HIGHLIGHTED TOPIC | Pulmonary Circulation and Hypoxia

Hypoxic pulmonary vasoconstriction

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Moudgil, Rohit, Evangelos D. Michelakis, and Stephen L. Archer. Hypoxic pulmonary vasoconstriction. J Appl Physiol 98: 390–403, 2005; doi:10.1152/japplphysiol.00733.2004.—Humans encounter hypoxia throughout their lives. This occurs by destiny in utero, through disease, and by desire, in our quest for altitude. Hypoxic pulmonary vasoconstriction (HPV) is a widely conserved, homeostatic, vasomotor response of resistance pulmonary arteries to alveolar hypoxia. HPV mediates ventilation-perfusion matching and, by reducing shunt fraction, optimizes systemic PO2. HPV is intrinsic to the lung, and, although modulated by the endothelium, the core mechanism is in the smooth muscle cell (SMC). The Redox Theory for the mechanism of HPV proposes the coordinated action of a redox sensor (the proximal mitochondrial electron transport chain) that generates a diffusible mediator [a reactive O2 species (ROS)] that regulates an effector protein [voltage-gated potassium (Kv) and calcium channels]. A similar mechanism for regulating O2 uptake/distribution is partially recapitulated in simpler organisms and in the other specialized mammalian O2-sensitive tissues, including the carotid body and ductus arteriosus. Inhibition of O2-sensitive Kv channels, particularly Kv1.5 and Kv2.1, depolarizes pulmonary artery SMCs, activating voltage-gated Ca2+ channels and causing Ca2+ influx and vasoconstriction. Downstream of this pathway, there is important regulation of the contractile apparatus’ sensitivity to calcium by rho kinase. Controversy remains as to whether hypoxia decreases or increases ROS and which electron transport chain complex generates the ROS (I and/or III). Possible roles for cyclic adenosine diphosphate ribose and an unidentified endothelial constricting factor are also proposed by some groups. Modulation of HPV has therapeutic relevance to cor pulmonale, high-altitude pulmonary edema, and sleep apnea. HPV is clinically exploited in single-lung anesthesia, and its mechanisms intersect with those of pulmonary arterial hypertension.

HPV shunts blood from poorly oxygenated areas to better ventilated lung segments, thereby optimizing ventilation-perfusion matching, reducing shunt fraction (115), and optimizing systemic O2 delivery in conditions such as atelectasis and pneumonia (24). HPV onsets within seconds of moderate hypoxia and reverses quickly on restoration of normoxic ventilation. Whereas, in pneumonia and atelectasis, HPV is a focal response limited to the diseased lung segment, with global hypoxia, as occurs at high altitude or with sleep apnea, HPV constricts PAs throughout the pulmonary circulation, increasing the pulmonary vascular resistance (PVR).

The first modern observation of HPV was made in 1894 when Bradford and Dean (22) described increases in pulmonary arterial pressure (Ppa) in response to asphyxia. Half a century later, HPV was recognized as an adaptive phenomenon by von Euler and Liljestrand (123). They ventilated anesthetized cats with either hypoxic (10% O2) or hypercapnic gas mixtures and found that Ppa increased, with minimal change in systemic PO2. They concluded that HPV might “increase the blood flow to better aerated lung areas, which leads to improved conditions for the utilization of alveolar air” (123). This review summar-

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ائز the properties of HPV, based on studies in humans and animals and on the comparative physiology of O2 sensing in simpler organisms, and presents fundamental properties that any proposed mechanism for O2 sensing must explain. The relevance of HPV in health and disease is discussed, and several proposed cellular and molecular mechanisms of acute HPV are presented.

PROPERTIES OF HPV
Lessons from Human Physiology

Substantial work defining the properties of HPV has been performed by integrative physiologists studying normal and diseased humans. Ignorance of this integrative physiology and overreliance on reductionist models, using vascular cells and rings, has created confusion in the quest for the molecular mechanism of HPV. In addition, because HPV is elicited by moderate airway hypoxia, rather than anoxia or low mixed-venous PO2, it is worthwhile to define “hypoxia” as it pertains to HPV. Asccent to the summit of Mount Everest defines the limit of hypoxia tolerated by humans and thus serves as a practical guide to what constitutes “physiologically relevant” hypoxia. At the summit of Mount Everest, the inspired and arterial PO2 values are nearly identical (~43 Torr), whereas the arterial PCO2 is 11 ± 2 Torr and arterial pH is 7.53 (74). If one uses relevant levels of hypoxia and remains mindful of the characteristics of HPV in vivo, the controversies in molecular HPV research will be reduced.

HPV in Humans

HPV increases PVR by 50 to 300%. The response to hypoxia onset in minutes, reaching a maximum within 15 min (20). In normal volunteers, inhalation of 12.5% O2 decreases systemic PO2 to below 50 Torr and increases PVR by 100–150% (88). HPV is not potentiated by repeated hypoxic challenges, nor does it decrease when sustained for hours (29, 35). This is also true in studies of HPV conducted in isolated, blood-perfused lungs (117). An interesting case report attests to the potential of segmental HPV to persist chronically. A patient with bronchial obstruction due to an adenoma had persistent, HPV-induced lung hypoperfusion that was normalized by resection of the tumor and restoration of distal airflow (48). In a dramatic demonstration that HPV depends on airway, not mixed-venous Po2, unilateral graded hypoxia (12–5% inspired O2) was delivered while the contralateral lung received 100% O2. This reduced the perfusion of the hypoxic lung from a normoxic value of 52 ± 2 to 30 ± 8% of total lung flow, without a significant fall in mixed-venous Po2 (52). In healthy volunteers, 8 h of hypoxia increased PVR from 1.2 ± 0.3 to 2.9 ± 0.3 Wood Units at 2 h and thereafter PVR remained constant, reversing on return to normoxia (35). Systemic vascular resistance (SVR) decreased in parallel. The intrinsic nature of these opposing responses to hypoxia is demonstrated by the ability to demonstrate simultaneous HPV and renal vasodilatation in the isolated, serially perfused lung-kidney model and even in isolated arterial rings (53, 81). HPV also occurs in children. In a study of children with congenital heart disease, mild hypoxic ventilation (15% inspired O2) increased the PVR/SVR ratio from 0.33 to 0.40 (124).

HPV is significantly impaired by hyperventilation and the resulting respiratory alkalosis (21), a clinical pearl that can be exploited to reduce pulmonary hypertension (PHT). HPV in vivo is not reduced by effective inhibition of eicosanoid synthesis (88). Likewise, the endothelin receptor antagonist BQ-123 does not impair HPV (62). In patients with chronic obstructive pulmonary disease (COPD), HPV is attenuated by nifedipine (27), a prototypic inhibitor of L-type, voltage-gated Ca2+ channels, recapitulating findings in isolated rat lungs (78). Pretreatment of COPD patients with nifedipine attenuates the hypoxia-induced increase in PVR by 50% without reducing SVR (27). This suggests that a large proportion of HPV results from Ca2+ entry via the L-type Ca2+ channel and, since endothelium lacks L-type calcium channels, indicates that HPV is largely endothelium independent.

HPV in Animal Models

If alveolar PO2 is maintained above 60 Torr, there is a little pulmonary vasoconstriction to hypoxemia, even when mixed-venous PO2 is reduced to 10 Torr (77), confirming that alveolar O2 tension, not blood PO2, is the major determinant of HPV (38). Micropuncture studies confirm that the small resistance PAs (<200 μm) are directly exposed to the alveolar PO2 and are the major site of HPV (64). HPV can be demonstrated in salt-perfused isolated lungs (78) and resistance PA rings denuded of endothelium (15). Indeed, isolated PASMC from resistance PAs constrict to hypoxia. In contrast, smooth muscle cells (SMCs) from carotid arteries or even conduit PAs do not constrict to hypoxia (73). This demonstrates that HPV is unique to the PA, particularly the resistance segment of the pulmonary circulation. As in humans, hypoxia dilates most systemic arteries in animals (53). These findings indicate that HPV cannot be explained by endocrine or paracrine vasoconstrictors that have concordant effects on the pulmonary and systemic circulation (e.g., endothelin, leukotrienes). We believe that the restricted occurrence of HPV to intrapulmonary arteries [and to a lesser extent veins (110)] reflects the localization of the molecular apparatus that mediates HPV, namely the mitochondrial redox sensor and the effectors (O2-sensitive PASMC K+ channels) to the resistance PAs.

Although HPV can be sustained for hours (117), the persistence of global hypoxia ultimately results in a selective downregulation of acute HPV, despite the occurrence of PHT and despite the finding that constrictor responses to other stimuli are preserved or enhanced (39, 80, 97). Exposure to hypoxia for as little as 3 h can elicit this selective suppression of HPV (49). Animals and humans that are genetically adapted to life at high altitude, such as the yak (39) or the native Tibetan (85), have weak or absent HPV.

There is disagreement about the nature of HPV in isolated PA rings. In animals, HPV causes a rapid increase in PVR that gradually plateaus and is sustained, much as occurs in humans. However, in isolated PA rings, some groups find that HPV is biphasic, consisting of an immediate, endothelium-independend constriction, which peaks in ~10 min (phase I) and a second, slowly developing endothelium-dependent sustained constriction that peaks at ~40 min (phase II) (126) (Fig. 1). Although respecting these findings, our group (7) consistently finds a monophasic PA constriction in resistance PA rings, even if they are denuded of endothelium. We believe that the role of the endothelium is to modulate HPV. Nitric oxide, produced in response to pulmonary vasoconstriction, sup-
presses HPV (14, 23), and endothelin, produced in response to hypoxia, enhances HPV (91).

**O₂ Sensing in Simple Organisms**

The use of “HPV-like” mechanisms for regulating O₂ distribution and optimizing O₂ uptake is evident in simpler organisms. In bacteria, the supply of O₂ is modulated by aerotaxis, locomotion of the organism toward the optimal environmental Po₂ (109). The heme sensor that mediates aerotaxis transduces its signal via a phospho-relay, involving soluble chemotaxis proteins, which, when phosphorylated, interact with the flagellar motor. In *Escherichia coli*, the Aer protein is the O₂ sensor and it has a flavin adenine dinucleotide (FAD) binding site at its amino terminus. The FAD is oxidized or reduced in response to redox changes in the bacterial electron transport chain (ETC). The resulting conformational change alters Aer and thus changes the phosphorylation of the chemotaxis proteins. In this example, much as in mammalian HPV, the initiating signal for the hypoxic response is dependent on a diffusible redox mediator.

The evolution of multicellular and compartmentalized organisms necessitated development of specialized oxygen transport structures (airways and blood vessels) with associated “sensors” to regulate flow through these conduits. In fish, the parallel development of specialized “membrane oxygenators” (gills) and a circulatory system that perfuses the gill (branchial arteries), necessitated evolution of a coordinating mechanism. In a manner analogous to the differential response of the mammalian pulmonary and systemic circulations to hypoxia, piscine branchial arteries constrict to hypoxia, whereas systemic arteries dilate (112). In fish, the gill’s hypoxic response is intrinsic to the arteries and is impaired by metabolic inhibitors (99), analogous to HPV’s localization to the resistance PA (73, 110) and its sensitivity to ETC blockade (5, 103) in mammals. Microvessels in frog’s skin, which in amphibians function like lungs, contract to hypoxia by a mechanism involving K⁺ channel inhibition in vascular SMCs (75), much as occurs in PASMCs (94).

**HPV in Health and Disease**

In a canine model of acute atelectasis (achieved without induction of systemic hypoxemia), PVR increases within 15 min, reaches a maximal value by 1 h, and then remains elevated, resulting in a “sustained diversion of blood flow away from the atelectatic lung and a generalized increase of pulmonary perfusion pressure” (47). HPV is also instrumental in the fetal pulmonary circulation. In the presence of the low arterial P/O₂ in utero (~20 Torr) PVR is high (105). Hyperbaric maternal oxygenation decreases PVR in the fetal lung by 38% (57). These observations suggest that the fetal pulmonary circulation is actively maintained in a vasoconstricted state by HPV.

HPV also contributes to the pathophysiology of numerous diseases. Perhaps the best-characterized disease where HPV has been implicated in the pathophysiology is high-altitude pulmonary edema (HAPE). The pathogenesis of HAPE entails a high Ppa, which subsequently leads to increased capillary pressure resulting in pulmonary edema (17). This hydrostatic leak of the arteries and the capillaries is associated with altered permeability to large proteins, resulting in edema and an inflammatory response (18, 131). Drugs that reduce HPV [nifedipine (17), hydralazine and phentolamine (51), and inhaled nitric oxide or O₂] lower Ppa and improve gas exchange in HAPE (107). This suggests that, under conditions of global hypobaric hypoxia, exaggerated or nonhomogeneous HPV contributes importantly to the mechanism of HAPE. In contrast to these adverse effects, HPV can be exploited clinically, in single-lung anesthesia, to reduce blood flow to a lung by rendering it hypoxic, thereby creating a dry operative field.

**Mechanism(s) of O₂ Sensing in HPV**

The final common pathway of all forms of pulmonary vasoconstriction involves activation of the contractile apparatus (actin and myosin). There is little evidence that the contractile apparatus in the PA is unique. Most agents that constrict PAs, with the notable exception of hypoxia and mitochondrial ETC inhibitors (5), cause concordant systemic vasoconstriction. What appears to account for the restriction of hypoxic vasoconstriction to the resistance PAs is the unique localization of a functional “O₂-sensing unit” within the PASMC. The sensor, which we believe to be the proximal portion of the mitochondrial ETC, detects falls in alveolar O₂ and responds by modulating production of a mediator. The mediator, which appears to be a diffusable reactive O₂ species
ROS, alters the function of one or more effector proteins (K+ and Ca2+ channels) through a process of reduction/oxidation (redox) chemistry in which transfer of electrons to and from key sulfhydryl groups alters channel function. In hypoxia, decreased ROS production and an associated increase in reduced redox couples inhibit redox-sensitive K+ channels. This increases cytosolic Ca2+ influx via the voltage-gated L-type Ca2+ channels. This increases cytosolic Ca2+, thereby activating the PASMC’s contractile machinery. Before reviewing the evidence for mitochondria as pulmonary vascular O2 sensors, it is useful to review the structure and function of the mitochondrial ETC. In so doing, it is essential to keep in mind that mitochondria are not generic, different tissues, and even different vascular SMCs have functionally distinct mitochondria (81). The recognition of mitochondrial diversity forces one to measure mitochondrial function and ROS generation in the tissue of interest, rather than extrapolating from other species and tissues.

**ETC**

The ETC is composed of four mega-complexes that mediate transfer of electrons down a redox potential gradient through a series of carriers, resulting in final acceptance of the electron by O2 producing ATP and water (Fig. 2). In the course of electron transport, H+ are translocated, creating a proton gradient that generates the mitochondria’s extremely negative $E_m(\Delta \Psi_m)$. This potential energy is subsequently dissipated in the synthesis of ATP. A small percentage of total electron flux involves unpaired electrons, resulting in the generation of ROS within the mitochondrion, notably superoxide radical. To detoxify ROS, the mitochondria express a unique, inducible isofor of superoxide dismutase (SOD) (manganese SOD, SOD2 or MnSOD). MnSOD transforms toxic superoxide radicals to hydrogen peroxide (H2O2), which may serve as a diffusible redox mediator in HPV (5, 12, 81). Although it has been stated that for superoxide to serve as a signaling molecule it must exit the mitochondria through an anion channel (127), it appears likely that this ROS is rapidly converted to diffusible H2O2 within the PASMC’s mitochondria, creating a signal that modulates the functions of enzymes and ion channels (81, 84).

Complexes I and III produce most of the mitochondrial ROS (37), although there is tissue heterogeneity in which complex predominates, complex I (16, 59) vs. complex III (30, 122). However, when considering the site of ROS generation, one must be mindful that each complex consists of many subunits. For example, complex I consists of 42 subunits, seven of which are encoded by mitochondrial DNA (37). Inhibition of a complex proximal to the site of ROS production site would decrease ROS production, whereas inhibition distal to this site would disrupt the flow of electron down the chain, diverting them to react with O2 and generate superoxide radical. MnSOD levels are inversely proportional to mitochondrial ROS production (16), presumably because this inducible antioxidant enzyme is elevated to protect the mitochondria. Differences in basal, normoxic mitochondrial ETC function and ROS generation appear to account for the observed heterogeneity in MnSOD expression in PA vs. renal artery mitochondria (81). The PA have less negative $\Delta \Psi_m$, generate more ROS, and have much higher MnSOD levels than do renal arteries (81).

Complex I of the ETC is an NADH ubiquinone oxidoreductase where NADH is reduced to NAD+ as it transfers its two electrons to ubiquinone, which is oxidized to ubiquinol (Fig. 2). Complex I is inhibited by rotenone, a naturally occurring isoflavonoid derived from the root of *Derris elliptica* (108). Complex II is a flavoprotein dehydrogenase, where succinate, an exogenous substrate, transfers electrons to FAD to form FADH2. Complex III of the ETC is ubiquinol-cytochrome c oxidoreductase. This complex transfers electrons from FADH2 (contained in ubiquinol) to cytochrome b, and subsequently to the final electron acceptor, cytochrome c. Although the traditional model holds that each complex is independently embedded in the lipid bilayer of the inner mitochondrial membrane...
and connected by diffusing coenzyme Q and cytochrome c. Bianchi et al. (19) recently showed that multicomplex units can be isolated, suggesting that direct electron channeling may occur between complexes I and III.

There is an intermediate step involving the cytochrome bc1 complex. In the bc1 complex, ubiquinol becomes oxidized to ubisemiquinone as it passes an electron to the Rieske iron-sulfur center. Gille and Nohl (46) postulate that it is impairment of electron flux from ubiquinol to cytochrome bc1 that leads to the generation of ROS. During normal, coupled electron flux, the ubiquinol/bc1 redox couple governs the transfer of one electron to the Rieske iron-sulfur protein and recycles the second electron via low potential cytochrome b into the Q-cycle (46). By altering membrane fluidity, thereby changing electron flux, ROS generation was increased. Gille and Nohl (46) concluded, “The regulator which controls leakage of electrons to oxygen (and generation of ROS) appears to be the electron branching activity of the bc1 complex.”

In heart mitochondria, ubisemiquinone is the only reduced electron carrier in complex III capable of reducing O2 to superoxide radical, and factors preventing the formation of ubisemiquinone also prevent H2O2 formation (120). Myxothiazol is an inhibitor of the electron transfer to the Rieske iron-sulfur center of the bc1 complex that inhibits oxidation of ubiquinol to the ubisemiquinone radical by the Rieske iron-sulfur center (119). Thus ROS formation within complex III can theoretically be dissected by using myxothiazol vs. antimycin A, which targets the more distal oxidation of the ubisemiquinone radical to ubiquinone. This technique was used by the Schumacker group to suggest that it is the proximal portion of complex III that is involved with HPV. Complex IV is the cytochrome oxidase, composed of two cytochromes, a and a3. This complex, which is inhibited by cyanide, accepts electrons from reduced cytochrome c and passes them to O2 creating H2O.

Mitochondria as O2 Sensors in PASMCs

The mitochondrion’s role as the primary site for O2 consumption, energy generation, and determinant of cellular redox state makes it an ideal candidate for O2 sensing. In addition, we now recognize that the mitochondria form filamentous networks that permeate the vascular SMCs (Fig. 3) (81). This proximity to the plasma membrane and sarcoplasmic reticulum
(SR) is ideal for an organelle that serves as a sensor. The evidence suggesting that the mitochondrial ETC is important in O₂ sensing is based in part on the concordant effects of certain ETC inhibitors and hypoxia. In 1981, Rounds and McMurtry (103) noted that five inhibitors of oxidative ATP production: azide, cyanide, dinitrophenol, antimycin A, and rotenone caused "a transient pressor response, followed by loss of vascular reactivity to hypoxia, angiotensin II, and chemical inhibitors." The magnitude of the vasoconstriction in these isolated lungs was proportional to the strength of HPV for all drugs, except azide. Moreover, like HPV, the pressor responses were inhibited by verapamil, indicating that activation of the contractile apparatus was largely dependent on Ca²⁺ influx via the L-type Ca²⁺ channel. They attributed the effects of these metabolic inhibitors to a presumed inhibition of ATP production. However, subsequent studies showed that the moderate hypoxia that elicits HPV has no acute effect on ATP production or phosphocreatine levels in perfused lungs (25, 68, 69). Although anoxia does cause ATP depletion, anoxia results in only transient pulmonary vasoconstriction with the predominant response being pulmonary vasodilatation. Inhibitors of the mitochondrial ETC also emulate hypoxia's effects on the carotid body (activation of neurotransmitter secretion) (36), the systemic vasculature (vasodilatation) (81), and the ductus arteriosus (vasodilatation) (82), providing strong circumstantial evidence that the mitochondria are O₂ sensors.

Mitochondrial Redox Theory of HPV

Building on the work of Rounds and McMurtry, we showed that inhibitors of complex I (rotenone) and complex III (antimycin A), but not of complex IV (cytochrome c oxidase), mimic hypoxia's effect in the pulmonary circulation (81). The discordant effects of azide (103) in the previous work and of cyanide (5), in our studies, suggested that something about mitochondrial function, other than ATP synthesis, was crucial to O₂ sensing, that being generation of ROS (3). We proposed an alternative explanation for the concordant effects of certain metabolic inhibitors and hypoxia on pulmonary vascular tone, the so-called Redox Theory of HPV (3). This theory proposed that the PASMC's O₂-sensitive mechanism involved the proximal portion of the mitochondrial redox cascade within ETC (3) and identified the production of ROS, a by-product of uncoupled electron transport, and/or the attendant upstream accumulation of electron donors associated with ETC inhibition, as the mediator. There is a tonic, normoxic production of ROS in the PASMCs, generated by mitochondria, that is inhibited rapidly by moderate hypoxia (3). Inhibitors that block the PASMC ETC upstream of ubisemiquinone, such as rotenone (5) and myxothiazol (127), tend to inhibit the formation of ubisemiquinone and thereby reduce the formation of ROS. Conversely, ETC inhibitors that act downstream, such as cyanide and azide, increase ROS levels by increasing the production of ubisemiquinone (5). Our data are consistent with hypoxia, rotenone, and antimycin acting upstream and inhibiting ROS formation (5, 81; Fig. 2).

Because ROS have been traditionally regarded as toxic by-products of metabolism or radiation that damage lipids, proteins, and DNA, the theory was initially extremely controversial and misunderstood. The Redox Theory invokes a physiological role for ROS that were produced at extremely low levels in proportion to normal electron flux. To protect against the potentially damaging effects of even this small physiological production of ROS, PASMCs possess cytosolic glutathione and several antioxidant enzymes, such as SOD (which reduces superoxide radical to H₂O₂ and catalase (which reduces H₂O₂ to H₂O and O₂). H₂O₂ is a stable molecule with long diffusion radius and is therefore a much better candidate as a ROS mediator in O₂ sensing than the superoxide radical from which it derives (28). There are currently two schools of thought regarding the changes in ROS levels and how they signal HPV. Several groups find that the higher the PO₂ the more ROS are produced (i.e., hypoxia is a low ROS state) (10, 41, 81, 92); others find exactly the opposite (i.e., hypoxia elevates ROS) (70, 127).

Radicals Decrease in Hypoxia

Higher normoxic level of ROS causes physiological oxidation of K⁺ channel in PASMCs. O₂-sensitive Kᵢn, in PASMCs are activated by oxidation and the resulting hyperpolarization maintains physiological vasodilatation (98). During alveolar hypoxia, ROS production falls in proportion to the inspired O₂ (5, 10, 92), and K⁺ channels are reduced and inhibited, depolarizing E_M (98). In support of this view, our laboratory used three different ROS detection methods [lucigenin-enhanced chemiluminescence, AmplexRed H₂O₂ assay, and 2′,7′-dichlorodihydrofluorescein diacetate (DCF)]. In endothelium-denuded, resistance PA rings, a concordant decrease in ROS levels during hypoxia or on exposure to ETC inhibitors was detected by all three methods (81). This has been also supported by other studies where a similar decrease in ROS production is observed with HPV (92, 97). Rotenone and antimycin A, which cause pulmonary vasoconstriction and inhibit subsequent hypoxic responses, each inhibit lung ROS production, whereas other types of vasoconstrictors, angiotensin II and KCl, have no effect (5, 12). An often overlooked validation of the lucigenin enhanced chemiluminescence technique is that it shows the expected results when used to measure ROS in a myocardial ischemia-reperfusion model: radical suppression during ischemia and overshoot during reperfusion (56). These data support the concept that ROS are generated in proportion to PO₂ and demonstrate that ROS generation with hypoxia (low ROS as in HPV) is similar to the ischemic phase of myocardial ischemia-reperfusion, but different than reperfusion phase, where ROS levels are pathologically high. Whereas rotenone and antimycin cause pulmonary vasoconstriction at micromolar doses (3, 103), cyanide must be given in millimolar amounts to elicit sustained vasconstriction. In contrast, at micromolar doses, cyanide increases ROS production and causes a transient constriction due to increased ROS production. Cyanide does not inhibit subsequent HPV (5, 127). Not only do the proximal mitochondrial ETC inhibitors lower ROS, they also inhibit whole cell K⁺ current (I_K) in PASMCs. As expected, these stimuli tend to increase I_K in renal artery SMCs, a pattern reminiscent of hypoxia and important for the unique occurrence of hypoxic contraction in the pulmonary circulation (81). Paky et al. (92) also found that hypoxia reduces lung ROS production, as measured using lucigenin. They noted that the lung ROS were inhibited by the SOD-mimetic tiron and by antimycin A. Likewise, Freeman and Crapo (41) noted that ROS production in rat lungs and...
mitochondria increases in direct portion to \( P_{O_2} \). Hool and Arthur (58) also found that mitochondrial-derived (myxothiazol sensitive) ROS (\( H_2O_2 \)), measured using 5- and 6-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester, is decreased by hypoxia (\( P_{O_2} 15 \) Torr) in adult guinea pig cardiomyocytes. The hypoxic inhibition of \( H_2O_2 \) production accounted for impaired basal activity of L-type \( Ca^{2+} \) channel and enhanced sensitivity to adrenergic stimulation (58), analogous to the mitochondrial-\( K^+ \) channel dialog evoked by the Redox Theory of HPV. Wolin’s group (50, 84) also has evidence that it is the withdrawal of normoxic ROS (\( H_2O_2 \)) that elicits HPV, although they attribute the source of the ROS to NADH oxidase. In summary, we find that, regardless of the technique of ROS measurement, both in isolated lungs and isolated PA rings, hypoxia decreases ROS production, and this is a reliable effect that occurs rapidly (i.e., preceding the onset of HPV) and is reversible (5, 10, 12).

**Radicals Increase in Hypoxia**

Marshall et al. (76) found an increase in ROS during hypoxia in isolated PA myocytes, although they attributed this to NADPH oxidase. Waypa et al. (127) did parallel experiments in isolated perfused lungs and PASMCs. The authors showed that, when these cultured PASMC were treated with hypoxia, intracellular DCF fluorescence was increased, and this was blocked by myxothiazol. On the basis of these results, they agreed that mitochondria indeed function as O2 sensors, but concluded that increased, instead of decreased, mitochondrial ROS production triggers HPV (127). Moreover, they observed that a variety of antioxidants (pyrrolidinedithiocarbamate, eb-selen, and the CuZn SOD inhibitor diethyldithiocarbamate) abolished HPV (127). Both the Schumacker and Sylvester groups (70, 127, 128) conclude that hypoxia increases mitochondrial-derived ROS production.

Unlike the groups that find hypoxia decreases ROS, Schu- macker’s group performed their ROS measurements in cul- tured PASMCs and used DCF to detect ROS. DCF is a suboptimal fluorescent probe to measure ROS, as discussed in a recent review on controversies in HPV (2). DCF detects nitric oxide more efficiently than ROS and can itself generate \( H_2O_2 \). However, it is unlikely that the use of cultured cells and DCF are the only explanations for the divergent findings among groups. In agreement with the Schumacker group, Liu et al. (70) recently reported that hypoxia increases ROS as measured using DCF fluorescence and “tended to increase lucigenin-enhanced chemiluminescence,” and “in some preparations produced electron paramagnetic resonance spectra consistent with hydroxyl and alkyl radicals.” Moreover, they found that SOD and catalase reduced or abolished the constriction induced by hypoxia in porcine PAs. The latter finding contradicts our previous report that SOD plus catalase enhance HPV when administered in liposomes so that they can enter the cell (11).

**Points of Agreement and Likely Causes for Disagreements**

Both camps conclude that it is not the production of ATP but the creation of ROS by the proximal ETC that accounts for the hemodynamic effects of hypoxia and drugs like rotenone. There is also consensus that the ROS relevant to \( O_2 \) signaling are derived from mitochondria, rather than nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox). We (5) concluded in 1993 that inhibition of complex I and III mimicked hypoxia and accounted for the signal (diminished ROS) that inhibited PASMC K\(^+\) channels leading to HPV. Waypa et al. (127) similarly stated in 2001, “HPV requires mitochondrial electron transport proximal to the ubisemiquinone site but does not require the entire mitochondrial ETC to be functional.”

Moreover, when one examines the data from the Schumacker group (127, 128), all the isolated lung data agree with our observations (i.e., rotenone increase normoxic pressure and inhibits subsequent HPV). Both groups find that cyanide fails to abolish HPV (3) or hypoxia-induced increases in cytosolic calcium in PASMCs, a surrogate for constriction (128). We were able to inhibit cyanide constriction with SOD (5), and they inhibited it with eb-selen, a synthetic form of glutathione peroxidase (127).

The Schumacker group finds, in isolated perfused lungs, that proximal ETC inhibitors [diphenylene iodonium (DPI) 10 \( \mu \)M, rotenone (50 ng/ml), and myxothiazol (50 ng/ml)] inhibited HPV; whereas antimycin A did not. Although they find that antimycin A (1 ng/ml) does not selectively inhibit HPV, at 10 ng/ml it does cause pulmonary vasoconstriction and inhibits HPV (127), much as we reported (5). However, at these doses it also inhibits constriction to U-46619 (127). Thus their expectations are that inhibition of the crucial complex from which ROS are generated to signal HPV should impair constriction to hypoxia, but not other vasoconstrictors, such as U-46619, and do so without itself causing vasoconstriction. In contrast, we would expect the ETC inhibitor to mimic hypoxia, and both cause vasoconstriction and impairment of subsequent HPV. Because metabolic inhibitors lower ATP levels over time, a fall in ATP and activation of ATP-sensitive \( K^+ \) channels would eventually occur and cause relaxation. Perhaps this occurred within the 30 min during which they observed con- traction in culture PASMCs and caused the observed reduction in U-46619 contraction that they observed with antimycin A. In our studies, where ROS and PVR were measured simulta- neously, the changes in PVR and ROS occurred within seconds or minutes of administration of hypoxia or ETC inhibitors, and the change in ROS preceded the change in tone. The finding that myxothiazol did not cause pulmonary vasoconstriction is confusing. Nonetheless, the work from these two groups sug- gests that ROS from complexes I and III are involved in the mechanism of HPV.

However, there are areas of disagreement. The Schumacker group (70, 76) finds that hypoxia increases ROS. This is supported by some other groups as well. It is likely that many of these differences relate to divergence in the techniques and tissue preparations used to measure ROS. Our group measures ROS using luminol, lucigenin, and AmplifiedRed (for \( H_2O_2 \)) in freshly isolated, resistance PA rings (81) and isolated perfused lungs (5, 9, 10). Notably, most of the measurements of con- traction in the Waypa (127) study are conducted in isolated PASMCs from microvessels (\( n = 3 \), passage number unspec- ified but \( >6 \) days in culture), and a surrogate for PVR (changes in length 30 min after a stimulus is initiated) is used. The Schumacker group measures ROS in cultured PASMCs using DCF. Because cultured cells rapidly lose ion channels and also have diminished \( O_2 \) sensitivity, at least in the ductus arteriosus, we believe that it is optimal to base conclusions about \( O_2 \) sensing on fresh tissue (82). In balance, however, the conclusions of these two groups (that HPV is sensed by mitochondria, that the
sensor is in the proximal ETC, that the mediator is an ROS, and that the mechanism resides in the PASMC) are more concordant than discordant. Although there is no consensus or clear explanation for the opposing findings, the clarity would be enhanced if all investigators agreed to the following.

Report the physiological conditions showing that temperature, pH, PCO2, and PO2 are in relevant ranges. Incubation of tissues for ROS measurement is too often done at 25°C and, without careful buffering and bubbling of the media, the tissue becomes alkalotic, which creates spurious ROS readings. This is particularly problematic for techniques requiring incubation with the reporter (e.g., AmplexRed DCF). Studies should be performed at ~37°C, pH 7.35–7.45, Pco2 30–40 Torr with a hypoxic PO2 of 35–50 Torr, and normoxic PO2 of 100–140 Torr.

Show representative traces and document the magnitude of the hypoxic response. Frequently the magnitude of HPV is very small, suggesting the preparations are nonphysiological. HPV should double PVR in isolated lungs, and no priming is required for HPV in healthy ring preparations.

Give attention to sample size. Sample sizes should exceed five to allow use of parametric statistics.

Address the uniqueness of proposed constrictor mechanism to the pulmonary circulation. A putative pathway explaining HPV should operate uniquely in the pulmonary circulation, compared with systemic arteries, which dilate to hypoxia.

Make measurements in vivo and in freshly isolated tissues whenever possible. The further one gets from the in vivo situation, the greater the likelihood of spurious results.

Define the entire pathway of HPV. The Redox Theory offers testable explanations for the sensor (mitochondria), the effector (Kv channels), and the mediator (decreased ROS). The downstream mechanism involved by those that propose an increase in ROS needs to be clarified. Large doses of ROS do cause pulmonary vasoconstriction but in so doing elicit pulmonary edema (4, 8, 26), which is not a characteristic of HPV.

Other Potential Sources for ROS

Another potential O2 sensor is NAD(P)H oxidase or one of the Nox isoforms. NAD(P)H oxidase contains b558-type cytochromes and subunits, resembling the phagocytic cell NADPH oxidase system. The flavocytochrome contains two membrane-bound subunits, the 91-kDa membrane subunit of the phagocyte oxidase (gp91phox) and p22phox, and two cytosolic proteins, p47phox and p67phox. NADPH oxidase [or a variant that preferentially uses NADH as a substrate (50)] produces ROS in proportion to the PO2 and therefore has been suggested as an O2 sensor (83). Production of ROS by Nox isoforms has been demonstrated in a variety of cells in O2-sensitive tissues, including the neuroepithelial body, PASMC, and carotid body. Much of the evidence favoring NADPH oxidase as an O2 sensor rests on the effects of a relatively nonspecific pharmacological probe, DPI. DPI is an inhibitor of the NADPH oxidase and does, in several respects, mimic hypoxia. It reduces normoxic ROS production in the PA and inhibits IK in PASMCs (130). After causing slight vasoconstriction, DPI decreases subsequent pressor responses to hypoxia (116). Unfortunately, DPI is not a good probe, because it nonspecifically inhibits flavoprotein-containing enzymes, including nitric oxide synthase and complex I of the mitochondrial ETC (43).

The development of mice deficient in the 91-phox containing NADPH oxidase (Nox-2) provided us with an opportunity to study the role of NADPH oxidase in HPV. In these mice, loss of a functional NADPH oxidase dramatically lowers ROS production (measured using luminol, lucigenin, or unenhanced chemiluminescence) (12). However, HPV and the O2-sensitive portion of PASMC IK are preserved (12). This suggests that gp91phox present in PASMC and PA endothelium is not required for O2 sensing in HPV. Moreover, rotenone constriction is preserved or enhanced, consistent with the mitochondrial O2 sensor hypothesis (12). Preserved O2 sensing has also been reported in the type 1 cell of the carotid body from these mice (104). Overall, these findings argue against the classical NADPH oxidase system as an O2 sensor in HPV, although they do not exclude a role for other novel oxidases.

EFFECTOR MECHANISMS OF HPV

K+ Channels

4-Aminopyridine (4-AP), an inhibitor of Kv channels, but not ATP-sensitive K+ channels, causes pulmonary vasoconstriction (55). Post et al. (94) showed that hypoxia inhibited Ik and depolarized Em in canine PASMCs. This initiated extensive research to quantify the contribution of K+ channels to HPV and determine the molecular identity of the O2-sensitive K+ channels. K+ channels are proteins consisting of four transmembrane-bound a-subunits and four regulatory b-subunits. The ionic pore, which determines the channel’s intrinsic conductance and ionic specificity, is created by the formation of tetramers of a-subunits. The Kv channels also have, in their S4 region, a voltage sensor. b-Subunits associate with many Kv channels and alter their expression and kinetics. There are several types of potassium channel a-subunits, including Kv1, inward rectifier, and twin pore channels. The Kv channels have emerged as a possible effector in HPV (97).

Kv channels are important determinants of equilibrium potential of vascular SMCs. Closure of Kv channels decreases the tonic efflux of K+ that otherwise occurs because of the intra-/extracellular concentration gradient (145/5 mM). Channel closure renders the cell interior relatively more positive (depolarized). At these more positive potentials (positive to ~30 mV), the open probability of L-type voltage-gated Ca2+ channels increases. This increases intracellular Ca2+ influx (down a 20,000/1 concentration gradient). Although less important than in cardiomyocytes, Ca2+ influx also cause release of intracellular stores, so-called calcium-induced calcium release, effectively increasing total calcium levels inside the cell. Increase in cytosolic Ca2+ not only activates contraction via the actinmyosin apparatus but also increases the activation of immediate early genes, inducing a proliferative response (93). Thus regulation of K+ channel activity and the subsequent regulation of Ca2+ may be important to maintain the pulmonary circulation’s low PVR and the thin-walled morphology of small PAs.

Although all K+ channels are somewhat sensitive to prolonged or severe O2 deprivation (because most require some basal phosphorylation and thus ATP), certain K+ channels are specially suited to O2 sensing, by virtue of possessing key cysteine and methionine groups. Reduction or oxidation of these residues by a redox mediator, such as ROS, can cause conformational changes in the channel, thereby altering pore
function (13). In this regard, some K_v channels, including K_v1.5, respond to reduction and oxidation by changing their gating and open-state probability (15). In the PASMCs, oxidants (e.g., H_2O_2, diamide, and oxidized glutathione) increase I_K, whereas reducing agents (e.g., reduced glutathione) and agents that facilitate electron shuttling (e.g., duroquinone, a synthetic coenzyme Q mimetic) inhibit I_K (98). In accordance with their electrophysiological effects, oxidants (e.g., diamide) dilate the pulmonary circulation (mimicking O_2), whereas reducing agents mimic hypoxia (129). Hypoxia and redox agents may alter the function of K^+ channels, directly (61) or by modulating levels of a diffusible redox mediator, such as ROS. It is unknown whether the electrophysiological effects of O_2 are direct or mediated via a redox mediator, as we propose. However, for a channel to respond to a diffusible redox mediator, it arguably must be intrinsically redox sensitive.

To date, nine families of K_v channel have been identified, each with multiple isoforms. The identification of the K_v channels involved in the E_s of PASMCs has been difficult. These channels activate in a nonlinear fashion with depolarization, and many are inhibited by the pore-blocking drug 4-AP. A variety of putative O_2-sensitive channels exist in the PASM (K_v1.2, K_v1.5, K_v2.1, K_v3.1b, and K_v9.3) (31). The importance of K_v1.5 and K_v2.1, 4-AP-, and redox-sensitive channels is emphasized by two models that manifest selective suppression of HPV, the chronic hypoxic PHT model and the K_v1.5 knockout mouse. In chronic hypoxia, impairment of HPV results from loss of K_v1.5, and to a lesser extent K_v2.1, expression with concordant suppression of O_2-sensitive I_K (97, 111). In chronic hypoxia, enhancing expression of K_v1.5, via K_v1.5 adenoviral gene transfer (95), restores K_v expression, O_2-sensitive I_K, and HPV. Mice with targeted K_v1.5 deletions also have impaired HPV and reduced PASMC O_2-sensitive I_K (7). In a recent study, we showed that preconstriction with the K_v blocker 4-AP eliminates subsequent HPV, although the PA constricts vigorously to phenylephrine under the same conditions (15). The strong parallel between constriction to the selective K_v1.x channel inhibitor correolide (15) and hypoxia suggests that, of the candidate K_v channels, a K_v1.x channel is central to the mechanism of HPV (15). Theoretically, correolide can inhibit K_v1.2; however, tityustoxin, an inhibitor of K_v1.2/K_v1.3, inhibits only a small portion of the hypoxia-sensitive I_K (15). Moreover, after intracellular dialysis of PASMCs with antibodies against intracellular domains of K_v1.5 causes membrane depolarization and with the combination of anti-K_v1.5 and anti-K_v2.1, little responsiveness to hypoxia remains (13, 15). Finally, although mRNA for many K_v channels is more abundant in resistance vs. conduit PAs, only K_v1.5 protein is expressed more abundantly in the resistance PAs, which are the main locus for HPV (15). Further support for the physiological relevance of K_v1.5 to HPV comes from studies of heterologously expressed K_v1.5. Human K_v1.5, cloned from normal PA, generates an outward K_v current at −65 mV, which is inhibited by hypoxia (15). Inhibition of K_v1.5, using immunoelectropharmacology, depolarizes PASMCs (15). Thus the consequence of enriched K_v1.5 expression in resistance PAs is a relative hyperpolarization of resistance vs. conduit PASMCs (−65 vs. −35 mV) that can be attributed to the localized expression of O_2-sensitive K_v channels in resistance PASMCs.

Intracellular Ca^{2+} Regulation

Intracellular Ca^{2+} plays an obligatory role in pulmonary vasoconstriction (44, 106); the question is the extent to which influx of extracellular Ca^{2+} vs. release of intracellular Ca^{2+} initiates HPV. There is no doubt that, as in all types of SMC, release of Ca^{2+} from intracellular pools, particularly the SR, is important to vasoconstriction. As with the differential function of O_2-sensitive K_v channels in PA vs. renal arteries, there are differences in handling of intracellular Ca^{2+} between SMCs in these circulations. In PASMCs, inositol triphosphate (IP_3) and ryanodine-sensitive Ca^{2+} stores are organized into spatially distinct compartments; in contrast, renal artery SMCs have Ca^{2+} stores that are spatially contiguous (60). Moreover PASMC manifest spontaneous, localized intracellular Ca^{2+} transients, sensitive to caffeine and ryanodine, much like Ca^{2+} sparks in cardiac myocytes (60). The constrictor pathway for certain agonists, including endothelin, likely involves Ca^{2+} sparks (100). In PASMCs, increasing Ca^{2+} influx, whether via depolarization or the L-type Ca^{2+}-channel agonist BAY K 8644, increases spark frequency (100). Enhancing Ca^{2+} sparks with caffeine precipitates membrane depolarization in PASMCs, whereas it promotes hyperpolarization in systemic arterial SMCs (100). These results suggest that PASMC sparks, like those in systemic arterial SMCs, originate from ryanodine receptors but indicate that they play a different physiological role in modulating E_M, particularly in response to certain agonists (100). The role of sparks in HPV, if any, is uncertain.

Hypoxia causes intracellular Ca^{2+} increase, reaching maximum level in 1–2 min, and this is sustained during hypoxia, reversing on return to normoxia (102, 106). Urena et al. (121) found that in conduit PASMC, hypoxia reduced basal intracellular Ca^{2+} and decreased Ca^{2+} spikes (121), consistent with the lack of significant HPV in conduit PAs (6). In resistance PAs, two subsets of PASMC were identified, one in which hypoxia increased cytosolic Ca^{2+}, a response mimicked by KCl and inhibited by nifedipine or the removal of extracellular Ca^{2+}, and another in which hypoxia decreased Ca^{2+} (121). These findings of longitudinal heterogeneity in Ca^{2+} homeostasis are in keeping with the previously identified K_v channel diversity in the pulmonary circulation (1, 6). Nonetheless, the predominant source of Ca^{2+} for HPV appears to be extracellular and is admitted to the PASMC via the L-type Ca^{2+} channels. For example, in 300-μm PAs, hypoxia (P_O_2 30–50 Torr) causes vasoconstriction and increases intracellular Ca^{2+} (54), both of which were blocked by verapamil, as occurs in humans (27) and rodents (78).

Interest in intracellular Ca^{2+} release is greatest in groups that evoke a role for the endothelium in HPV. Even among groups that find HPV has two phases, there is disagreement about the relative role of Ca^{2+} influx vs. intracellular Ca^{2+} release. Zhang et al. (133) report that, in Ca^{2+}-free solutions, both phase I and II hypoxic contractions in PA rings are reduced to 11 and 32% of their normal magnitude. Moreover, amiodipine, an L-type Ca^{2+} channel antagonist, largely inhibited phase I of HPV (133). Adding to the credibility of this report, they noted a greater HPV in resistance vs. conduit PAs. Many other studies have shown that phase I of HPV is inhibited by calcium channel blockers or by depletion of ryanodine-sensitive SR Ca^{2+} stores (32, 33, 106). Furthermore, preincubation of PA with SR Ca^{2+} ATPase antagonist cyclo-
piazonic acid and the nonspecific inhibitor of extracellular Ca\(^{2+}\) entry lanthanum also abolishes phase I of HPV without affecting the sustained phase II hypoxic constriction (33, 102). Several groups have reported that, unlike phase I, phase II is not dependent on extracellular Ca\(^{2+}\) (34, 132). Studies with Ca\(^{2+}\)-free solution have shown the presence of phase II HPV responses in both endothelium-intact and endothelium-denuded PAs (33). In addition, depletion of Ca\(^{2+}\) release from SR stores by preincubation with caffeine or ryanodine abolishes phase II HPV (33, 34). Furthermore, inhibition of RyRs has been shown to attenuate or partially inhibit hypoxia-induced Ca\(^{2+}\) increases in PASMCs and reduce constriction in isolated perfused PA and lungs (44, 71, 86). This suggests that Ca\(^{2+}\) stores are important in mediating phase II of the HPV response at least in this model.

Controversies Regarding Ca\(^{2+}\) Pools That Mediate HPV

As in the case of the O\(_2\) sensor, there are controversies regarding the Ca\(^{2+}\) pools that mediate HPV. Our unpublished data confirm that inhibition of SR Ca\(^{2+}\) handling does inhibit HPV but only in a nonselective manner (i.e., doses of ryanodine or thapsigargin sufficient to inhibit HPV also inhibit constriction to angiotensin II and KCl). More importantly, we do not observe "2 phases" in vivo in our isolated perfused lung or resistance PA ring studies (15). Some groups find essentially no role for the L-type Ca\(^{2+}\) channel in HPV (102), and others find a contribution from both Ca\(^{2+}\) release and influx (106). It is likely that, while influx of calcium via the L-type channel is crucial, SR calcium release reinforces HPV; however, the suggestion that HPV is independent of extracellular calcium is at odds with a substantial body of work in vivo [in humans (27)] and in isolated lungs (27, 65, 78, 96), where specific inhibitors of L-type Ca\(^{2+}\) channels inhibit and agonists of the L-type Ca\(^{2+}\) channel enhance HPV (79, 118). It should also be noted that several early, key papers indicating a crucial role for early release of intracellular calcium in the mechanism of systemic vasoconstriction and HPV (44, 45) have been brought into question by the retraction of many figures on which such theories were initially based at the order of the Office for Research Integrity due to falsification of data (http://ori.dhhs.gov/html/misconduct/gelband.asp).

Cyclic Adenosine Diphosphate Ribose

A potential mediator implicated in the mechanism of HPV is the production of cyclic adenosine diphosphate ribose (cADPR; Refs. 33, 34, 40, 132). cADPR in PASMCs sensitizes SR Ca\(^{2+}\) release via RyRs (42) (Fig. 4). cADPR production correlates with β-NADH levels, and it is postulated that in hypoxia increasing β-NADH levels increases cADPR and thus SR Ca\(^{2+}\) release. Inhibition of β-NADH oxidase by hypoxia, with subsequent β-NADH accumulation, has been postulated to represent an O\(_2\)-sensing pathway. β-NADH induces a concentration-dependent increase in cADPR production from β-NAD\(^{+}\) (132). Moreover, the range over which a change in the β-NADH/β-NAD\(^{+}\) ratio augments cADPR accumulation is within the physiological and was observed in extracts from PA during normoxia and moderate hypoxia. cADPR is at least one order of magnitude higher in tissue homogenates of PASMCs compared with aortic and mesenteric SMC (132). The level of the enzyme involved in accumulation of cADPR was inversely related to the diameter and was increased in hypoxia (132). However, there is significant evidence that does not support this theory. First, inhibition of this mechanism, using 8-bromo-cADPR, has no effect on the endothelium-dependent phase I of HPV (33). It is surprising that an important mechanism for eliciting HPV would be ineffective in blocking the initial phase of HPV. Second, the levels of cADPR generated with increased β-NADH was twofold, whereas hypoxia generated a 10-fold increase in cADPR accumulation, suggesting other mechanisms contribute to the generation of cADPR (33). For proponents of ROS increasing in hypoxia, the cADPR theory is appealing because superoxide has been implicated as a signal for increasing cADPR synthesis by directly activating ADP-ribose cyclase (67, 90). Finally,

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**Fig. 4.** Proposed schematic representation of pathways involved in HPV. The Redox Theory proposes that the proximal mitochondrial ETC senses hypoxia and decreases ROS production. A decrease in superoxide radical or H\(_2\)O\(_2\) in turn inhibits effector O\(_2\)-sensitive K\(_{\text{c}}\) channels. K\(_{\text{c}}\) channel inhibition depolarizes the membrane and activates Ca\(^{2+}\) entry via L-type Ca\(^{2+}\) channels. Other theories propose NADPH oxidase as a sensor, cyclic adenosine diphosphate ribose (cADPR) as a redox activated mediator that causes intracellular Ca\(^{2+}\) release by activating ryanodine receptors, and Rho-kinase, which sensitizes the contractile apparatus to Ca\(^{2+}\). E\(_{\text{m}}\), membrane potential; MnSOD, manganese superoxide dismutase; SR, sarcoplasmic reticulum.
although 8-bromo-cADPR inhibits phase II HPV, this is a very nonspecific proof. Many vasodilators achieve this, including prostaglandins, nitric oxide, adrenomedullin, etc. Thus this is a potentially important pathway but perhaps not specific to the mechanism of HPV, and certainly further study is warranted.

Rho Kinase and the Contractile Apparatus

Elevation of intracellular Ca\(^{2+}\) elicits contraction primarily via activation of Ca\(^{2+}\)-calmodulin-dependent myosin light chain kinase (MLCK) and resultant phosphorylation of a 20-kDa MLC (MLC\(_{20}\)). Phosphorylation of MLC\(_{20}\) increases the intrinsic ATPase activity of myosin, thereby enhancing the velocity and force of the actin-myosin cross-bridging cycle (113). Vascular smooth muscle tone is primarily determined by the phosphorylation/dephosphorylation ratio of MLC\(_{20}\), which in turn is regulated by the relative activities of MLCK and myosin light chain phosphatase (MLCP) (113). The dynamic balance is such that increases in activity of MLCK or decreases in MLCP activity will increase MLC\(_{20}\) phosphorylation and contraction. Rho-kinase is a pivotal mediator of Ca\(^{2+}\) sensitization and vascular SMC (114). Upon binding of the small monomeric G protein RhoA, Rho-kinase inhibits MLCP, resulting in Ca\(^{2+}\)-independent phosphorylation and activation of CPI-17, a phosphoprotein inhibitor of MLCP (66) (Fig. 4). In vivo evidence that Rho-kinase may be involved in HPV, and specifically in the Ca\(^{2+}\) sensitization implicated in the development of sustained HPV, comes from studies of isolated arteries and in situ perfused lungs of the rat (101). Y-27632, an inhibitor of Rho-kinase, preferentially inhibits sustained HPV while having a minimal effect on the transient phase of hypoxic contraction in vivo, ex vivo, and in vitro settings (125). Hypoxia can activate Rho-kinase in a Rho-dependent manner, and inhibition of either RhoA (via exoenzyme C\(_3\)) or Rho-kinase (via Y-27632) attenuates the hypoxic increase in MLC\(_{20}\) phosphorylation (125). However, given the fact that Rho-kinase is present in other vascular beds, such as aorta (89) and coronary arteries (63, 87), the role of this kinase in eliciting a specific hypoxic pressor response is unclear.

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