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A mitochondrial redox oxygen sensor in the pulmonary vasculature and ductus arteriosus¹

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Abstract

The mammalian homeostatic oxygen sensing system (HOSS) initiates changes in vascular tone, respiration, and neurosecretion that optimize oxygen uptake and tissue oxygen delivery within seconds of detecting altered environmental or arterial PO₂. The HOSS includes carotid body type 1 cells, adrenomedullary cells, neuroepithelial bodies, and smooth muscle cells (SMC) in pulmonary arteries (PA), ductus arteriosus (DA) and fetoplacental arteries. Hypoxic pulmonary vasoconstriction (HPV) optimises ventilation-perfusion matching. In utero, HPV diverts placentally-oxygenated blood from the non-ventilated lung through the DA. At birth, increased alveolar and arterial oxygen tension dilate the pulmonary vasculature and, constrict the DA, respectively, thereby transitioning the newborn to an air-breathing organism. Though modulated by endothelial-derived relaxing and constricting factors, O₂-sensing is intrinsic to PA- and DASMCs. Within the SMC's dynamic mitochondrial network, changes in PO₂ alter the reduction-oxidation state of redox couples (NAD⁺/NADH, NADP⁺/NADPH) and the production of reactive oxygen species, ROS (e.g. H₂O₂) by Complexes I and III of the electron transport chain (ETC). ROS and redox couples regulate ion channels, transporters, and enzymes, changing intracellular calcium [Ca²⁺]_i and calcium sensitivity and eliciting homeostatic responses to hypoxia. In PASMC, hypoxia inhibits ROS production and reduces redox couples, thereby inhibiting O₂-sensitive voltage-gated potassium (K_v) channels, depolarizing the plasma membrane, activating voltage-gated calcium channels (Ca_L), increasing [Ca²⁺]_i and causing vasoconstriction. In DASMC, elevated PO₂ causes mitochondrial fission, increasing ETC Complex I activity and ROS production. The DASMC's downstream response to elevated PO₂ (K_v channel inhibition, Ca_L activation, increased [Ca²⁺]_i and rho kinase activation) is similar to the PASMC's hypoxic response. Impaired O₂-sensing contributes to human diseases, including pulmonary arterial hypertension and patent DA.

Keywords

Hypoxic pulmonary vasoconstriction; ductus arteriosus; mitochondria; oxygen-sensitive potassium channels; pulmonary arterial hypertension; patent ductus arteriosus

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Introduction

Although human life requires oxygen, all humans begin life in the hypoxic intrauterine environment. The foetus receives oxygen from the mother through the placenta. With the first breath, at the moment of birth, we are exposed to elevated oxygen concentrations in the environment. Thereafter, we live in an environment in which oxygen is usually abundant. However, as we proceed through life we may be exposed to hypoxia both as a consequence of changes in our environment, such as exposure to high altitude, or through disease (e.g. pneumonia, atelectasis). In the modern era of artificial ventilation we may also experience hyperoxia.

Oxygen is required for mammalian life because mammals have high energy demand. Oxygen's ability to receive electrons permits it to participate in an elegant cascade within the mitochondria that ultimately produces energy-rich phosphates (ATP). Situated at the distal end of a redox cascade (the mitochondrial electron transport chain, ETC), oxygen receives electrons and in doing so, is reduced, forming water. The reductive journey of electrons down a redox gradient begins with electron donors nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂), which are produced by mitochondrial metabolic cycles, notably, the Krebs cycle. These donors pass their electrons down a redox potential gradient that is spread over 4 mega-complexes (ETC Complexes I-IV) spanning the inner mitochondrial membrane. This electron transport powers the pumping of hydrogen ions across the inner mitochondrial membrane and in so doing, generates the potential energy to power ATP synthesis.

The range of physiologically optimal oxygen tension is narrow; too much or too little oxygen can result in disease. Evolution has endowed mammals with a network of specialized tissues that sense oxygen in their local environment. These tissues couple an upstream mitochondrial O₂-sensor mechanism with downstream redox-responsive effectors (ion channels and enzymes). Upon sensing small changes in PO₂, these effectors initiate compensatory responses that optimize the uptake and delivery of oxygen to our tissues. This network is called the Homeostatic Oxygen Sensing System (HOSS) [95]. The components of the HOSS are strategically distributed in the body to rapidly optimize oxygen uptake and distribution in response to small changes in airway oxygen levels (FiO₂) or blood oxygen levels (PO₂).

The HOSS includes type 1 cells in the carotid body, and smooth muscle cells (SMC) in small pulmonary arteries (PA), fetoplacental arteries in the placenta, and the ductus arteriosus (DA). HOSS components can increase ventilation to enhance oxygen uptake (the type 1 cells in the carotid body), match ventilation to perfusion in the lung to avoid perfusing hypoxic lung segments (PASMC in small resistance-level pulmonary arteries), and redistribute blood to hypoxic organs by causing vasodilatation (SMC in the DA or systemic arteries). Additional components of this homeostatic system include the adrenomedullary chromaffin cells in the adrenal glands, which can release catecholamines to counteract hypoxic stress at birth, and neuroepithelial bodies, neuroendocrine cells in the airways, the function of which is less well understood. The HOSS should arguably be considered one of

the body's major physiologic systems, on equal footing with the cardiovascular, nervous, or endocrine systems.

Attributes of HPV

Since its first description 120 years ago by Bradford and Dean [16], and subsequent more extensive description by von Euler and Liljestrand [24], the search for the mechanism of HPV has been the subject of much research [10, 11, 64, 66–68, 93–95, 97]. The field has also seen substantial debate [88, 96]. To help clarify the study of HPV, it is essential to understand its physiologic phenotype. The basic attributes of HPV include its rapid onset. HPV onsets within seconds of exposure to hypoxia [42] and reaches a maximum within minutes [13]. HPV is also completely reversible upon restoration of normoxia [82]. In addition, HPV is primarily elicited by alveolar hypoxia, rather than pulmonary arterial hypoxemia. Consistent with this responsiveness to alveolar hypoxia, HPV is strongest in the resistance pulmonary arteries (PA), which are exposed to alveolar oxygen tensions (FiO_2). The fact that HPV is reversible and localized to the region of the lung that is hypoxic is illustrated in Figure 1. In this patient, airway hypoxia caused by a mucus plug in the left lung's main bronchus led to corresponding left lung HPV, thereby reducing left lung perfusion and optimizing systemic PO_2 . HPV was sustained until the mucous plug was removed by means of bronchoscopic aspiration.

Hypoxic constriction is unique to the PA; systemic arteries, such as the mesenteric arteries [101] and renal arteries [62], relax in response to hypoxia. Likewise, at the cellular level, PASMC from PA constrict to hypoxia while cerebral artery SMC relax [51]. There is a longitudinal heterogeneity in the magnitude of HPV in cats, with HPV being greater in small, resistance PA (diameter $< 300\mu\text{m}$) and less in large, conduit PA (diameter $> 500\mu\text{m}$) arteries. Indeed, larger PA behave like systemic arteries, exhibiting neither significant hypoxic constriction nor hypoxic PASMC depolarization [50]. Similar localization of HPV to resistance PA is noted in the rat [7, 9]. On a molecular level, the downstream constrictor mechanism in HPV is well accepted to result, in large part, from a rise in intracellular calcium in PASMC [57].

Role of potassium and calcium channels in HPV

The effector mechanism of HPV involves the coordinated activity of redox- and voltage-sensitive K^+ [74] and Ca^{2+} channels [93]. McMurtry et al showed that verapamil, an inhibitor of large-conductance, voltage-gated calcium channels (Ca_L) virtually eliminated HPV in an isolated perfused rat lung. This indicates that the calcium required for HPV is largely derived from the extracellular pool and enters the PA via Ca_L channels [57], a finding reinforced by Harder in studies conducted in isolated PA rings [35]. Conversely, HPV is enhanced by Ca_L channel agonists, such as BAY K8644 [59, 86]. The sensitivity of Ca_L channels to changes in oxygen tension varies longitudinally along the pulmonary vasculature. Resistance PA have increased Ca_L channel current density and greater I_{Ca} hypoxia-sensitivity, as compared to conduit PA [27].

Ca_L channels are voltage-dependent and cell membrane depolarization favours their activation (although the Ca_L can be intrinsically O_2 -sensitive [27]). Potassium (K^+) channels

are known to regulate membrane potential (E_m) in multiple cell types, including SMC. In pharmacology experiments using isolated perfused rodent lungs, Hasunuma et al showed that the K^+ channel blockers, tetraethylammonium (TEA) and 4-aminopyridine (4-AP), cause pulmonary vasoconstriction [36]. Post et al used the whole-cell patch clamp technique to directly demonstrate that outward potassium current (I_K) creates the PASMC's relatively negative E_m (< than $-50mV$). This electronegativity prevents opening of the Ca_L channels, which have their own a voltage sensor that favours channel opening at more positive, depolarized, membrane potentials (i.e. $> -20mV$) [74]. The PASMC's I_K is inhibited by hypoxia, leading to membrane depolarization and vasoconstriction. Post also demonstrated that K^+ channel antagonists, notably the voltage-gated K^+ channel (Kv) inhibitor 4-AP, mimicked the hypoxic response in isolated rat lung as well as PA rings, increasing pulmonary artery pressure (PAP). 4-AP inhibits the hypoxia-sensitive portion of I_K in isolated rat PASMC [74]. Although blockers of the Ca_L eliminate the majority of HPV [35, 57], hypoxia also increases intracellular calcium by enhancing release from the sarcoplasmic reticulum (SR) [79]. Hypoxia can also directly enhance calcium entry through the Ca_L [28].

Initially, pharmacology and electrophysiology were used to suggest the identity of the oxygen-sensitive K^+ channels involved in HPV. These studies showed that the PASMC's hypoxia-sensitive I_K current was sensitive to 4-AP, but not TEA or charybdotoxin (CTX), inhibitors of large conductance, Ca^{2+} -sensitive K^+ channels (BK_{Ca}). The hypoxia-sensitive I_K was predominant in PASMC isolated from resistance PA, whereas a BK_{Ca} -predominant I_K was evident in PASMC derived from conduit PA (which dilate in response to hypoxia) [7]. Subsequently, correolide, an inhibitor of Kv1.x channels, provided further specificity showing that much of the hypoxia-sensitive I_K in PASMC was conducted by member of the Kv1.x channel family.

Molecular studies ultimately identified specific voltage-gated, O_2 -sensitive K^+ channels that elicit HPV, including Kv1.5 and Kv2.1 [9]. Kv1.5 mRNA and protein levels are increased in resistance PA (relative to conduit PA), and the majority of I_K in these arteries is inhibited by correolide. Inhibition of Kv1.5 and Kv2.1, achieved by administering antibodies against these channels to the intracellular space using a patch pipette, results in additive inhibition of cell depolarization. Superimposition of hypoxia after addition of the anti-Kv antibodies does not result in further membrane depolarization. This suggests that these two channels contribute a majority of the hypoxia-sensitive I_K . Consistent with this, Platoshyn et al demonstrated that expression of Kv1.5 is proportional to sensitivity to hypoxia, and that heterogeneity in the expression of Kv1.5 channels amongst PA segments accounts in part for regional heterogeneity in the magnitude of HPV [73]. Finally, HPV is impaired in mice lacking Kv1.5, even though expression of other putative oxygen-sensitive Kv channels (Kv 1.1, 1.2, 2.1, 3.1, and 4.3) is unaltered [8].

It is noteworthy that ion channels may be "redox-sensitive" due to their intrinsic amino acid sequence (i.e. the presence of redox-sensitive sulfhydryl groups) [21]. Certain amino acids, such as histidine, cysteine, methionine, and tryptophan are particularly sensitive to modification by ROS. Thus, ROS may cause redox changes in key amino acids, thereby altering channel conformation and function. In addition, Kv channels are tetramers of α -subunits and may assemble as heterotetramers, often with associated β -subunits. The

heterotetrameric composition of a channel or its association with β -subunits may alter its O_2 sensitivity. For example, when expressed in PASMC, heterotetrameric channels comprised of Kv1.5 plus Kv1.2 or Kv2.1 plus Kv9.3 α -subunits, display enhanced oxygen sensitivity compared to similar expression of homomeric channels [41].

Another line of evidence for the role of O_2 -sensitive K^+ channels in the mechanism of HPV comes from studies of rats exposed to chronic hypoxia. Chronic hypoxia attenuates acute HPV while leaving other forms of vasoconstriction unaltered or enhanced [58], and ultimately causes a form of pulmonary hypertension that is associated with decreases in the expression of both Kv1.5 and Kv2.1 [77, 102]. This loss of acute HPV in chronic hypoxic pulmonary hypertension can be reversed by infection of the lung with Kv1.5, delivered by a serotype 5 adenovirus. Kv1.5 gene therapy also reduces pulmonary hypertension and regresses both right ventricular and PA hypertrophy [75].

Other voltage-gated channels, including Kv3.1b, have been implicated in the mechanism of HPV. Osipenko et al used patch clamp techniques to investigate the effect of hypoxia on Kv3.1b. They reported that hypoxia inhibited Kv3.1b, but not Kv1.x channels, and that addition of TEA inhibited the electrophysiological effects of hypoxia in PASMC [69] – a finding discordant to observations reported by both Hasunuma [36], Post [74], and Archer [8].

Some debate exists surrounding the role of Kv channels versus other types of K^+ channels in HPV. This debate revolves around consideration of the PASMC's resting E_m . Two-pore K^+ channels (K_{2P} channels) have greater open-state probability at more negative E_m than do Kv channels. Unlike Kv channels, they do not show desensitization with prolonged or repeated depolarization. Thus, it has been argued that they may be more relevant to HPV since they would be expected to be active at the PASMC's resting E_m . However, it should be noted that, under proper experimental conditions (i.e. studying PASMC at physiological temperature and PO_2) using a patch clamp protocol that does not include prolonged exposure of the cell to depolarization or extreme depolarization steps, Kv channels are also active at resting E_m . Candidate K_{2P} channels include TASK channels (TWIK-related, acid-sensitive K^+ channel). TASK channels conduct a background current that is voltage-independent [22]. Additional 2-pore K^+ channels, including TASK-2, THIK-1 (tandem pore domain halothane-inhibited K^+ channel 1), TREK-2 (TWIK-related K^+ channel 2), and TWIK-2 (tandem pore domain weak inward rectifier K^+ channel 2) have been detected in the PASMC [29]. However, when evaluating the potential role of K^+ channels in HPV, one would expect observations to be concordant with established attributes of HPV. While it is expected that inhibition of a putative candidate channel should elicit vasoconstriction (mimicking hypoxia), blockade of K_{2P} channels, with anandamide or bupivacaine, did not significantly increase pulmonary vascular tone [29].

Although there is no clear explanation for these divergent findings, the majority of research surrounding TASK channels occurred using proximal rather than resistance PA [32]. Moreover, one can envision heterogeneity in the channels involved in HPV amongst individuals and between species. In addition, each channel activates and inactivates at specific voltages, and therefore, there may be a rolling progression of channels inhibited by

hypoxia. Those channels active at resting membrane potential would be first to be inhibited by hypoxia, but the resulting depolarization would bring into play other channels that function at more positive potentials. Other factors that result in heterogeneous reports include the failure to define PO_2 and pH in many studies. Anoxia and hypoxia are distinct, and it is important to be precise when defining study conditions and ensure they replicate conditions relevant to HPV. Finally, there is diversity in the quality of the cell used when examining the mechanism of HPV. Some laboratories use conduit PAs (which are easier to harvest but lack HPV), while others use PASMC that have been passaged in culture. Cell culture down-regulates the expression of many Kv channels and attenuates hypoxia-sensitive I_K . The healthier the PASMC preparation, the more negative the resting membrane potential (usually $\sim -60mV$). In evaluating the quality of the evidence for a channel's role in HPV one should consider whether the cells being used are primary isolates from resistance PAs, whether they manifest a robust response to hypoxia, whether they have a demonstrable hypoxic response (cellular constriction or a rise in $[Ca^{2+}]_i$) and whether the studies were performed at optimal temperature and pH.

The downstream portion of the HPV mechanism is shared with other tissues in HOSS, albeit the specific ion channels involved and the consequence of channel inhibition varies amongst tissues. For example, hypoxic inhibition of I_K and depolarization of the plasma membrane activates Ca_L channels leading to calcium influx in the type 1 cell of the carotid body [47, 72], the adrenomedullary cells [65], and the neuroepithelial body [99]. However, in each of these tissues, the rise in calcium triggers secretion of mediators, rather than vasoconstriction. In the DASMC, the downstream electrical cascade is similar to that in the PASMC, but is triggered by increased PO_2 [87].

Mitochondria – the O_2 sensor mediating HPV

While the identity of HPV's effector mechanism is reasonably well established, the sensor and mediator components of the O_2 -sensing system are the subject of ongoing investigation (and some controversy [88, 96]). Although there is certainly controversy, our worthy opponents in a debate on the mechanisms of HPV noted:

“Currently, the best documented hypothesis for HPV proposes that Ca^{2+} entry is mediated primarily via voltage-dependent L-type channels, as a result of hypoxia-induced inhibition of voltage-gated K^+ channels (Kv channels) and consequent depolarization. According to this scheme, Kv channel inhibition is caused by a decrease in the ambient intracellular concentration of H_2O_2 which results when mitochondrial electron transport, and consequently the production of superoxide ion, falls due to the lack of O_2 . There is an enormous body of evidence supporting this hypothesis, which has been presented in a number of authoritative reviews” [1].

Although the O_2 -sensitive ion channels respond to changes in PO_2 , they are not themselves sensors. In fact, systemic and pulmonary arteries have a similar complement of K^+ and Ca^{2+} channels, and respond similarly to Kv channel blockers such as 4-AP with vasoconstriction, and to Ca_L inhibitors, such as verapamil and nifedipine, with vasodilation. Rather, it is the

vascular oxygen sensor and the sensor's susceptibility to initiate PO_2 -dependent changes in production of redox mediators that differs between these vascular beds.

Mitochondria are leading candidates for the role of PA O_2 sensors. Although mitochondria rely on molecular oxygen to produce ATP the PASMCM's oxygen sensor does not rely on ATP depletion, which would be a late and extreme signal of hypoxia. Instead, the sensor monitors mitochondrial electron transport and the related production of ROS and changes in redox couples, which are perturbed by hypoxia before ATP is depleted. Recent advances in live cell imaging using mitochondrial-targeted fluorophores reveal that the PASMCM's mitochondria are arrayed in an extensive network [15, 52] (Figure 2). Recognition of the structure and mobility of mitochondria offers biologic plausibility that these organelles are positioned to rapidly convey redox signals to ion channels in the plasma membrane as well as cytosolic enzymes within vascular SMC [63].

Rounds and McMurtry demonstrated that various ETC inhibitors increased pulmonary vascular resistance, mimicking the effects of hypoxia in a crystalloid-perfused isolated rat lung model [80]. They postulated that the constrictor effect related to energy depletion. However, further investigation showed that within the rapid time frame of the vasoconstriction, there was no energy depletion or change in PO_2 . Rather, these ETC inhibitors were changing cytosolic redox chemistry and mitochondrial ROS production. Moreover, while rotenone and antimycin A (specific inhibitors of complex I and complex III, respectively) recapitulated the effects of hypoxia, cyanide, an inhibitor of complex IV, did not [62]. These observations are consistent with the Redox Theory of HPV, which has gradually been refined since its initial publication in 1986 [3], reviewed in [96, 97]. The Redox Theory (Figure 3) postulates that the mitochondrial ETC is an important oxygen sensor that varies production of ROS in direct proportion to physiologic changes in PO_2 [64]. This is plausible, since a small percentage of electron transport involves unpaired electrons that yield superoxide anion. Superoxide anion is rapidly converted by mitochondrial superoxide dismutase 2 to H_2O_2 , a less toxic redox mediator with a wider diffusion radius. Thus, the more electron flux, the more uncoupled electrons and ROS production [5]. Conversely, reduced electron flux results in fewer radicals and less H_2O_2 production. This applies not only to the PA and ductus, but also the heart (where ischemia-reperfusion is well established to reduce and increase ROS production, respectively) [38]. Evidence for diffusible mitochondrial-derived ROS being the mediator of HPV has been demonstrated through many experimental strategies. First, there are concordant effects of hypoxia and inhibitors of the proximal ETC on the pulmonary vs. systemic vasculature (Table 1). Perfusion of isolated rabbit lungs with lucigenin (a detector of superoxide, O_2^-) demonstrated endogenous production of O_2^- in the lung that was inhibited by superoxide dismutase (SOD, an enzyme responsible for detoxification of O_2^- to H_2O_2), but not catalase (an H_2O_2 scavenger) or dimethylthiourea (DMTU, an hydroxyl radical scavenger) [70]. These experiments have also been conducted using isolated, saline-perfused rat lungs. In this preparation, hypoxia also decreases the chemiluminiscent signal. This decrease in ROS production (whether measured with luminol or lucigenin) precedes the increase in PAP, as would be expected if it were the signal connecting the sensor to the downstream effectors [4] (Figure 4). Likewise, proximal ETC inhibitors (antimycin A and rotenone) also cause pulmonary vasoconstriction and decrease ROS formation in isolated perfused rat lungs [6].

In addition, PA from rats exposed to chronic hypoxia not only have reduced HPV but display a concordant decrease in constriction to ETC inhibitors (whereas their constrictor responses to phenylephrine is enhanced). This selective impairment of oxygen sensing in response to chronic hypoxia is recapitulated in the fawn hooded rat, a model of spontaneous PAH in which there is mitochondrial fragmentation and decreased expression of ETC complex I [15] (Figure 5). H_2O_2 is known to be a pulmonary vasodilator, as demonstrated by Wolin and Burke. Although, in bovine pulmonary arteries, at least some of this vasodilatation relates to activation of guanylate cyclase [98]. Apart from inhibitors of ETC complexes I and III, the only other class of chemicals which is known to mimic hypoxia (without changing PO_2) are redox agents - agents that can reduce (donate an electron) or oxidize (remove an electron). Treatment of rat PA and aortic rings with diamide (a sulfhydryl oxidant) or oxidized glutathione (GSSG) causes relaxation of PA rings and (in parallel patch clamp experiments) opens K_v channels, as measured by increases in whole-cell I_K . Conversely, antioxidants such as co-enzyme Q10, duroquinone, or reduced glutathione (GSH) elicit dose-dependent, endothelium-independent vasoconstriction [76] and inhibit I_K in PASMC (mimicking hypoxia) [76, 93] (Figure 6). Based on the concordant effects of reducing agents and ETC inhibitors in the pulmonary vasculature, it appears that states of reduction (like authentic hypoxia itself) cause pulmonary vasoconstriction, while oxidants (like normoxia itself) cause vasodilatation.

The use of comparative physiology, contrasting the pulmonary to systemic vasculature, has also been helpful in identifying the role of the mitochondrial redox O_2 sensor. Compared to isolated RA rings, PA rings have greater normoxic production of H_2O_2 and, unlike RA rings, decrease ROS production upon exposure to both hypoxia and inhibitors of the proximal ETC (e.g. rotenone or antimycin A). As a result, whereas hypoxia decreases I_K in the PASMC and causes vasoconstriction, it increases I_K and elicits vasodilation in the RASMC [62].

While there is agreement in the literature that the mitochondrion is likely the oxygen sensor responsible for producing mediator ROS, whether hypoxia lowers or elevates ROS production is a source of lingering controversy [88, 96]. The hypothesis that ROS production decreases with hypoxia is based on the notion that, with decreased O_2 , there is reduced electron flux. Since ROS are produced by the 3% of electron flux involving uncoupled electrons, hypoxia would be predicted to decrease ROS. Conversely, the hypothesis that hypoxia increases ROS is based on the supposition that in the absence of O_2 , there is distal obstruction of the ETC and a retrograde accumulation of electrons leading to autoxidation of the chain and ROS production [18, 33, 89, 90, 92]. Interestingly, there is no disagreement that ROS increase with elevation of PO_2 in the ductus [78], nor is there disagreement that ROS production is decreased during oxygen deprivation that accompanies cardiac ischemia [37], as recently reviewed [43].

Whether physiologic hypoxia increases or decreases mitochondrial ROS depends in part on whether there is input block concomitant with hypoxia. A reduction in electron influx into the ETC during hypoxia (resulting from simultaneous impairment of the Krebs cycle) would promote a decrease in ROS production (as we hypothesize). Conversely, ongoing input of electrons during hypoxia might favour increased ROS if the terminal electron receptor (O_2)

were absent. Differences in the quality of the tissue preparation in which HPV is assessed, the use of different techniques for measuring ROS, and the use of anoxia (as opposed to hypoxia by some groups) may account for divergent results. Suggestions for overcoming these differences in experimentation have been previously published [66]. It is noteworthy, that over time there has been some movement towards consensus, with the group that reports hypoxic increases in ROS conceding that mitochondrial ROS production is decreased with hypoxia, as measured with mitochondria-targeted RoGFP [91]. Interestingly, the main proponent of ROS increasing during hypoxia in the lung also noted that ROS increased during ischemia (prior to reperfusion) in the heart, noting: “*Superoxide generation occurs during ischemia before reperfusion from the ubisemiquinone site of the mitochondrial electron transport chain*” [14]. Interestingly, they performed their studies in isolated cardiac myocytes. We have performed similar studies of cardiac ischemia/reperfusion, but did so measuring ROS in the Langendorff perfused heart model. We found, as do most groups, that the ischemic period is one of low, not high ROS and that high ROS occurred with reperfusion [38]. Consequently, these groups, whether studying the left ventricle or the pulmonary vasculature, seem to disagree about the effect of oxygen deprivation on ROS production. One suspects the difference relates to the severity of hypoxia (versus anoxia), the use of cells versus organs and the duration/severity of oxygen deprivation.

The distal half of the pathway proposed to mediate redox regulation of pulmonary vascular tone during normoxia (normoxic vasodilatation caused by H₂O₂-induced Kv channel opening) is operant in systemic arteries [71].

The ductus arteriosus (DA)

The DA is a large conduit artery that connects the left pulmonary artery to the descending aorta. In utero, where the foetus' systemic PO₂ is < 40mmHg, the DA is maintained in a state of hypoxic vasodilatation. This ductal patency diverts placentally-oxygenated blood ejected from the right ventricle away from the unventilated foetal lungs, and instead directs it to the systemic vasculature. Upon birth, as the infant breathes oxygen-rich air, the FiO₂ rises and systemic PO₂ increases, leading to DA constriction. DA constriction is regulated by endothelial-derived vasodilator and vasoconstrictor mediators, including various prostaglandins [45, 56] and endothelin [20]. Anatomical patency of the ductus arteriosus is considered physiological during the first three days of life [83], but persistence of ductal patency (PDA) subsequent to this can lead to pulmonary hypertension and congestive heart failure [34]. The prevalence of PDA is estimated to be 57 per 100 000 live births, with considerably higher incidence in preterm infants (birth weight 501–1500g) [39]. Epidemiological findings highlight the importance of oxygen sensing in promoting PDA, including the increased incidence of PDA in infants born into environmental hypoxia at high altitude (4000+ m) compared to babies born at sea level [2].

Oxygen sensing in the DA

An early theory for oxygen-induced DA constriction is summarized in the endothelin hypothesis [19]. In this theory, a rise in PO₂ elicited increased synthesis of this potent peptide vasoconstrictor, resulting in DA constriction. Invoking a circulating vasoconstrictor

mediator to explain DA constriction is similar to early theories of HPV, where the proposed mechanism involved oxygen-sensitive release of vasoconstrictive circulating mediators, such as endothelin [23, 46] or leukotrienes [54]. However, like HPV, the core of oxygen-induced DA constriction is intrinsic to the vasculature and requires neither the endothelium nor endothelin itself to occur. Like HPV, O₂-induced DA constriction is mediated by a mitochondrial O₂ sensor within the SMC that regulates K⁺ and Ca²⁺ channels, as well as rho kinase, to initiate and sustain DA constriction.

In 1996, Tristani-Firouzi et al, using foetal rabbits and employing a similar strategy to Post et al in studies of HPV [74], demonstrated that the Kv channel inhibitor 4-AP induced DA constriction and that O₂ caused little additional constriction, suggesting a Kv-channel-dependent mechanism of constriction [87]. They showed that the O₂-sensitive I_K in DASMC is a Kv current, which, when inhibited, causes membrane depolarization and activation of the Ca_L channel, thereby elevating intracellular calcium and causing vasoconstriction. Roughly two thirds of the net rise in DASMC [Ca²⁺]_i originated from the extracellular compartment and entered the DASMC via the Ca_L channel.

O₂ elicits constriction of the DA even in absence of endothelium [25], suggesting that, similar to the PA, the O₂-sensing mechanism is intrinsic to the SMC. Indeed, in human DA rings, simultaneous inhibition of prostaglandin synthase, endothelin receptors, endothelin converting enzyme and nitric oxide synthase failed to attenuate O₂-induced DA constriction. However, this cocktail did inhibit constriction to exogenous endothelin and lower endothelin synthesis by the ductus. Targeted inhibition of L-type Ca²⁺ channels in human DA blocks normoxic constriction of the vessel [61]. Preterm DA rings exhibit reduced O₂-mediated constriction and, as would be predicted, manifest reduced O₂-sensitive I_K density. Thus, the preterm DA is an interesting model, as both the sensor and constrictor mechanisms are immature and can be targeted individually. The decrease in O₂-sensitive I_K in preterm DASMC is associated with decreased expression of Kv1.5 and Kv2.1 (with preserved expression of the L-type Ca²⁺ channel). Gene transfer of Kv1.5 or Kv2.1 channels improves O₂-constriction in preterm rabbit DA rings. When this therapy is made more precise and targeted solely to the SMC, gene transfer of Kv1.5 also enhances O₂-mediated constriction in isolated human DA rings [85].

The mitochondrion is also the upstream O₂ sensor in the DA, and similar to resistance PA, DASMC mitochondria alter ROS production (specifically, H₂O₂) in proportion to PO₂ (Table 1). Quantification of DA ROS production (measured using luminol and lucigenin chemiluminescence) revealed increased ROS levels in normoxic versus hypoxic DA. This increase in ROS was inhibited by catalase, confirming that it reflected H₂O₂. Likewise, removal of H₂O₂ by inclusion of catalase in the patch clamp pipette solution increased outward I_K in isolated, hypoxic, DASMC, studied in the whole cell configuration. Conversely, H₂O₂ supplementation using tert-butyl hydroperoxide decreased I_K, suggesting that increased ROS levels inhibit K⁺ channels within the DA. Additionally, oxidizing agents can initiate constriction of dilated hypoxic DA rings, and dilation of constricted normoxic rings can be triggered by antioxidants [78]. Regulation of K⁺ channels by O₂ occurs in human DA in a manner opposing that seen in adult pulmonary arteries [61]. Exposure of human DA rings to chronic normoxia (*ex vivo*) decreases expression of Kv channels

(including Kv1.5 and Kv2.1), and dampens DA sensitivity to 4-AP or O₂-induced vasoconstriction. Adenoviral transfer of O₂-sensitive Kv channel genes restores 4-AP and O₂ sensitivity. In a final example of the reverse response of the DA versus the PA to changes in redox state, proximal ETC inhibitors mimic hypoxia in the DA, increasing outward I_K, decreasing ROS production and reversing O₂-mediated vasoconstriction [63].

Recently we have explored how changes in mitochondrial form controls mitochondrial function. Mitochondria exist in dynamic networks that are rapidly joining through a process called fusion, and dividing through a process called fission. The movement and division of mitochondria are collectively referred to as mitochondrial dynamics. Mitochondrial dynamics are regulated by a relatively small number of conserved, large GTPases that promote fission (dynamin related protein 1, Drp1) or fusion, (mitofusin 1 and 2, Mfn1 and Mfn2; and optic atrophy 1, OPA1), reviewed in [12]. Hong et al demonstrated that mitochondrial fission is required for O₂-induced DA constriction and subsequent closure [40]. Using DA from both human infants and full term rabbits, expression of the mitochondrial fission gene Drp1 was measured under hypoxic and normoxic conditions. Upon exposure to normoxia, both Drp1 activation and mitochondrial fission increase in term DASMC. Fission increases mitochondrial complex I activity and ROS production [40]. Inhibition of mitochondrial fission using mdivi-1 selectively inhibits O₂-induced ROS production and DA constriction, without altering constriction to other agonists. The changes in the structure of the mitochondrial network occurred in seconds, prior to changes in ROS production or tone, suggesting that fission is an obligatory initial step in oxygen sensing in the DA [40].

Future Directions

Numerous aspects of the oxygen-sensing mechanism require further elucidation. While the opposing response of ion channels to ROS in PASMC (constriction in response to decreased ROS) and DASMC (constriction in response to increased ROS) is well documented, the basis for this opposing response requires further study.

Also, the relative importance of the various O₂-sensitive K⁺ channels remains somewhat controversial and warrants additional investigation. Certainly, mutation of TASK channels, one of the channel types implicated in oxygen sensing, is one newly recognized cause of heritable PAH [48].

Although it is well established that the calcium driving DA and PA constriction originates in large part from the extracellular compartment and enters the cells via Ca_L channels, the relative significance of oxygen-sensitive calcium release from either the SR or store operated channels remains unclear.

Finally, the recognition that disorders of oxygen sensing can result in human diseases such as PAH and PDA offers new insight into disease pathogenesis [10, 31]. In chronic hypoxic pulmonary hypertension (a model for WHO Group 3 pulmonary hypertension in humans), the suppression of both mitochondrial-derived H₂O₂ production and expression of Kv1.5 is evident, and can be ameliorated with Kv1.5 gene therapy [75]. Likewise, the preterm ductus arteriosus does not constrict well to oxygen and is deficient in the expression of Kv1.5 and

other O₂-sensitive Kv channels. Augmentation of Kv channel expression is therapeutic in models of patent preterm DA [85]. It is noted that Kv1.5 is present in numerous tissues other than the pulmonary vasculature, including atrial myocytes [26], the ventricle [53], pancreatic islets [49], and the brain and spinal cord [81]. There is great variation in the electrophysiologic properties of Kv1.5 amongst tissues. This relates tissue-specific expression of different splice variants of Kv1.5. There is also heterogeneity in the composition of Kv1.5 channels based on variable α -subunit composition of the channel tetramer and variation in the associated channel β -subunits.

Systemic induction of Kv1.5 as a form of therapy would certainly elicit unwanted effects. For example, Kv1.5 over-expression has anti-apoptotic effects in the brain [100], but pro-apoptotic effects in pancreatic beta cells [44]. Thus, site-specific manipulation of Kv1.5 is likely required for any potential therapeutic application. This approach has been used successfully in preclinical studies of diseases of the pulmonary vasculature [75], ductus arteriosus [85], and the heart [17]. Indeed, downregulation of Kv1.5 is a hallmark of PAH, seen in the PASMC in all animal models of PAH, as well as human PAH.

Targeting the mitochondria to reduce ROS production has been investigated as a potential therapy for numerous pathologies; specifically, MitoQ, an antioxidant quinone moiety cross-linked to triphenylphosphonium cation, can pass through both the plasma membrane and mitochondrial membrane, where it accumulates within the mitochondrial matrix and is adsorbed and is continually recycled by Complex II of the ETC [84]. MitoQ has been tested as a potential therapy for Alzheimer's disease [55], hypertension and associated cardiovascular disease [30], and metabolic syndrome [60]. It should be noted, however, that **increased ROS** is pathological in these cases, whereas diminished ROS production is observed in chronic hypoxic pulmonary hypertension and PDA, at least by some investigators. Further investigation into targeting the mitochondria to produce physiological levels of H₂O₂ without stimulating potentially pathological over-production might be warranted to restore oxygen sensing. As an alternative to targeting the mitochondria to alter ROS production, the proteins associated with mitochondrial dynamics, specifically, Drp1 may hold therapeutic promise. Inhibiting Drp1 in preclinical studies has resulted in decreased fission and cell proliferation in human PASMC from PAH patients as well as in rodent PASMC [52]; suggesting regulators of the mitochondrial network are viable therapeutic targets for diseases of impaired oxygen sensing.

Conclusions

The ability of specialized tissues to sense changes in oxygen and initiate homeostatic responses that optimize oxygen uptake and delivery is integral to normal development and environmental adaptation. In all tissues within the HOSS, a redox O₂ sensor (likely within the mitochondrion) alters production of diffusible mediators (including ROS, such as H₂O₂). This redox signal alters the function of downstream effector ion channels (i.e. voltage-gated K⁺ and Ca²⁺ channels) and enzymes (e.g. rho kinase), which mediates vascular tone in the PA and DA. The clinical significance of oxygen sensing is three-fold: *physiological*, as the optimization of oxygen uptake is crucial to both development in utero as well as adaptation to changing environmental oxygen tension; *medical*, as HPV can be exploited in patients

undergoing single-lung anaesthesia to maintain systemic oxygenation [68]; and *pathological*, as subversion of the oxygen-sensing pathways is relevant to disease pathogenesis in conditions such as PAH and cancer.

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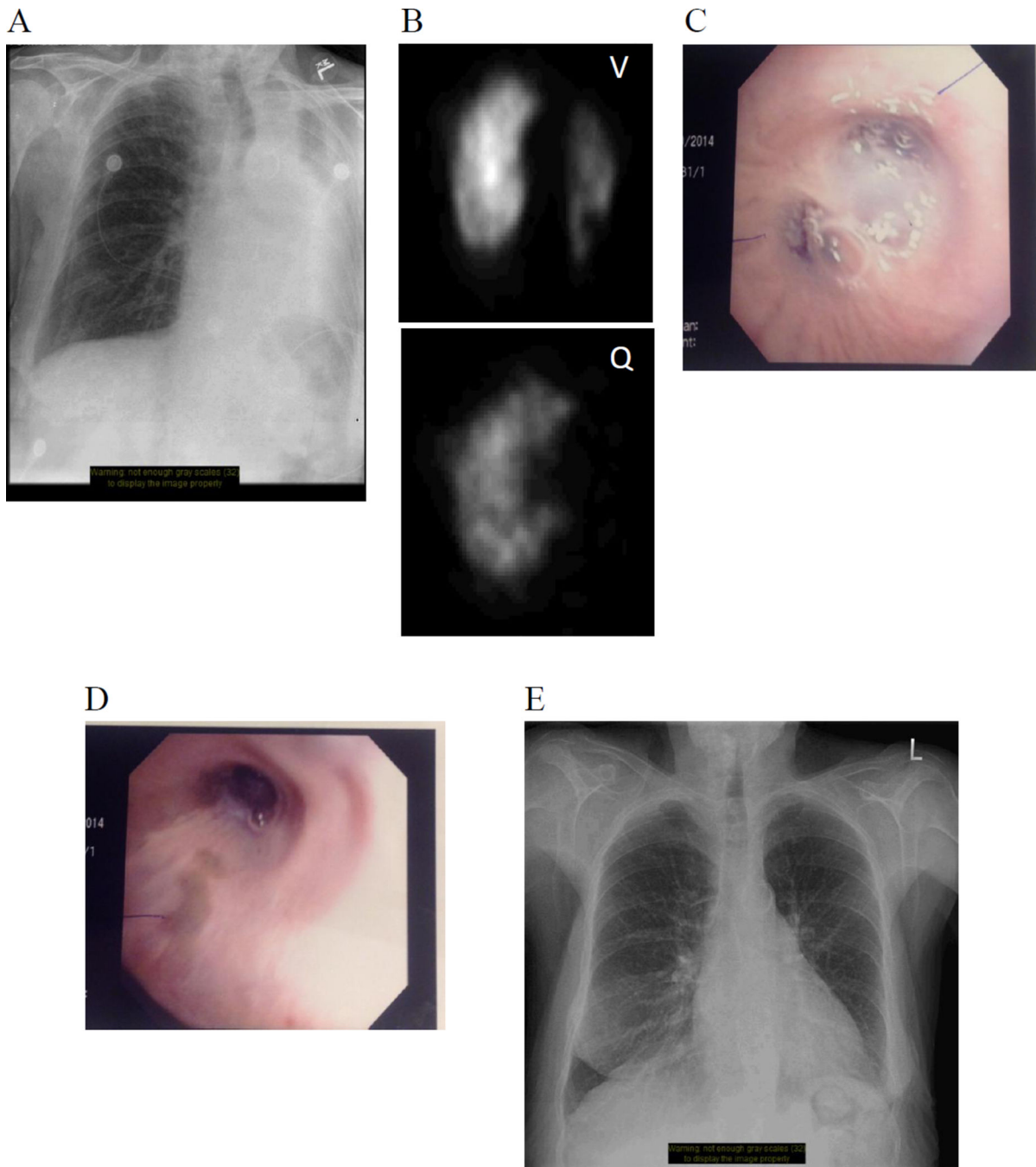


Fig. 1. Hypoxic vasoconstriction by ventilation/perfusion scintigraphy

Image series of a 77-year-old woman with chronic obstructive pulmonary disease and peripheral vascular disease who experienced transient, acute hypoxia on the hospital ward while awaiting removal of an ischemic toe. A) Plain anteroposterior film displaying left upper lung zone volume loss suggestive of bronchial obstruction. B) Ventilation (upper) and perfusion (lower) (V/Q) scintigraphy scans confirming diminished ventilation to the left lung, absent perfusion to the left upper lobe (LUL), and decreased perfusion to the left lower lobe (LLL), demonstrating appropriate hypoxic vasoconstriction of the LUL vasculature.

The relatively preserved perfusion to the LLL despite absent ventilation is known as “reverse ventilation-perfusion mismatch,” and is indicative of inadequate hypoxic vasoconstriction reflex to that area. C) Bronchoscopy revealing mucous plugging of the left superior and inferior lobar bronchi. D) Post-bronchoscopic aspiration of mucous plug. E) Plain anteroposterior film displaying complete re-expansion of left lung post-aspiration

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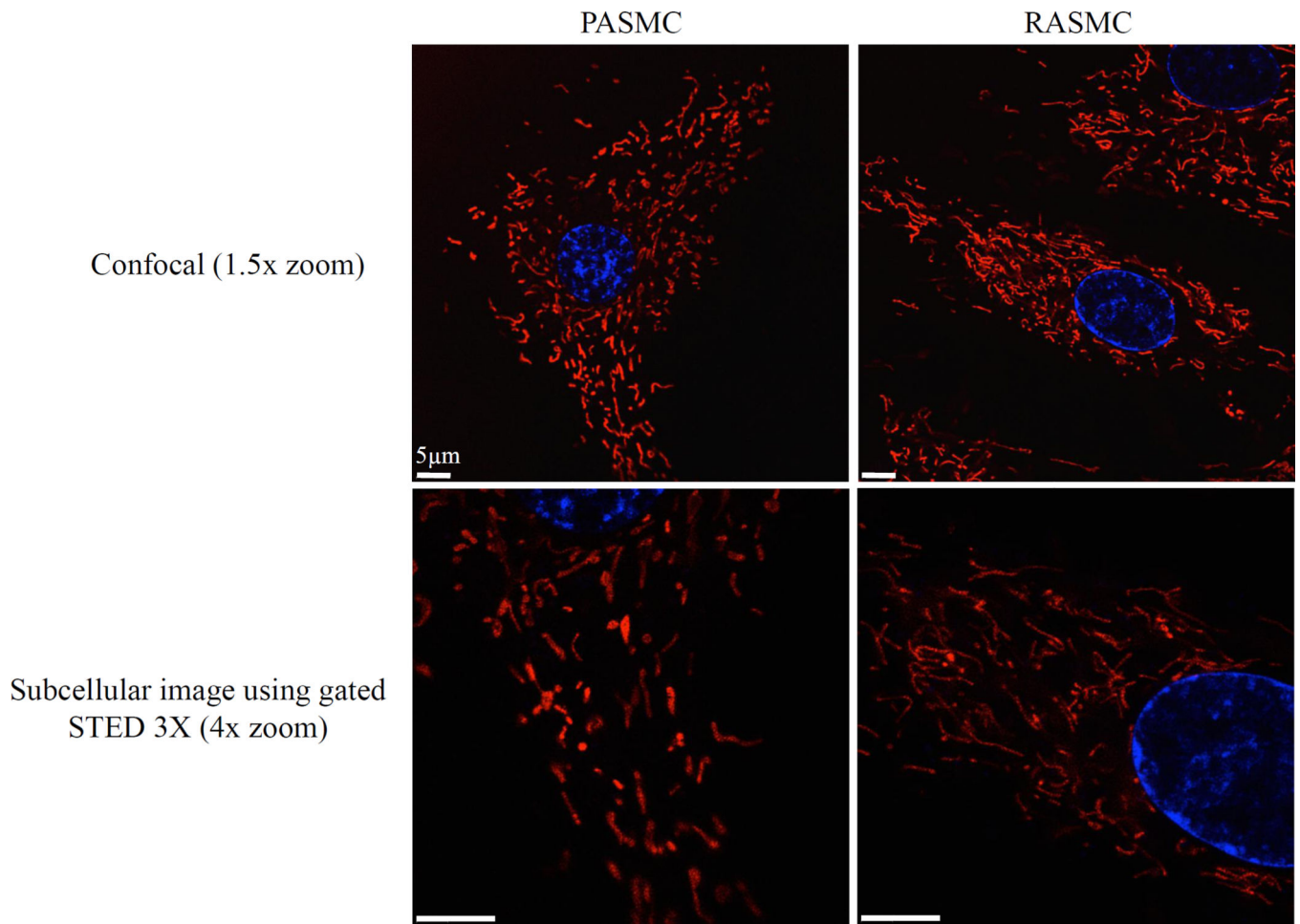


Fig. 2. Smooth muscle cell mitochondria are arranged in an extensive network

Standard confocal (top) and super resolution (bottom) images of rat arterial smooth muscle cells. Cells stained with 50nM MitoTracker® Red FM and Hoechst 33342 (Molecular Probes, Life Technologies; Waltham, MA) for 20 min at 37°C and imaged using a Leica TCS STED 3X confocal microscope with and without stimulated emission depletion (STED); all images 100X oil with 1.5x or 4.0x zoom. A) Confocal image (1.5x) and B) STED confocal image (4.0x) of pulmonary artery smooth muscle cells. C) Confocal image (1.5x) and D) STED confocal image (4.0x) of renal artery smooth muscle cells

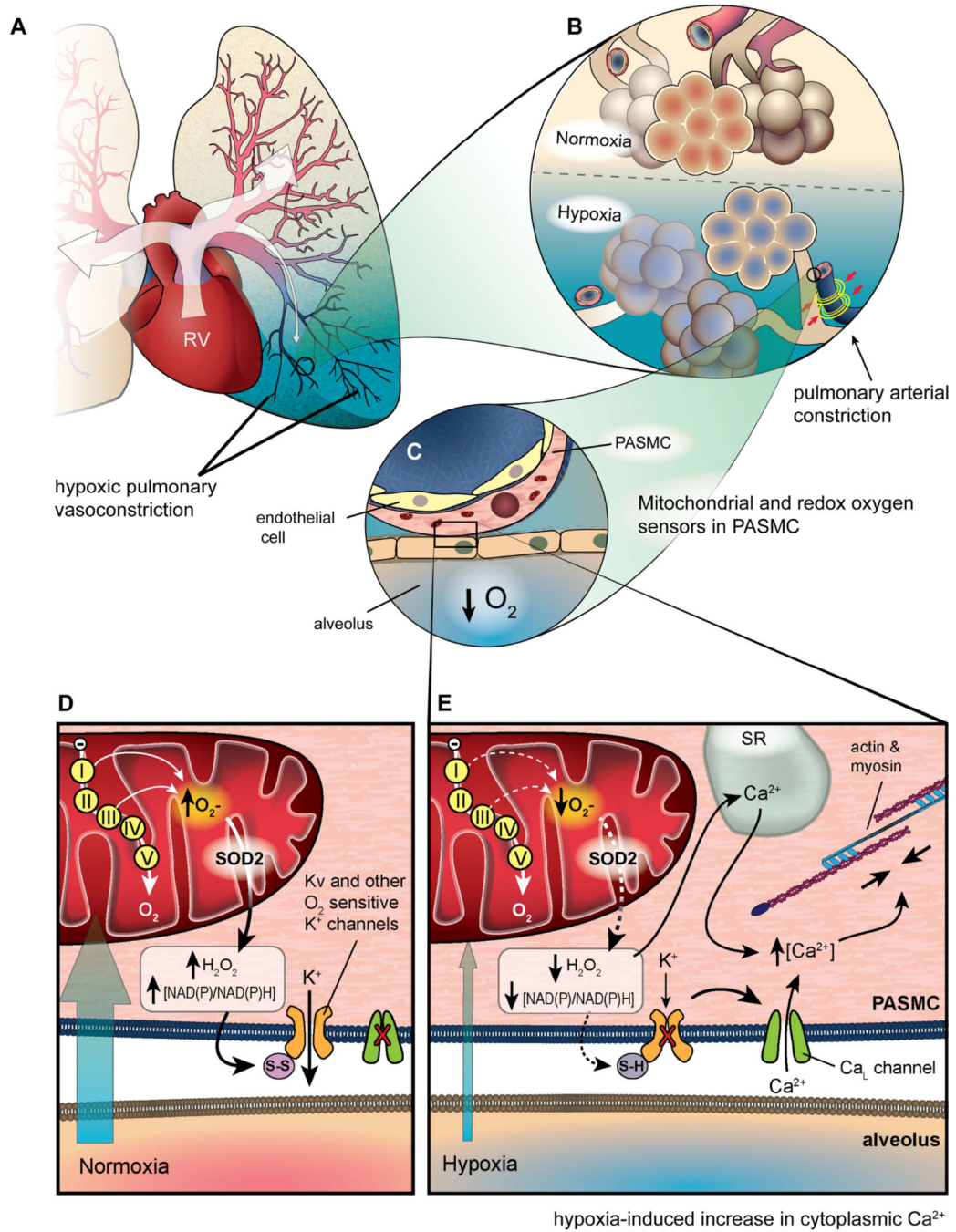


Fig. 3. The Redox Theory of HPV

A) Illustration of a left lung experiencing regional hypoxia in the left lower lobe. Well-ventilated portions of the lung have pink blood vessels and increased blood flow (indicated by thick arrows), whereas the hypoxic region of the lung is blue with small, constricted, blue blood vessels and diminished blood flow (indicated by a thin arrow). B) Inset of lung illustrating oxygenated alveoli and dilated blood vessels in a normoxic region of the lung versus hypoxic alveoli and constricted pulmonary arteries in a hypoxic region of the lung. C) Inset of pulmonary artery illustrating the proximity of PASMC mitochondria to a hypoxic

alveolus. D) Under normoxic conditions, mitochondrial ROS production and elevated NAD(P)/NAD(P)H ratio contribute to an oxidative environment, resulting in oxidized sulfhydryl groups on K^+ channels, keeping them open, while the Ca_L channel remains closed. E) During hypoxia, diminished ROS production and reduced NAD(P)/NAD(P)H ratio result in depolarization of the cell, closing of K^+ channels and subsequent opening of Ca_L channels. Influx of Ca^{2+} triggers Ca^{2+} release from the sarcoplasmic reticulum (SR), stimulating actin and myosin and triggering vasoconstriction

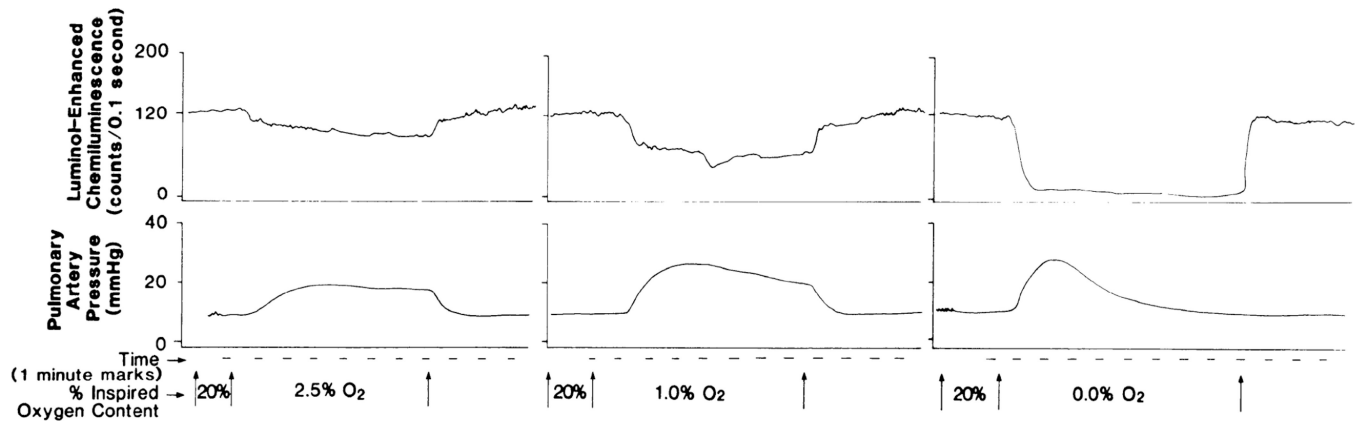


Fig. 4. Simultaneous measurement of ROS production via chemiluminescence and mean pulmonary artery pressure (PAP) during hypoxia in isolated rat lung

Hypoxia-induced change in luminol-enhanced chemiluminescence begins earlier and peaks sooner than simultaneously measured change in mean pulmonary artery pressure during hypoxia ventilation. Magnitude of change in chemiluminescence is proportional to inspired O₂ content. This is a representative recording from single lung. *From [4], reproduced with permission.*

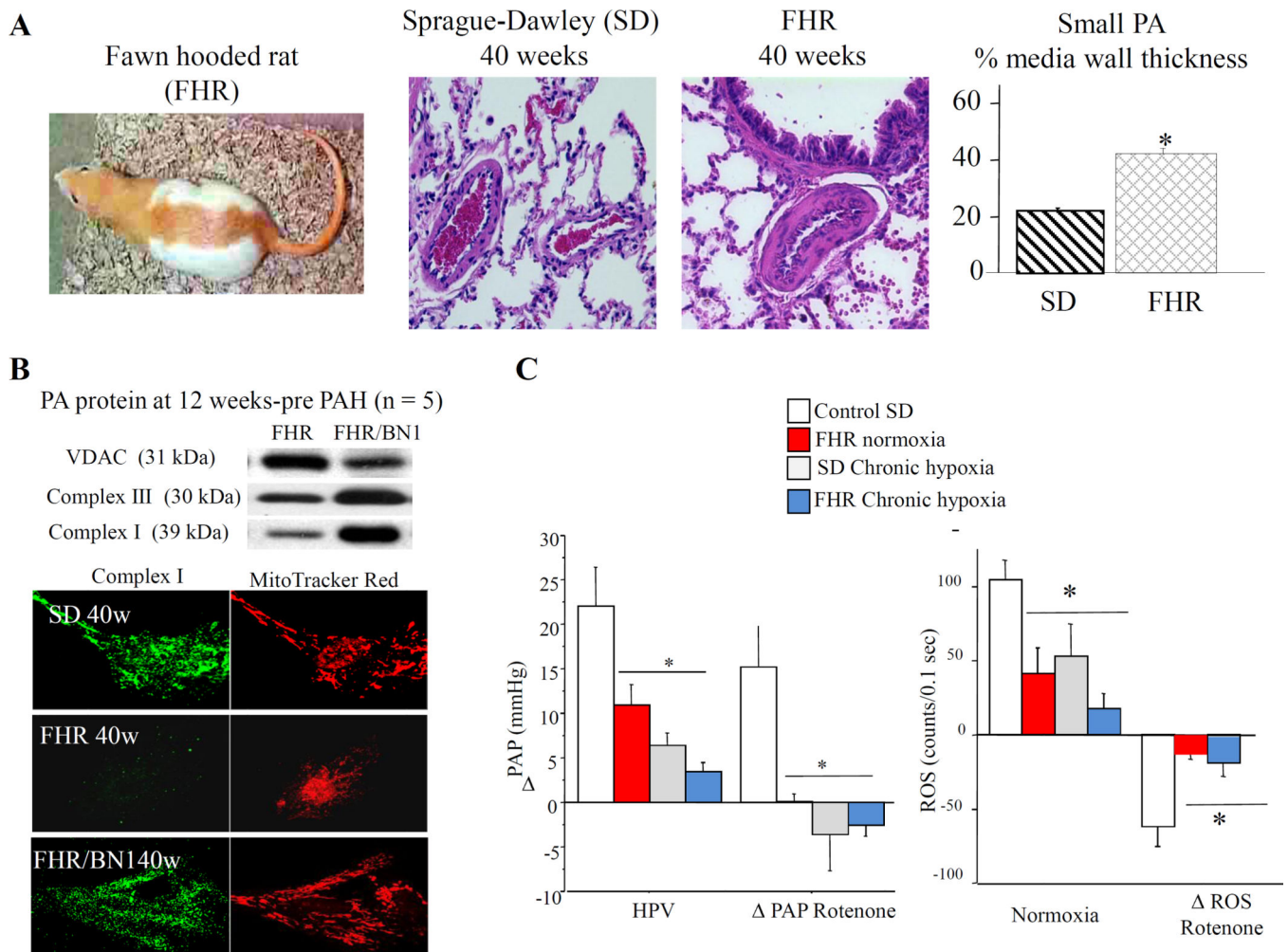
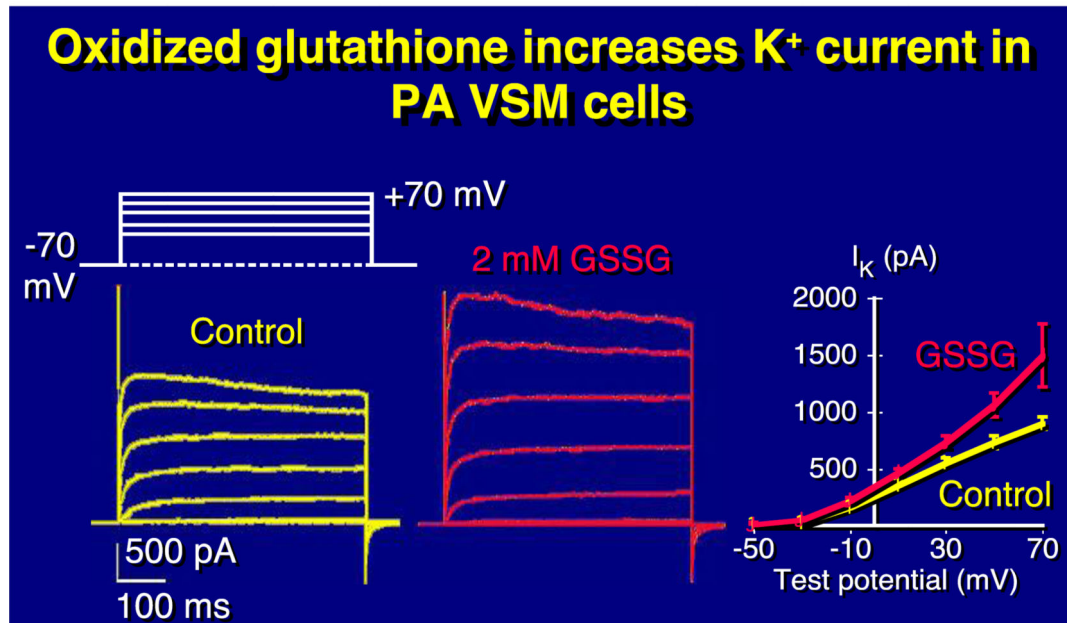


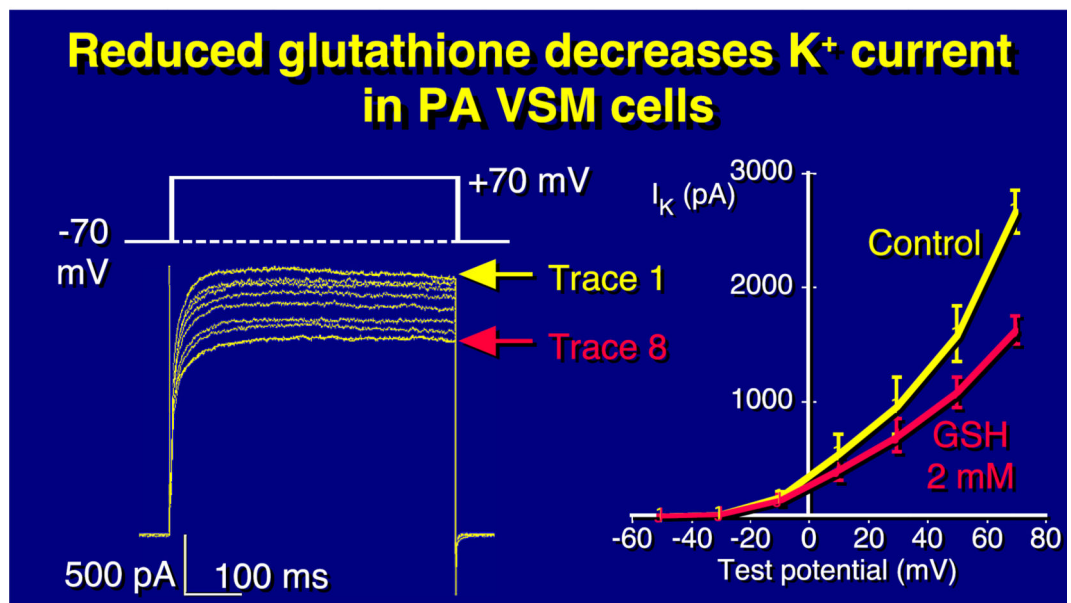
Fig. 5. Similarities between Sprague-Dawley rats exposed to chronic hypoxia and the fawn hooded rat that exhibits spontaneous PAH

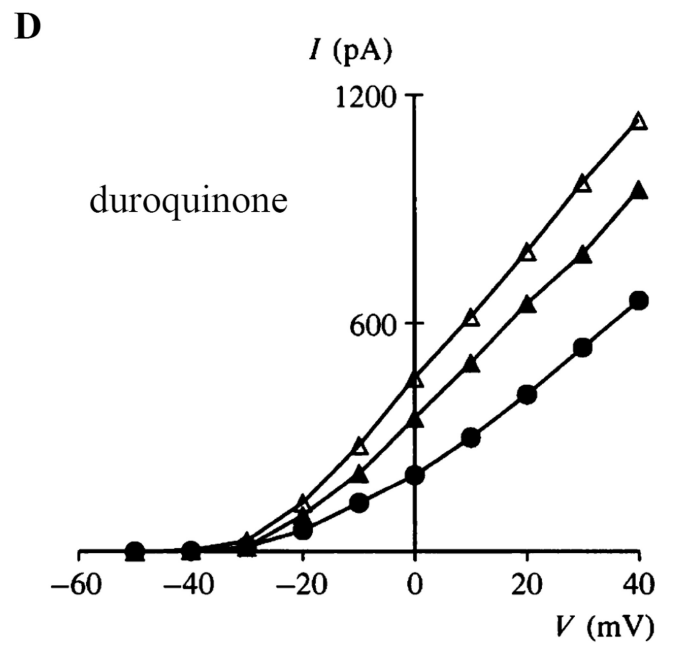
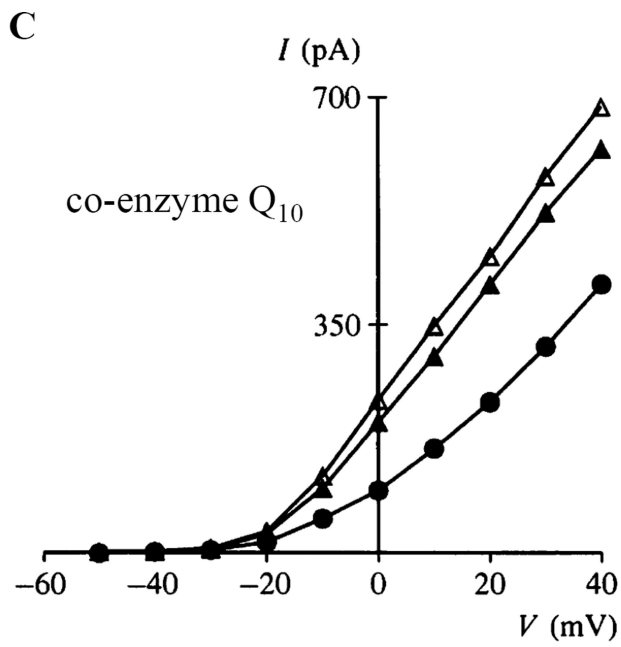
Early loss of ROS precedes spontaneous PAH in FHR. A) Lung histology reveals that 40-week-old FHRs have increased medial hypertrophy of small pulmonary arteries vs 40-week-old Sprague-Dawley rats ($*P < 0.05$). B) Expression of electron transport chain complexes is decreased in FHR pulmonary arteries before PAH (voltage-dependant anion channel expression is increased). FHR PASMCs express less electron transport chain complex I and have fewer mitochondria arrayed in a less organized reticulum than FHR/BN1 PASMCs. C) Hypoxic and rotenone vasoconstriction and lung ROS levels (luminol) are similarly impaired in normoxic FHRs and chronically hypoxic Sprague-Dawley rats vs normoxic Sprague-Dawley rats ($*P < 0.05$). From [15], reproduced with permission

A



B





- △ Before application (control)
● During application (treated)
▲ After wash

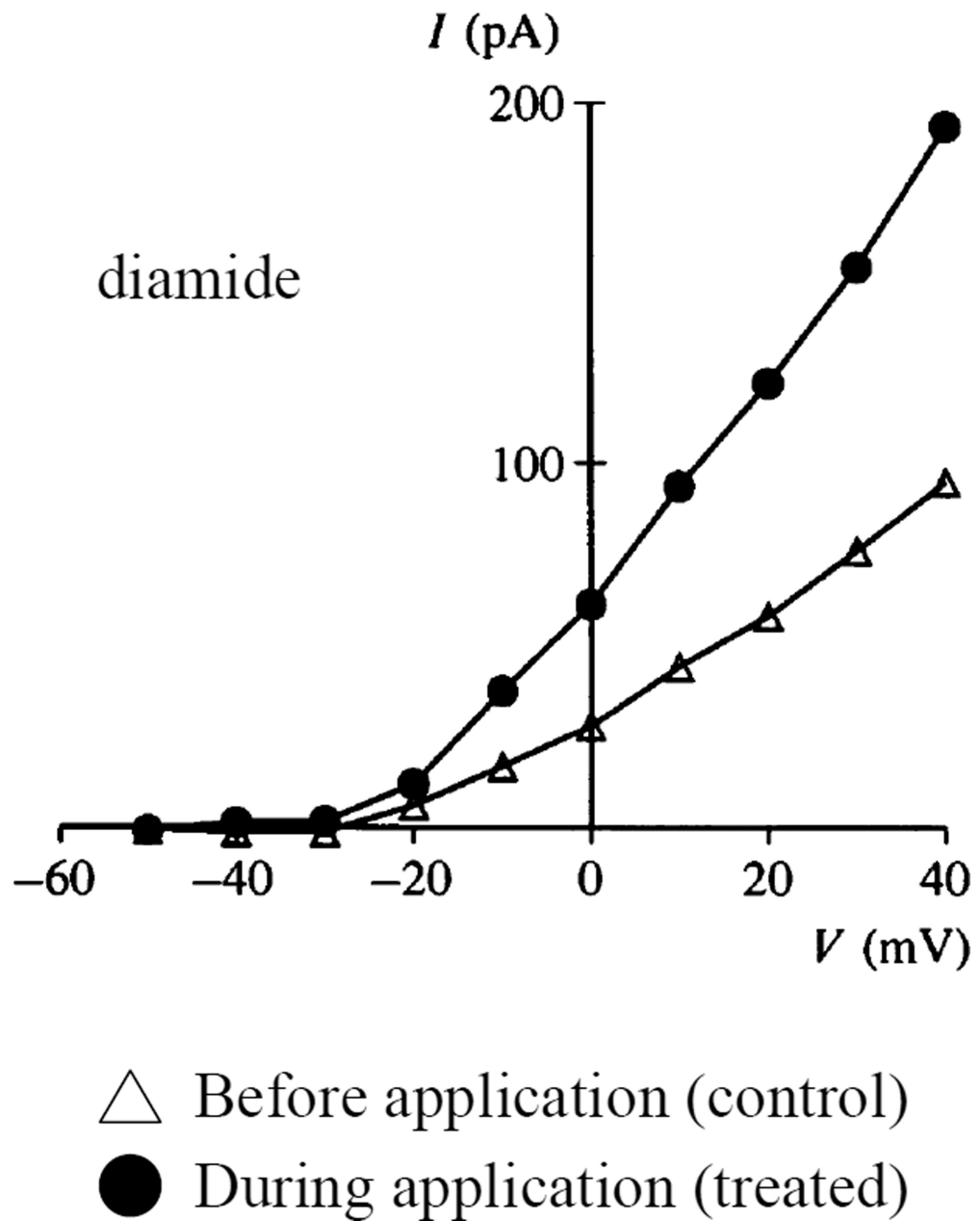
E

Fig. 6. Reducing agents inhibit and oxidants enhance PASM K⁺ current

Effect of intracellular oxidants and reducing agents on whole-cell potassium currents in rat pulmonary artery smooth muscle cells. A) Family of K⁺ currents elicited by voltage steps before (left) and after (middle) 5 min dialysis of oxidized glutathione (GSSG) and current-voltage relationship for mean (\pm SEM) potassium current in the presence or absence of 2mM GSSG (right). B) Series of K⁺ currents elicited by a repetitive voltage step from -70 to $+70$ mV, every 20s after start of dialysis of reduced glutathione (GSH) (left) and current-voltage relationship for mean (\pm SEM) potassium current in the presence or absence of 2mM GSH

(right). Graphical representation of current-voltage relationships for mean (\pm SEM) potassium current in the presence or absence of C) 100 μ M co-enzyme Q₁₀, D) 100 μ M duroquinone, E) 100 μ M diamide. *5A and 5B from [93], 5C-5E from [76], reproduced with permission*

Table 1

Similarities between hypoxia, proximal, and distal electron transport chain (ETC) inhibitors on pulmonary artery smooth muscle cells (PASMC)

	Hypoxia	Proximal ETC Inhibitors	Distal ETC Inhibitors
Redox couples (e.g. NAD ⁺ /NADH)	reduced	reduced	
Voltage-gated K ⁺ current	↓	↓	↔
ROS levels in PASMC	(controversial) ↓ Per Archer lab	↓	↑
Cytosolic Ca ²⁺	↑	↑	↑*

*-the distal ETC inhibitor cyanide induces increased cytosolic Ca²⁺ via a mechanism discrete of that elicited by hypoxia

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