markedly. One model proposes that hypoxia decreases the generation of superoxide in the PA, thereby decreasing cellular oxidant stress and shifting the cytosol to a more reduced state. This allegedly affects redox-sensitive thiol...
groups in regulatory subunits of voltage-dependent potassium (Kv) channels, causing the channels to close. The decrease in K⁺ current triggers membrane depolarization causing voltage-gated (L-type) calcium channels to open, leading to the influx of extracellular calcium. A second model proposes that hypoxia paradoxically initiates an increase in ROS signaling in the PA. The resulting shift in the redox status to a more oxidized state triggers the release of intracellular calcium stores, recruitment of calcium channels in the plasma membrane, and activation of contraction. Progress in resolving whether ROS levels increase or decrease with hypoxia has been hindered by disagreements over the interpretation of data obtained from different oxidant-sensitive probes. Pending the development of more specific tools for assessing intracellular redox signals, investigators have used other means to acquire a better understanding of the O₂ sensor underlying HPV. In this article we will discuss the two models of O₂ sensing that are currently in vogue and comment on their applicability to the recent developments in HPV research.

THE HPV RESPONSE

It is well established that the HPV response is local to the lung, because it is retained in excised lungs and in rings of pulmonary artery studied in an organ bath (4, 32, 57, 59, 84, 121, 122, 129). Even isolated PA smooth muscle cells (PASMC) contract under hypoxia, indicating that these cells possess an O₂ sensor and the signaling mechanisms necessary to activate a contractile response (110, 128). Hypoxic constriction is preserved if PA vessels are denuded of endothelium, although the response is markedly attenuated (8). This indicates that the endothelium probably amplifies, rather than initiates, HPV. It has also been established that the HPV response is biphasic (22, 23, 51, 80, 82, 83) and consists of an initial transient constriction response (phase 1) followed by a slow sustained constriction (phase 2). However, it should be noted that some investigators witness a monophasic response (5), which may be due in part to the masking of the transient relaxation that occurs after phase 1 in their experimental model. Phase 1 occurs even in the absence of the endothelium (128) and is referred to as the acute phase because it begins within seconds after hypoxic challenge. Phase 1 appears to involve the release of calcium from the intracellular stores, the closure of Kv channels, membrane depolarization, and the opening of voltage-gated L-type calcium channels (8, 22, 23, 67, 73). By contrast, phase 2 does not appear until minutes after hypoxic challenge, but it can last for hours to days during chronic hypoxia. Phase 2 requires an intact endothelium, which suggests that an endothelium-derived promoting factor may be involved (1, 23, 50, 51, 53, 55, 109). Early studies suggest that endothelin-1 (ET-1) may contribute to the endothelium-dependent HPV amplification (55). Consistent with that idea, the expression and release of ET-1 by endothelial cells has been shown to increase during hypoxia (12, 36, 124). Moreover, studies using BQ-123, an ET-1 receptor subtype A antagonist, show an attenuation of the phase 2 component of HPV in intact PA vessels (55, 85). However, more recent studies suggest that ET-1 might be accompanied by additional unidentified, endothelium-derived factors that contribute to an increase in the calcium sensitivity of the contractile mechanism during phase 2 (1, 79, 83). The actions of these endothelium-derived factors are discussed below.

EVIDENCE IMPLICATING A DECREASE IN ROS SIGNALING DURING HPV

Early studies suggested that hypoxia decreases extracellular oxidant levels, based on measurements using lucigenin or luminol chemiluminescence in perfused lungs (4, 6, 65). More recent studies in endothelium-denuded rings of distal PA also show a decrease in ROS release using chemiluminescence, Amplex Red, and DCF fluorescence during hypoxia (61). These findings suggest that a tonic level of ROS generation occurs during normoxia, which decreases during hypoxia due to the lessened availability of O₂. If this is the case, then what is the site of the ROS generation during normoxia? Initially it was hypothesized that a constitutively active NAD(P)H oxidase system was responsible (3, 10). That system would presumably decrease its production of superoxide during hypoxia, due to a fall in the availability of O₂ as a substrate. However, mice with targeted deletion of the gp91 (Nox-2) subunit of NADPH oxidase still exhibit an intact HPV response (7), so that mechanism has been ruled out. It is conceivable that alternative isoforms (Nox-1 or Nox-4) to the gp91 (Nox-2) subunit are involved (49), but definitive studies testing this hypothesis have not yet appeared. Some investigators suspect that mitochondria are the source of constitutive ROS production and that these organelles decrease oxidant production during hypoxia (3). This idea arose from their finding that the mitochondrial inhibitors antimycin A and rotenone mimicked the hypoxic response, presumably by decreasing tonic mitochondrial ROS generation (3, 4).

On the basis of these results, Archer and colleagues (4, 61) proposed a model in which hypoxia decreases mitochondrial electron transport, thus decreasing ROS production by complex I and/or complex III while increasing cytosolic [NAD(P)H] (Fig. 1A). The decrease in NADH utilization by mitochondria would shift the cytosolic redox balance to a more reduced state, therefore increasing the [NADH]/[NAD⁺] ratio and the glutathione pool ratio ([GSH]/[GSSG]) (114). Such a redox shift has been postulated to cause an inhibition of membrane K⁺ current via its effects on redox-sensitive Kv channel subunits. In that regard, Rettig et al. (76) identified a cysteine residue in the β-subunit of the Kv channel that is sensitive to oxidation. When the cysteine is reduced, the NH₂-terminal inactivating domain of the β-subunit binds to the inner mouth of the channel pore formed by the α-subunits of the Kv channel, causing the inactivation of the channel. The resulting inhibition of membrane K⁺ current causes membrane depolarization, leading to the opening of L-type, voltage-gated Ca²⁺ channels and subsequent myocyte contraction. In support of this hypothesis, diamide, a chemical that oxidizes sulfhydryls in the cell, caused vasodilation in the isolated perfused lung (115), whereas reducing agents caused decreases in K⁺ currents in pulmonary artery smooth muscle cells (68, 70, 75, 116). In addition, hypoxia was reported to cause inhibition of steady-state K⁺ currents and membrane depolarization in rat PASMC (69, 70). However, this sole necessity for Kv channel inhibition during HPV has met with some criticism. Sham et al. (90) and Hasunuma et al. (34) demonstrated that pharmacological inhibition of Kv channels had no effect on HPV and in some
cases it augmented the response. This suggests that Kv channel inhibition alone cannot account for the HPV response. Rather, Kv channels may represent a downstream amplification step that contributes to HPV.

In a complementary model based on a hypoxia-induced decrease in ROS signaling, Wolin and colleagues (64, 72) proposed that a PA smooth muscle microsomal NADH oxidoreductase acts as the O2 sensor during the HPV response (Fig. 1A). Analogous to an NAD(P)H oxidase, the microsomal NADH oxidase was proposed to generate \( \text{H}_2\text{O}_2 \) under normoxic conditions. This \( \text{H}_2\text{O}_2 \) would then interact with catalase to activate soluble guanylyl cyclase, causing an increase in the production of the vasodilator cGMP. This tonic production of cGMP during normoxia would maintain a basal state of smooth muscle relaxation, resulting in low pulmonary vascular resistance (64, 72). During hypoxia, decreases in the production of \( \text{H}_2\text{O}_2 \) would cause an attenuation of the vasorelaxant signal, thereby increasing pulmonary vascular resistance (72). Therefore, according to this model, HPV involves an attenuation of a normoxic vascular relaxation process.

Initial concerns arose with respect to these models when it was recognized that autooxidation and other methodological limitations may have interfered with the ability of chemiluminescence measurements to accurately detect intracellular oxidant stress (96). Furthermore, other investigators report that the mitochondrial inhibitor rotenone decreases ROS production, whereas antimycin A augments oxidant generation (19–21, 29, 66, 101). Therefore, according to the model of Archer and colleagues (4, 61), the former compound should have activated HPV, whereas the latter should have blocked it; this was not the case. Reeve et al. (75) and Olschewski et al. (70) demonstrated that the exogenous administration of reducing agents under normoxic conditions mimic HPV in isolated PA vessels and that oxidizing agents can reverse PA constriction induced by either reducing agents or hypoxia in accordance to the Archer model. However, other investigators have shown that antioxidants appear to block the response to hypoxia in intact lungs, isolated PA vessels, and isolated PASMC without causing a sustained increase in pulmonary vascular tone (54, 110, 111). The basis for these contradictory findings may involve the methodology and site of action for the various agents. Finally, it is now recognized that the cytosol normally maintains a highly reduced environment (30), making signaling via further reductive stress unlikely. It should be noted, though, that definitive measurements of reduced glutathione (GSH) vs. oxidized glutathione (GSSG) in PASMC have yet to be performed. However, increased ROS generation as a means of signaling in vascular cells has been demonstrated in response to angiotensin II, growth factors, mechanical strain, and hypoxia (2, 21, 49, 54, 57, 71, 110). Collectively, these observations bring into question the idea that hypoxia-induced decreases in ROS trigger HPV.

**EVIDENCE IMPLICATING AN INCREASE IN ROS SIGNALING DURING HPV**

An alternative to the hypoxia-induced decrease in ROS signaling was initially suggested by Marshall et al. (57), who argued that hypoxia activates an NAD(P)H oxidase in PASMC, thereby signaling hypoxia with a paradoxical increase in ROS production as measured by chemiluminescence in isolated PA smooth muscle cells (Fig. 1B). Pulmonary arteries contain the Nox-2 subunit of that oxidase (7, 49, 57), and ROS production by cultured PA myocytes (and HPV in intact lungs) is inhibited by diphenylene iodonium (DPI) (32, 57, 100, 110). However, that compound broadly inhibits flavoproteins, including nitric oxide synthase, mitochondrial Complex I, and some ion channels (7, 56, 118). Further studies...
were not pursued by that group, so the significance of that model is still unresolved.

Other studies suggest that hypoxia triggers an increase in ROS signaling in PASMC (110), as evidenced by an increase in oxidation of the intracellular probe 2',7'-dichlorofluorescin diacetate (DCFH). That response is attenuated by myxothiazol (110), a mitochondrial inhibitor that blocks electron flux into Complex III of the mitochondrial electron transport chain (ETC). Similar evidence of a hypoxia-induced increase in a ROS production was reported by Liu et al. (54) and by Killilea et al. (45), who found increases in DCFH oxidation in hypoxic PASMC. Although these studies support the idea that increased ROS signaling is critical for HPV, those results have been challenged because DCFH oxidation is nonspecific (89, 99), and the site of its oxidation in the cell is not known. Interestingly, Liu et al. also found evidence of increases in radical production using lucigenin-derived chemiluminescence and electron paramagnetic resonance (EPR) spectrometry. The involvement of an increasing ROS signal is further evident by the fact that exogenous H$_2$O$_2$ mimics HPV in PASMC in isolated lungs (110, 111), although it should be noted that H$_2$O$_2$ can also release Ca$^{2+}$ from mitochondria (78) and also potentially trigger nonspecific effects (42, 77). Nevertheless, in both isolated perfused lungs and cultured PA myocytes, cell permeable antioxidants blocked contraction in response to hypoxia, without affecting alternative methods of inducing contraction (54, 110, 111). In addition, overexpression of catalase in the cytosol was found to attenuate both hypoxia-induced and H$_2$O$_2$-induced increases in cytosolic Ca$^{2+}$ without affecting the response to angiotensin II (110). Taken together, these findings support the idea that an increase in an ROS signaling occurs during HPV.

Involvement of mitochondria in the HPV response is indicated by the ability of myxothiazol to inhibit the increase in ROS signaling during hypoxia (110) (Fig. 1B). Further evidence of mitochondrial participation comes from studies using other site-specific mitochondrial inhibitors (Fig. 2). These studies suggest that HPV requires electron transport in the proximal (but not the distal) region of the ETC (51, 110, 111, 119). For example, when the ETC was inhibited at Complex I by rotenone or DPI, HPV was abrogated, yet contraction in response to receptor-mediated thromboxane A$_2$ analog U46619 was preserved (110, 111, 119). Leach et al. (51) extended this observation by demonstrating that rotenone blocked hypoxic contraction of PA vessels and by showing that HPV could be restored after rotenone blockade by using succinate to shuttle electrons into Complex III via Complex II, bypassing the rotenone inhibition at Complex I (51). In cultured PA smooth muscle cells, we measured the contractile response to hypoxia using time-lapse measurements of cell dimensions (110). In those studies, the same mitochondrial inhibitors that abolished HPV in the intact lung were found to block contraction of the myocytes during hypoxic superfusion. By contrast, inhibitors of the distal region of the ETC (cyanide or antimycin A) do not abolish HPV, which suggests that a fully functional ETC is not required for HPV (51, 110, 111). The site specificity of ETC inhibition can be explained by the observation that distal inhibitors do not prevent hypoxia-induced increases in ROS production from the proximal region of the ETC, especially complex III (16). Cyanide, which inhibits the terminal cytochrome oxidase in the ETC, increases ROS production at complex III, induces constriction during normoxia (110, 111), and triggers cytosolic calcium ([Ca$^{2+}$]$_c$) increases in PASMC (106, 111). This response was blocked by myxothiazol, which prevents electron flow into Complex III and by overexpression of catalase (111), which augments the scavenging of H$_2$O$_2$. Roudns and McMurtry (84) were the first to show that antimycin A elicited vasoconstriction in normoxic lungs. Although that study preceded the ROS hypothesis, it now seems clear that antimycin A, an inhibitor that blocks ubiquinone binding at the Qi site in complex III, increases contraction by augmenting the production of superoxide at complex III. This occurs because antimycin A prolongs the lifetime of ubisemiquinone at the Qo site in complex III, enhancing the opportunity for superoxide generation. Interestingly, Archer et al. (4) found that cyanide induced vasoconstriction during normoxia and augmented HPV without affecting the response to angiotensin II or KCl. Collectively, these findings are consistent with a role for increased mitochondrial ROS production in HPV.

A weakness of many of these studies is their reliance on pharmacological inhibitors, which may have unexpected effects. To begin to address this, $\rho^0$-PASMC were generated through the depletion of mitochondrial DNA by culturing the cells in the presence of ethidium bromide (46). These cells lack a functional ETC and are incapable of generating ROS during hypoxia (18). Interestingly, these cells lost their hypoxic response, yet retained the ability to respond to U46619 (110). However, additional studies using more selective genetic tools are needed to determine more specifically the sources of these oxidant signals.

**WHICH MITOCHONDRIAL COMPLEXES CONTRIBUTE TO ROS GENERATION DURING HYPOXIA?**

During respiration, reducing equivalents generated in glycolysis or the Krebs cycle are passed along the ETC (Fig. 3). Protons are extruded from the mitochondrial matrix at Complexes I, III, and IV, generating an electrochemical gradient...
Fig. 3. Mitochondrial ETC. During respiration, reducing equivalents are passed along the ETC. Protons are extruded from the mitochondrial matrix at Complexes I, III, and IV, generating an electrochemical gradient ($\Delta\Psi_m$) across the inner membrane. That $\Delta\Psi_m$ is used by the F$_0$F$_1$ ATP synthase to generate ATP. During respiration, most of the O$_2$ consumed is reduced to H$_2$O at Complex IV (cytochrome oxidase). However, superoxide can be generated at sites upstream from Complex IV when single electrons escape from the various transport proteins (15). Superoxide and its degradation product H$_2$O$_2$ have traditionally been viewed as toxic byproducts of the ETC (11, 14), but a growing body of data implicates them as participants in signal transduction. Oxidant generation can occur at Complex I, when electrons can escape from its flavoprotein (FMN) to be captured by O$_2$ (102), although the precise mechanism of ROS generation at that site is not fully understood (48). ROS production can also occur at Complex II, which consists of four polypeptide subunits A-D. Consistent with Complex II’s ability to generate ROS, Paddenberg et al. (71) suggested that ROS generation from this complex may be involved in HPV. Complex II functions as succinate dehydrogenase in the Krebs cycle, oxidizing succinate to fumarate. According to this model, reversal of complex II activity (fumarate reductase) results in an increase in ROS production during hypoxia. However, it is not yet clear how a respiring cell with a functioning Krebs cycle can have succinate dehydrogenase functioning in both directions at the same time. Hence, additional studies are required to more fully understand this potential mechanism.

Complex III has received the greatest attention in terms of its potential for generating ROS (13, 20, 74, 101). A predominant site of superoxide generation involves the Qo site, near the outer surface of the inner mitochondrial membrane. Ubiquinol, carrying two electrons obtained from Complex I or II binds to Complex III at the Qo site. After transferring one of its electrons to the Rieske iron-sulfur protein, ubisemiquinone is formed (20, 38, 63, 97). Ubisemiquinone normally transfers its remaining electron to the b cytochromes in Complex III. However, this free radical can alternatively transfer an electron to O$_2$, yielding superoxide. Inhibitors like myxothiazol or stigmatellin inhibit the binding of ubiquinol to the Qo site, thereby preventing the generation of ubisemiquinone. Hence, these “upstream” inhibitors limit HPV by attenuating the generation of the ubisemiquinone, which is the source of superoxide. ROS do not appear to be generated by Complex IV (cytochrome oxidase) (27), due to the high-affinity trapping of O$_2$ at the binuclear center (103). This would explain why the Complex IV inhibitor cyanide does not inhibit HPV. However, cyanide does induce smooth muscle cell contraction during normoxia most likely 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.

To trigger HPV, ROS arising from the ETC would need to exit from the organelle and reach the cytosol. Escape of superoxide from the outer surface of the inner mitochondrial membrane (i.e., the Qo site) is likely, but escape of that radical to the cytosol is hindered by superoxide dismutase (which dismutates superoxide to H$_2$O$_2$) and cytochrome c (which oxidizes superoxide directly) in the intermembrane space. Because overexpression of catalase in the cytosol blocks HPV in PA myocytes, it is likely that H$_2$O$_2$ escape is sufficient to trigger the downstream responses. It is yet to be determined whether the hypoxia-induced increase in ROS is a result of increased ROS generation by one or more of the mitochondrial complexes or a result of an increase in the egress of ROS from the mitochondria or a combination of both.
PHASE 1 OF THE HPV RESPONSE

During phase 1, PA rings exhibit a rapid increase in [Ca\(^{2+}\)]. This response is retained after removal of the endothelium, indicating that the O\(_2\) sensor triggering phase 1 is intrinsic to the PASMC (54). An increase in [Ca\(^{2+}\)] could potentially arise from the entry of extracellular Ca\(^{2+}\) through voltage-dependent, store-operated, and/or receptor-regulated Ca\(^{2+}\) channels (9, 43, 44, 73, 82, 105). However, recent studies suggest that intracellular release of Ca\(^{2+}\) may occur first and then possibly trigger extracellular entry (22, 23, 67) (Fig. 1B). 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 and/or inositol 1,4,5-trisphosphate (IP\(_3\)) receptors. Ryanodine receptors contain redox-sensitive cysteine thiols (25), so ROS released from mitochondria could trigger Ca\(^{2+}\) release by activating these channels. Alternatively, as proposed by Evans and Dipp (22, 26), phase I may reflect SR Ca\(^{2+}\) release through stimulation of ryanodine receptors via generation of the Ca\(^{2+}\)-mobilizing β-NAD\(^+\) metabolite, cyclic ADP-ribose (cADPR). Hypoxia causes an increase in cADPR accumulation, which appears to depend on hypoxia-induced increases in β-NADH levels (123). Presently, it is not known how β-NADH may modulate cADPR production, but it is possible that β-NADH may inhibit cADPR hydrolyase (26, 123). Another possibility is that cADPR accumulation may be the result of an increase in β-NADH-dependent ROS signaling (47). β-NADH feeds into complex I of the ETC, hence supplying a source of electrons for the formation of superoxide by the mitochondrial complexes during hypoxia. By this mechanism, cADPR-dependent Ca\(^{2+}\) release from the SR could occur as a downstream consequence of hypoxia-induced increases in ROS. Alternatively, activation of IP\(_3\) receptors could trigger Ca\(^{2+}\) release from the SR, based on hydrogen peroxide’s ability to activate phospholipase C (PLC) (31). PLC generates diacylglycerol (DAG) and IP\(_3\) from phosphatidylinositol bisphosphate (PIP\(_2\)). This would suggest a mechanism by which the hypoxia-induced ROS signal could cause Ca\(^{2+}\) release from IP\(_3\)-sensitive intracellular stores. However, Jin et al. (40) were not able to detect changes in IP\(_3\) levels in isolated pulmonary arteries during hypoxia, so the involvement of this pathway remains speculative.

The hypoxia-induced Ca\(^{2+}\) release from the SR is then thought to trigger capacitative calcium entry (CCE) and/or voltage-dependent calcium entry, thus increasing [Ca\(^{2+}\)], and therefore amplifying phase 1 tension generation (73, 82, 94, 98, 105) (Fig. 1B). Inhibition of extracellular calcium entry with 4-aminopyridine (4-AP)-sensitive Kv channels. The resulting decrease in K\(^+\) current caused membrane depolarization and the opening of voltage-gated L-type Ca\(^{2+}\) channels leading to increased [Ca\(^{2+}\)]. When voltage-dependent calcium entry was blocked by the L-type Ca\(^{2+}\) channel inhibitor nifedipine, phase I of the HPV response was only partially attenuated (82). This result suggests that voltage-dependent calcium entry and capacitative calcium entry may have a synergistic relationship in the HPV response.

Another potential organelle responsible for the hypoxia-induced increase in [Ca\(^{2+}\)] is the mitochondria. However, the mitochondrial membrane potential is ~150 to 200 mV negative to the cytoplasm, which provides a strong incentive for cytosolic Ca\(^{2+}\) to enter the mitochondrial matrix through a uniporter, rather than to escape to the cytosol (24). Hence, the mitochondria seem more likely to play a role in buffering the rise in cytosolic Ca\(^{2+}\) than adding to it. Hypoxia, or depolarization of the mitochondrial membrane potential with the uncoupler carbonyl cyanide 4- trifluromethoxy-phenylhydrazone (FCCP), augments a CCE-dependent increase in [Ca\(^{2+}\)] (43, 44). This observation led Kang et al. (43, 44) to propose that during hypoxia, the mitochondrial membrane depolarizes, decreasing mitochondria’s ability to buffer Ca\(^{2+}\) and thereby augmenting the hypoxia-induced rise in [Ca\(^{2+}\)] via SR release and CCE. However, additional studies are needed to clarify the potential contributions of mitochondrial Ca\(^{2+}\) buffering to the hypoxia-induced rise in [Ca\(^{2+}\)].

PHASE 2 OF THE HPV RESPONSE

If phase 1 is the spark of the HPV response, then phase 2 is the roaring fire. From a clinical standpoint, phase 2 is particularly important because lung diseases associated with chronic alveolar hypoxia lead to prolonged elevation of PA pressures, pulmonary vascular remodeling, and right ventricular hypertrophy. Severe increases in pulmonary arterial pressure may then progress to right heart failure. Little is presently known about the O\(_2\) sensor-underlying phase 2 of the HPV response, whether phase 2 is a downstream response of phase 1, or whether phase 2 results from the activation of an alternative O\(_2\) sensor.

Current research suggests that the pulmonary endothelium is critical for phase 2 of HPV. Removal of the endothelium significantly attenuates phase 2 in isolated pulmonary vessels (8, 22, 23, 51, 55, 80, 109, 123). This initially led a number of investigators to speculate that the endothelium releases factors that induce PA vasoconstriction during phase 2 of the HPV response (92). Yet Dipp et al. (23) and Leach et al. (51) were able to demonstrate in endothelium-denuded PA vessels that although hypoxia-induced tension declines after phase 1, it plateaus above baseline and remains at this level throughout the period of hypoxia. Robertson et al. (79) took this a step further by demonstrating that removal of the endothelium had no effect on [Ca\(^{2+}\)], at
any point during HPV. This suggests that the hypoxia-induced increase in \([Ca^{2+}]_i\) is intrinsic to PA smooth muscle and that the endothelium may generate additional factors that augment the sensitivity of the smooth muscle cell to the hypoxia-induced rise in \([Ca^{2+}]_i\). If so, then phase 2 could involve two components: one that maintains an increase in PA smooth muscle \([Ca^{2+}]_i\); above baseline levels and a second endothelium-dependent part that increases the sensitivity of the contractile response to the hypoxia-induced rise in \([Ca^{2+}]_i\).

**INCREASED \([Ca^{2+}]_i\) IN PA SMOOTH MUSCLE CELLS DURING PHASE 2**

PA constriction during phase 2 is dependent on a small initial rise in \([Ca^{2+}]_i\), (23, 41, 82), yet this rise does not require extracellular calcium entry (23, 82). This led Dipp et al. (22) to propose that phase 2 requires only the calcium released from intracellular, ryanodine-sensitive stores. They suggest that after initiating phase 1, continued hypoxia-induced cADPR synthesis during phase 2 could trigger the tonic release of low levels of \(Ca^{2+}\) from the SR through constant stimulation of the ryanodine receptor. Depletion of calcium from intracellular stores as well as inhibition of ryanodine-sensitive calcium release by the cADPR antagonist, 8-bromo-cADPR, abolishes tension generation during phase 2 of the HPV response in support of this idea (22, 82).

**ENDOTHELIUM-DERIVED FACTORS AMPLIFY PA CONSTRUCTION DURING PHASE 2**

Increased tension generation during phase 2 is dependent on endothelium-derived factors (55, 80, 109). Endothelin (ET-1) is a 21-amino acid peptide secreted by endothelial cells and is considered a contributing mediator of phase 2. At low doses (10⁻¹⁰ M), ET-1 has been shown to increase the sensitivity of the contractile response to the hypoxia-induced increase in \([Ca^{2+}]_i\), and at high doses (10⁻⁸ M), it increases \([Ca^{2+}]_i\) in PA smooth muscle cells (93). Whether endothelium-derived ET-1 increases Ca²⁺ sensitivity and/or increases \([Ca^{2+}]_i\) in PA smooth muscle cells during hypoxia has yet to be resolved. Nevertheless, hypoxia has been shown to increase ET-1 expression and secretion from cultured endothelium (36, 124). Moreover, BQ-123, an ET-1 receptor A (ETₐ) antagonist, attenuates phase 2 of HPV in isolated vessels and whole lungs (54, 85). This suggests that an endothelial \(O_2\) sensor detects the fall in \(O_2\) and activates transcription of ET-1. Hu et al. (36) found that the proximal promoter of the ET-1 gene contains a hypoxia-inducible factor 1 (HIF-1) binding site (HRE) on the antisense DNA strand, positioned at −118 to −125 bp upstream from the transcription start site. HIF-1 is the principal \(O_2\)-responsive factor underlying increased expression of glycolytic enzymes, glucose transporters, and other genes during hypoxia (86–88). HIF-1α is rapidly degraded during normoxia due to its hydroxylation by prolyl hydroxylase, thereby targeting it for ubiquitin-mediated degradation (37, 39). HIF-1α is stabilized during hypoxia via inhibition of that process, allowing it to heterodimerize and activate transcription (125). HIF-1α⁺/⁻ mice show attenuated pulmonary hypertension, right ventricular hypertrophy, and vascular remodeling during chronic hypoxia (126). Chronic changes in HIF-1 also affect the HPV response, in that PASMC from heterozygous mice show attenuated electrophysiological responses to hypoxia (91). Interestingly, mitochondrial ROS have been proposed to trigger the stabilization of HIF-1α during hypoxia (16, 17). This suggests that the same mechanism of \(O_2\) sensing may function in PA myocytes and endothelium, with the sensor activating cell-specific responses that, in combination, result in the HPV response.

The possibility that endothelium-derived factors in addition to ET-1 could exist was suggested by studies involving the inhibition of ETₐ and combined ETₐ/ETₐ receptors, which did not always attenuate the sustained HPV response (1, 50, 79, 83, 109). Presently, the identity of these additional hypoxia-induced, endothelium-derived factors is not known; however, one candidate was partially purified from hypoxic, isolated perfused rat lungs using stepwise acetone-tritile elution from Sep-Pak C₁₈ cartridges (83). This factor caused a slow and sustained constriction of small PA without a corresponding increase in \([Ca^{2+}]_i\) and was unaffected by combined treatment with BQ-123 and BQ-788 (an ETₐ receptor antagonist) (83). This led Robertson et al. (79, 83) to suggest that this endothelium-derived factor was not ET-1, yet it demonstrated \(Ca^{2+}\)-sensitizing properties, which could help maintain increased PA constriction even in the presence of slightly elevated intracellular calcium. Further research is needed to determine the identity of this putative mediator and to determine how hypoxia triggers its release.

**INCREASED \(Ca^{2+}\)-SENSITIVITY AUGMENTS PA CONTRACTION DURING PHASE 2**

The small elevation in \([Ca^{2+}]_i\) in PA smooth muscle cells, combined with the significant increase in PA constriction during phase 2, suggests that increased \(Ca^{2+}\) sensitivity may be an important contributor in chronic hypoxia. Various transduction pathways including protein kinase C (PKC) and RhoA kinase can regulate \(Ca^{2+}\) sensitivity in PASMC. The specific PKC inhibitor Ro-31–8220 was shown to have no effect on the development of tension during phase 2, apparently ruling out a role for PKC (80, 83). By contrast, the RhoA kinase antagonist Y-27632 significantly attenuated the HPV response during phases 1 and 2, making this a viable candidate (28, 81, 107, 108). Hypoxia activates RhoA in PA smooth muscle and endothelial cells, leading to the activation of RhoA kinase (81, 107, 108). RhoA kinase appears to increase \(Ca^{2+}\) sensitivity by inactivating myosin light chain (MLC) phosphatase through phosphorylation of its myosin binding unit (95). During smooth muscle contraction, \(Ca^{2+}/\text{calmodulin}\) activates MLC kinase, which in turn causes MLC phosphorylation and hence contraction. Smooth muscle contraction is regulated by a balance between the actions of MLC kinase (contraction) and MLC phosphatase (relaxation). Therefore, the downregulation of MLC phosphatase by RhoA kinase results in an increase in MLC phosphorylation, which augments tension generation at a given level of \([Ca^{2+}]_i\) and MLC kinase activity.

ET-1 can increase \(Ca^{2+}\)-sensitivity via a RhoA/RhoA kinase pathway in the basilar artery of rabbits (60), but whether a similar mechanism is present in the pulmonary...
arteries is not known. PA contraction induced by Robertson’s endothelium-derived factor was unaffected by the PKC inhibitor Ro-31–8220 (83); however, its effect on RhoA activation in PA smooth muscle cells was not reported. Therefore, further investigation is required to determine whether a link exists between these hypoxia-induced, endothelium-derived factors and the hypoxia-induced RhoA-dependent increase in Ca²⁺ sensitivity.

CONCLUDING REMARKS

Presently there is no definitive answer to the question of whether hypoxic pulmonary vasoconstriction is triggered by a decrease or an increase in mitochondria-dependent ROS. On the basis of current research, there is increasing evidence that a paradoxical increase in mitochondria-dependent ROS signaling is involved, which initiates a chain of events resulting in both an increase in [Ca²⁺]i in PA smooth muscle cells and an endothelium-derived increase in Ca²⁺ sensitivity (Fig. 4). How can these differences be resolved? First, more robust methods for assessing the thiol redox status in the cell need to be developed to be able to assess changes in redox in living cells in real time. In that regard, preliminary reports show that redox-sensitive cytosolic fluorescence resonance energy transfer sensors detect an increase in oxidant stress in PA myocytes during hypoxia (112). Second, studies are needed to assess the subcellular sources of these ROS, and the various compartments were oxidants or reductant signals regulate calcium and calcium sensitivity. It is possible that hypoxia could cause a decrease in ROS production from NADH oxidase, whereas it causes a simultaneous increase in ROS release from the mitochondrial ETC. Depending on which methods are used to assess the oxidant signal, it is possible that very different conclusions would be reached (89). Finally, much of the present disagreement is based on the different responses to pharmacological inhibitors used in different preparations at different doses. Given the availability of genetic tools to modulate ROS production and ROS scavenging, we remain optimistic that more definitive information regarding this important mechanism will soon be forthcoming.

GRANTS

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Fig. 4. Possible mechanisms underlying the biphasic constriction (phase 1 and 2) of the pulmonary arteries in response to hypoxia. HIF-1, hypoxia-inducible factor-1; ET-1, endothelin-1; ETA, endothelin receptor subtype A; RhoK, Rho kinase; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; AC, ADP-ribosyl cyclase; CH, cADPR hydrolase; SR, sarcoplasmic reticulum; SOCC, store-operated calcium channel; RyR, ryanodine receptor.
REFERENCES


Invited Review

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