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Review

Redox-Based Mechanisms of O_2 Sensing in Hypoxic Pulmonary Vasoconstriction; Where Are We Now?

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Abstract

Hypoxic pulmonary vasoconstriction (HPV) is a rapid and reversible constrictor response of the pulmonary vasculature, and especially its small muscular precapillary arteries, which is initiated by episodes of local alveolar hypoxia. Acting as a protective homeostatic vasomotor mechanism, HPV enables maximal gas exchange by diverting blood from poorly ventilated alveoli in to those rich in oxygen, thereby optimizing oxygen uptake and the ventilation-perfusion (V/Q) ratio so as to maintain the arterial oxygen partial pressure (P_aO_2) within the physiological range. HPV is an intrinsic mechanism of pulmonary artery smooth muscle cells (PASMC), and requires an *O_2 sensor* which acts through *mediator(s)* to trigger *effector mechanisms* within these cells to evoke constriction. Whereas HPV effector mechanisms are reasonably well-defined, the nature of the O_2 sensor and mediators remain in dispute. The three most comprehensive models of O_2 sensing in HPV share a focus on the concept that hypoxia activates effector mechanisms by inducing a change in the PASMC cytoplasmic redox state. According to the Redox Theory, first proposed by Kenneth Weir and Stephen Archer in 1995, hypoxia inhibits mitochondrial production of reactive oxygen species (ROS), thereby causing the cytoplasm to become more reduced. This inhibits ongoing vasorelaxation maintained by the opening of voltage-gated K^+ channels. In contrast, according to the Mitochondrial ROS hypothesis, introduced by Paul Schumacker and Naveen Chandel in 2001, hypoxia increases mitochondrial ROS production, causing an oxidizing shift in the cytoplasmic redox poise which activate several vasoconstricting pathways. In a third scenario, developed by Michael Wolin and Sachin Gupte, hypoxia evokes contraction by causing a fall in H_2O_2 production by NADPH oxidase, and by activating the pentose phosphate pathway. These effects inhibit basal vasorelaxation maintained by the guanylate cyclase and protein kinase G and also stimulate vasoconstricting mechanisms. In this comprehensive review, we summarize the key studies contributing to the development of these proposals and then subject the evidence supporting them to critical appraisal, based in part on how well they accord with the wider literature and recent developments in our understanding of how cells shape and deploy redox mechanisms in order to regulate cell function.

Keywords: O_2 sensing; hypoxia; hypoxic pulmonary vasoconstriction; K_V channel; mitochondria; NADPH oxidase; reactive oxygen species; redox; hydrogen peroxide; NADH

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Section 1: Introduction; Definition and Description of HPV

1.1. Basic Properties of HPV

Hypoxic pulmonary vasoconstriction (HPV) is a rapid and reversible constrictor response of the pulmonary vasculature, and especially its small muscular precapillary arteries, to episodes of local alveolar hypoxia [1–3]. Acting as a protective vasomotor mechanism which enables maximal gas exchange by diverting blood from poorly ventilated alveoli in to those rich in oxygen content on a breath-to-breath basis, HPV optimizes oxygen uptake and the ventilation to perfusion (V/Q) ratio to maintain the arterial oxygen partial pressure (PO_2) within the physiological range [4]. In parallel with HPV, acute hypoxia provokes systemic arterial vasodilation, thereby promoting oxygen delivery to body tissues by increasing blood flow [5].

Studies in isolated perfused whole lungs or lung lobes from a range of species indicate that HPV begins to develop when PO_2 falls below ~80 Torr, becomes half-maximal at ~50 Torr, and reaches its maximum amplitude at ~20 Torr [3]. Depending on the experimental model and conditions used, HPV generally manifests as a rapidly developing and sustained increase in pulmonary artery pressure (PAP) or pulmonary vascular constriction which in many studies exhibits a biphasic profile in which an initial transient development of force (Phase 1) is superimposed upon or followed by a slow and progressive increase in tension (Phase 2). The biphasic time course is typically observed when more severe levels of hypoxia are used to evoke the response [3]. In humans, HPV evoked when end-tidal PO_2 (a measure of the alveolar PO_2) was halved demonstrated an initial rapid increase in pulmonary vascular resistance which reached a steady state within minutes. This was followed by a second slower increase that began after 30 – 60 minutes and plateaued after ~3 hours [6,7]. HPV is a function of the PO_2 in both the alveoli and the blood perfusing the pulmonary arteries (PA), with the former being of greater importance [8]. It does not require, but is modulated by, the autonomic nervous system and diverse vasoactive factors [9] [3].

The mechanisms responsible for the two phases of HPV appear to be, at least to some extent, different [10,11]. Phase 1 is mediated by an increase in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) due to a rapid decrease in K^+ channel activity leading to membrane depolarization and opening of L-type voltage-gated Ca^{2+} channels, [10,12], as well as release of Ca^{2+} from the sarcoplasmic reticulum and the resulting stimulation of store operated Ca^{2+} entry (SOCE) [10,13,14] in pulmonary artery smooth muscle cells (PASMC). Most studies of HPV in isolated perfused lungs or PA have used short periods of hypoxia (≤ 15 minutes), suggesting that their conclusions apply mainly to Phase 1. Studies of O_2 sensing in isolated or cultured PASMC have also typically employed short hypoxic challenges, but the extent to which their results are representative of one or the other phase of HPV in more intact systems is unclear.

Phase 2 HPV is less well characterized but probably involves multiple pathways contributing to an increase in $[Ca^{2+}]_{cyt}$ as well as rho kinase-mediated Ca^{2+} sensitization [15]. Unlike phase 1, sustained HPV has generally (but not always) been found to be endothelium-dependent [16]. The evidence for an involvement of membrane depolarization in Phase 2 HPV is mixed [10,14,17,18]. Both phases are reversible, but if global hypoxia lasts longer than 24 hours, persistent HPV of the entire pulmonary circulation causes a sustained increase in PAP. This, together with phenotypical, biochemical and functional changes in each pulmonary vascular cell type, gradually leads to pulmonary vascular remodeling, which can be irreversible.

1.2. Proposed Models of O_2 Sensing in HPV

HPV is conceptualized as requiring an *O_2 sensor* which is coupled through *mediator(s)* to *effector mechanisms* that evoke constriction by raising PASMC $[Ca^{2+}]_{cyt}$ and/or causing Ca^{2+} sensitization [19] . It is generally (but not universally [20]) thought that all of these components of the response reside within the PASMC, although extrinsic influences (e.g. endothelial factors) can modulate HPV [3].

This review focuses on describing current theories which propose that the mitochondria and/or NADPH oxidase (Nox) are O_2 sensors which, acting through effects on reactive oxygen species (ROS)

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or cellular redox couples as mediators, modify the function of contractile effectors to cause HPV. We do not cover either the extensive set of known or putative HPV effectors, which have been described in detail elsewhere (e.g. [3] [21] [22] (except to discuss those which are proposed to be regulated by redox mechanisms where appropriate), or the role of ROS/redox mechanisms in pulmonary hypertension resulting from chronic pulmonary hypoxia (see e.g. [23–31] for reviews of this subject).

The term 'reactive oxygen species' has been criticized in light of evidence that the various types of ROS exert their own specific actions, typically on different sets of biomolecules, with 'oxidants' suggested to be a better alternative when the relevant oxidizing species has not been identified [32]. However, to be consistent with other reviews of O_2 sensing in HPV [21,33–35], and because the term 'oxidants' also encompasses reactive sulfur species, we prefer to refer to changes in superoxide/H_2O_2 production as increases or decreases in ROS, rather than oxidants.

There are currently three well-established proposals for O_2 sensing in PASMC in which ROS play pivotal roles. The bulk of this review is devoted to a description of the investigations which contributed to the development of these hypotheses, and an evaluation of what we feel are their strengths and weaknesses.

1. According to the Redox theory, introduced by Kenneth Weir and Stephen Archer in 1995 [36], hypoxia decreases the production of ROS by the mitochondrial electron transport chain (ETC), leading to a fall in [ROS] and/or the reduction of redox couples in the cytoplasm. This evokes the closure of voltage gated K+ (KV) channels which are activated by basal ROS production under normoxic conditions, thus causing membrane depolarization, the opening of voltage-gated Ca2+ channels (VGCC), and therefore contraction. Although the Redox Theory stresses the importance of KV channels, there are also other effectors which could potentially respond to a fall in ROS or a reduction of cytoplasmic redox couples in such a way as to cause HPV. For example, there is evidence that the reduction of Cys42 on PKG1a (protein kinase G a1), which may exert a tonic vasorelaxing influence on PASMC, diminishes its activity [37].
2. A proposal which we will refer to as the Mitochondrial ROS hypothesis, developed by Paul Schumacker, Naveen Chandal and colleagues and first described in 2001 [38], proposes that hypoxia causes an increase in mitochondrial ROS production, leading to a higher [ROS], and/or oxidation of redox couples, in the cytoplasm. This hypoxia-induced rise in cytoplasmic ROS may be supplemented by a PKCϵ-mediated stimulation of Nox which is triggered by the mitochondrial ROS [39]. The rise in cytoplasmic [ROS] might evoke contraction through multiple effector pathways, potentially including Ca2+ release from the sarcoplasmic reticulum, an increase in store operated Ca2+ influx, and RhoA/ Rho kinase-mediated Ca2+ sensitization [13–15].
3. The processes responsible for O2 sensing and HPV have also been the subject of an extensive series of papers by the laboratories of Michael Wolin and Sachin Gupte (see [40] for a review). In agreement with the Redox theory, these authors propose that hypoxia causes contraction by removing a normoxic vasodilating influence, although this is seen as being maintained largely by oxidation-induced activation of soluble guanylate cyclase and protein kinase G (PKGa1) rather than by the opening of KV channels. In addition, they have presented evidence that hypoxia activates the pentose phosphate pathway, thereby increasing the production of NADPH, and that this contributes to the inhibition of PKGa1 and also activates other Ca2+-dependent and -independent contractile mechanisms.

Two additional O_2 sensing mechanisms have been suggested to bring about HPV. According to the *AMP kinase* (AMPK) hypothesis, introduced by Mark Evans and co-workers[41] [42], HPV is caused by a small but meaningful inhibition of mitochondrial respiration which results in an increase in the cellular [AMP]/[ATP] ratio. This activates AMPK, which causes PASMC contraction through several mechanisms, the best defined of which is phosphorylation -induced inhibition of $K_V1.5$ channels, leading to membrane depolarization and Ca^{2+} influx [43,44]. Furthermore, Kenneth Olson's laboratory [45–48] has suggested that O_2-dependent metabolism of hydrogen sulfide (H_2S; aka sulfide) constitutes an O_2 sensor in many tissues, including pulmonary arteries and carotid body

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chemoreceptor cells (CBCC). According to this proposal, hypoxia suppresses the oxidation of sulfide, causing increased cellular concentrations of sulfide and/or reactive sulfur species. These activate effector mechanisms which cause, for example, HPV. Neither of these hypotheses as proposed by its originators incorporates the involvement of ROS. However, a possible role for ROS in both schemes is possible, as discussed in Sections 6.2 and 6.3.

1.3. Normoxia, Physoxia and Hypoxia

The O_2 concentrations (18-21 % O_2) which are used almost invariably in physiological experiments to simulate 'normoxia' are substantially higher than 'physoxic' concentrations (*i.e.* those experienced under baseline conditions by cells *in vivo*) [49]. There is a growing literature [50–52] attesting as to how this can distort many aspects of cell phenotype. Additional problems inherent in the routine use of effectively hyperoxic conditions in studies of acute O_2 sensing have been discussed in excellent reviews by Alva et al [53] and Olson [47], the former of which also presents an authoritative and perceptive discussion of many of the other issues around the role of ROS in O_2 sensing which we cover in this paper. The intra-alveolar PO_2 is ~13.3%, and that in arterial blood averages 12.3%. Measurements of lung tissue PO_2 in anesthetized animals breathing room air have yielded lower values. For example, O_2 electrodes placed in peri-bronchial lymph nodes in dogs reported PO_2 levels of ~53Torr, [54] and ~38 Torr [55], and an electron paramagnetic resonance sensor inserted into lung tissue of rats also detected a PO_2 of ~38 Torr, which fell to ~26 Torr in animals breathing 10% O_2 [56]. It is not clear why the PO_2 in lung tissue is so much lower than it is in the alveoli, but one can speculate that this could be due to the influence of the PO_2 in PA, which enter the lung containing blood with a mixed venous PO_2 of ~40 Torr. Importantly, it was shown in anesthetized dogs that the mixed venous PO_2 exerts an marked influence on HPV [8], presumably because the blood within a substantial fraction of the PA responsible for HPV has not yet been fully oxygenated and is affecting the PO_2 within the vascular wall. The effective PO_2 stimulus for HPV (P_SO_2) in this study was calculated to be determined by the alveolar and mixed venous O_2 concentrations according to the equation: $P_SO_2 = (P_AO_2^{0.59} \times P_{mv}O_2^{0.41})$. A similar weighting was observed in isolated perfused rat lung [57]. In this case, the P_SO_2 *in vivo* when breathing room air would be roughly 70 Torr, suggesting that this might be an appropriate PO_2 to use in experiments to simulate physoxia under normoxic conditions when conducting studies of HPV. The mitochondria and Noxs, which are likely to be the two main sources of superoxide/hyperoxide in PASMC, produce ROS in a manner which is linearly (mitochondria[58]) or hyperbolically (Nox [53,59]) dependent on the PO_2. For example, Nox4, which is thought to be constitutively active and therefore could be an important source of H_2O_2 in PASMC under basal conditions [60], has a K_mO_2 of ~136 Torr [59]. Thus, PASMC and other cells in preparations used to study O_2 sensing in HPV are likely to have been undergoing a degree of oxidative stress under experimental 'normoxic' conditions.

A related issue is that the O_2 concentration experienced by cultured cells in standard O_2-controlled incubators is usually supra-physoxic when the PO_2 is set to 18%. Even so, under certain conditions (e.g. high cell density) the PO_2 in the medium can be much lower than the physoxic level due to cellular O_2 consumption [61]. Cellular respiration can have an especially marked effect on the pericellular PO_2 in cells in culture [62], an effect which has been shown to be inhibited by block of the electron transport chain (ETC) [63]. This is a concern because experimental interventions used to study hypoxia often decrease the activity of the ETC, in which case they may also increase the pericellular PO_2 and therefore diminish the degree of hypoxia experienced by cells. Unfortunately, the extent to which these factors might have had an impact on the results of the investigations we will discuss is unclear.

The question of the range of levels of hypoxia which are appropriate for studying HPV has probably not been taken as seriously as it should have been in some papers. In many cases, for example, the level of hypoxia is defined as the % O_2 content in the gas mixture used to bubble the solution or in the incubator housing the cell chamber, with no information provided about the PO_2 in the solution. In an influential review of HPV, Moudgil et al [19] stressed the necessity of using

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physiological levels of hypoxia in studies of HPV, suggesting that the level of hypoxia at the summit of Mount Everest (8488 meters; alveolar and arterial PO_2 of ~43Torr recorded during the Operation Everest II expedition [64] might be an appropriate guideline, since the PO_2 in the small PA which are the main site of HPV should be very close to that in the alveoli.

On the other hand, if one takes into account the influence of the mixed venous PO_2 on HPV as described above, and factors in the resting $P_{mv}O_2$ (22 Torr) observed in another study by the Everest II expedition team [65] at a simulated altitude of 8488 meters, the P_SO_2 for HPV at this height would be ~33 Torr. Moreover, other studies have indicated that elevation has a larger effect on the arterial and alveolar PO_2; for example, P_AO_2 and $P_{mv}O_2$ values obtained at 8488 meters during an earlier expedition to Everest were 35 and 21 Torr, respectively [66], and Grocott et al [67] obtained arterial PO_2 values of 48, 42, 34 and 24 Torr at elevations of 5300, 6400, 7100 and 8400 meters, with an alveolar PO_2 of 30 Torr at 8400 meters, although the corresponding $P_{mv}O_2$ levels were not reported. Taking into account also that even moderate levels of activity further lower the $P_{mv}O_2$ at altitude [65], it can be argued that lower levels of hypoxia, perhaps down those approximating the PO_2 range at which HPV reaches its maximum amplitude (~20 Torr) are also suitable for studying physiologically relevant O_2 sensing mechanisms in HPV. Nonetheless, since the mechanisms responsible for O_2 sensing in PA may well vary according to the level of hypoxia, as is thought to occur in chemoreceptor cells of the carotid body (CBCC) [68], ideally the mechanisms of O_2 sensing in HPV should be assessed over a range of low PO_2 levels. This has not been done in any organized way.

Section 2: Reactive Oxygen Species as Signaling Molecules

2.1. ROS Definition and Function

ROS include oxygen radicals such as superoxide ($O_2^{\cdot-}$), hydroxyl (·OH) and peroxyl ($RO_2^{\cdot}$), as well as oxidants such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HClO). These are formed by cell metabolism and can oxidize biological molecules, although their reactivity varies between species [69,70]. $O_2^{\cdot-}$, the first species formed by single- or multi-enzyme reactions through the single-electron reduction of water, rapidly dismutates to H_2O_2, a process which occurs spontaneously and is also catalyzed by superoxide dismutase (SOD). ·OH , which can be formed by the reaction of H_2O_2 with Fe^{2+} (Fenton reaction) or of water with Fe^{3+} [71], is extremely reactive and is scavenged immediately upon its production. H_2O_2 is relatively stable and is able to cross membranes *via* aquaporins [72], properties which allow it to act as a signaling molecule. $O_2^{\cdot-}$ can also react with nitric oxide (NO) to form the reactive nitrogen species peroxynitrite ($ONOO^-$), which has a signaling function [73]. The two cellular sources of ROS which have received the most attention with regard to O_2 sensing and HPV are the mitochondrial electron transport chain (ETC) and Nox.

ROS can cause oxidative modifications of nucleic acids, carbohydrates, lipids and protein. Their excessive production therefore results in deleterious effects on cells, a situation referred to as *oxidative stress*. However, cells use smaller spatiotemporally controlled increases in H_2O_2 to regulate their normal function and initiate adaptive responses (oxidative *eustress*) [74], with HPV being a possible example.

2.2. H_2O_2 Signaling

H_2O_2 signaling depends on its ability to react with thiolates (S^-), the anionic form of cysteine sulfhydryl (-SH groups), leading to modifications of protein structure and therefore function (Figure 1). Since the pK_a value of the thiol in free cysteine is ~8.5, the fraction of cysteines in the reactive thiolate form should be small at the physiological cytoplasmic pH (e.g. ~7.3 in PASMC, [75]). However, the pK_a of cysteine thiols can be lowered by their proximity to positively charged amino acids [76] and by other factors related to their local environment within the protein structure [77,78]. Thus, a fraction of cysteine residues is able to react with H_2O_2 and other oxidizing species including reactive nitrogen or sulfur species, which cause S-nitrosation and S-sulfhydration, respectively. Reactive cysteines can also undergo S-thiolation, most importantly by oxidized glutathione (GSSG),

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causing s-glutathionylation. It is thought that disulfide bond formation and s-glutathionylation are the most important oxidative modifications for H_2O_2 signaling [79]. These modifications can alter three-dimensional protein structure, leading to protein translocation [80,81] or substrate targeting [80,82], ultimately causing rapid functional changes that can induce or fine-tune cell signaling during acute responses.

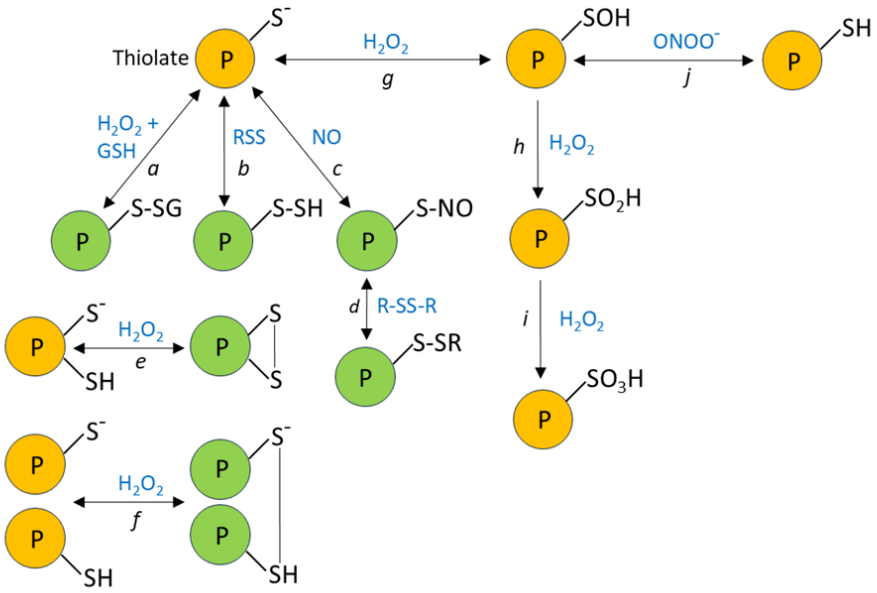

Figure 1. Oxidative modifications of reactive cysteines. Deprotonated cysteine thiols (thiolates) can react with a variety of oxidizing species, leading to the posttranslational modifications which can affect protein function. Reactions of thiolates with glutathione (GSH), reactive sulfur species (RSS) and nitric oxide (NO) leads to their (*a*) glutathionylation, (b) persulfidation and (*c*) nitrosylation, which can further lead (*d*) to a thiol exchange reaction leading to glutathionylation or the formation of an intra-or inter-protein disulfide bond. Thiolate oxidation by H_2O_2 can also lead to the formation of intra-or inter-protein disulfide bonds (e and f, respectively). The reaction of H_2O_2 with thiolates can also produce sulfenic acid (g). Although not shown in the diagram, cysteine sulfenic acid can react with GSH, RSS and NO, leading to its glutathionylation, persulfidation and nitrosylation respectively, and can also react with thiols to form disulfide bonds [83] (not shown). Notably, cysteine sulfenic acid can be formed (*j*) by the oxidation of thiols with peroxynitrite ($ONOO^-$), or by the reaction of peroxynitrous acid (ONOOH) with thiolates. These oxidative modifications can be reversed by cellular antioxidant mechanisms. Under more highly oxidizing conditions, sulfenic acid is further converted to (h) sulfinic acid and then (i) sulfonic acid; these reactions are largely irreversible. The types of oxidative modifications of protein tholates depicted in green have been shown to alter the activities of many proteins (e.g.[84]).

In order to minimize its potential damaging effects on cells, basal cytoplasmic concentrations of H_2O_2 are maintained in the low nanomolar range by the thioredoxin (Trx) and glutaredoxin (Grx) antioxidant systems, acting in concert with peroxiredoxins (Prxs), and Grx-dependent glutathione peroxidases (Gpx) (Figure 2) [85,86]. These systems, which are also largely responsible for terminating redox signaling by reversing oxidative thiol modifications, ultimately require the oxidation of NADPH, which is regenerated from $NADP^+$ by the pentose phosphate pathway and other enzymatic mechanisms [87]. The Prx family, comprising six isoforms which are differentially expressed in the various cell compartments, is generally thought to be particularly important for the removal of H_2O_2; it was estimated, for example, that 78% and 21% of H_2O_2 scavenging in cells is dependent on Prx and Gpx, respectively [88]. Intriguingly, however, a recent study using the novel NADPH sensor NAPStar3b has shown that that the glutathione system plays the predominant role in antioxidant defence in HEK293 cells [89]. H_2O_2 is also scavenged by the enzyme catalase, which is

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located mainly but not exclusively in peroxisomes. It is believed that the antioxidant effect of catalase, which does not require cofactors, is more important at higher H_2O_2 concentrations, at which the activities of Prx and Gpx may be limited by the necessity for their recycling by Trx and Grx [90].

The extent to which the direct reactions between H_2O_2 and protein thiolates described above can account for redox signaling has been questioned on kinetic grounds. Scavenging of H_2O_2 by Prx is rapid, with a 2nd order rate constant inthe range of 10^5–10^8 M^{-1} [88,91], whereas H_2O_2 reacts much more slowly with cysteine thiolates ($k \approx 10^1$ - 10^2 M^{-1}).

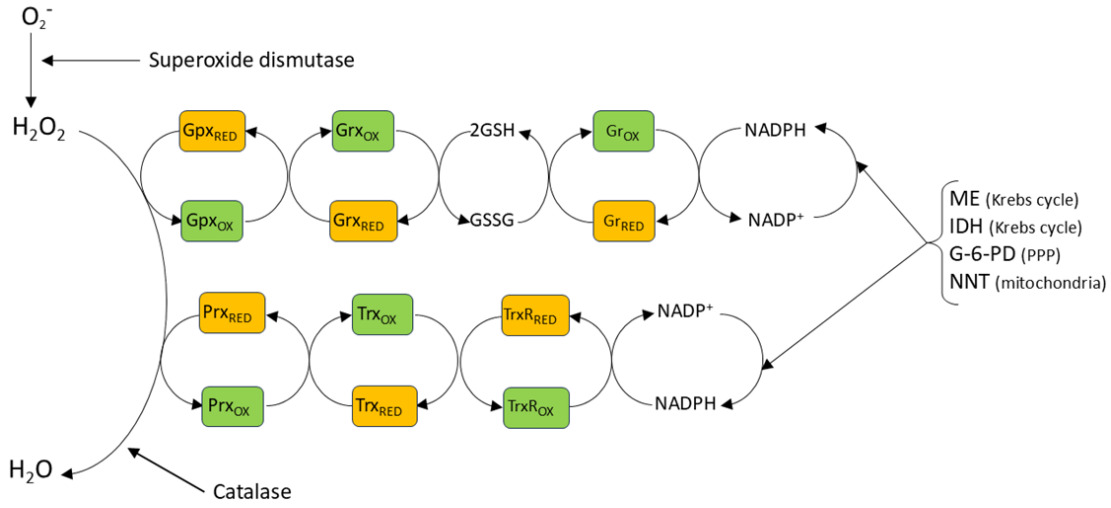

Figure 2. Antioxidant mechanisms. Superoxide spontaneously dismutates to H_2O_2 at a high rate, but this process is reinforced by cellular (and extracellular) SODs. Once formed H_2O_2 is thought to have a half-life of ~10 s. It is reduced to H_2O, most importantly by peroxiredoxins (Prxs), with the oxidizing equivalent then being passed ultimately to NADPH *via* thioredoxin (Trx) and thioredoxin reductase (TrxR). H_2O_2 is also reduced by glutathione peroxidase, which also passes the oxidizing equivalent to NADPH, in this case *via* glutathione peroxidase (Gpx), glutaredoxin (Grx), glutathione (GSH) and glutathione reductase (Gr). $NADP^+$ is reduced back to NADPH by several enzymes, including malic enzyme (ME) and isocitrase dehydrogenase (IDH) in the mitochondria and by glucose-6-phosphate-dehydrogenase (G-6-PD) and also 6- phosphogluconate dehydrogenase in the cytoplasm. $NADP^+$ reduction to NADPH in the mitochondria is also coupled to the oxidation of NADH to NAD^+ by the NAM nucleotide transhydrogenase in the inner mitochondrial membrane, a reaction which is driven by the protein gradient across this membrane (see Section 3.1.1.1). These processes ensure that the $NADP^+$: NADPH redox couple is very highly reduced in both cytoplasm and mitochondria [87], thereby maintaining the effectiveness of the NADPH requiring antioxidant systems. At higher concentrations, H_2O_2 is also scavenged by catalase, an enzyme mainly expressed in lysosomes. Moreover, Prx is present in micromolar concentrations in cells while H_2O_2 concentrations probably remain submicromolar during signaling [74]. Consequently, antioxidant mechanisms are predicted to scavenge H_2O_2 before it can react with thiolates on target proteins.

Despite these constraints, there is evidence that cells can use H_2O_2 as a highly specific signaling molecule if it is formed very close to its target protein (e.g. [92]). Longer range specific targeting can also be achieved by way of the formation of *thiol peroxidase-based redox relays* (Figure 3) in which Prx (or Gpx) is used to convey oxidizing equivalents from H_2O_2 to a target protein, rather than sending them down an antioxidant pathway to be consumed by NADPH [93,94]. In a one-step relay, once oxidized by H_2O_2, Prx oxidizes the target protein rather than Trx. In a two-step relay, Prx oxidizes Trx, which then oxidizes the target protein rather than oxidizing Trx reductase (TrxR). The fact that Prx is able to act in both antioxidant and peroxidase capacities can reconcile the apparent paradox

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that H_2O_2, despite being subject to swift and efficient scavenging by cellular antioxidant pathways, can initiate the rapid and reversible oxidization of relatively unreactive thiols. The initial reaction between H_2O_2 and Prxs is very fast, with rate constants in the range of 10^7-10^8 M^{-1} s^{-1} for all five isoforms; similar rate constants are also seen for the reaction of Prxs and peroxynitrite [95,96]. The factors which determine the balance between the antioxidant and oxidant/signaling activities of Prxs are still being worked out. One possibility described in an elegant study by Portillo-Ledesma et al [95] is that the antioxidant capacity of Prx becomes saturated at a certain threshold concentration of oxidant (e.g. H_2O_2), above which it can oxidize target proteins. They showed that this threshold is particularly low (4 nM H_2O_2) for the cytoplasmic isoform Prx2, suggesting that it can oxidize target protein at relatively low H_2O_2 concentrations while the antioxidant activity of Prx isoforms with higher thresholds (e.g. Prx1) continues. The activity of Prx is also regulated by several post-translational modifications [97].

The specificity of Prx dependent redox relays is fostered by the rapid association of Prxs, once they are oxidized, with discrete sets of target proteins. This occurs through the formation of disulfide bonds at its active site, which happens during target protein oxidation. For example, in experiments carried out in HEK293T cells, Px1 and Prx2, which are both expressed in the cytoplasm, were shown to associate with 735 and 165 proteins, respectively [98]. Of these, only 53 bound to both isoforms. This highlights the specificity of these interactions, which was shown to depend on the local amino acid sequence around the target cysteine. Of the 1233 proteins found to bind to one or more of the five Prx isoforms expressed in these cells, ~80% had previously been identified [76] as harboring redox-sensitive cysteines, suggesting that Prx-dependent oxidative modifications of protein thiols probably play a widespread role in H_2O_2-signaling. It has also been proposed that redox relays which utilize Gpx and GSSG to cause S-glutathionylation of target cysteines are important in the redox regulation of many proteins, especially within mitochondria [99].

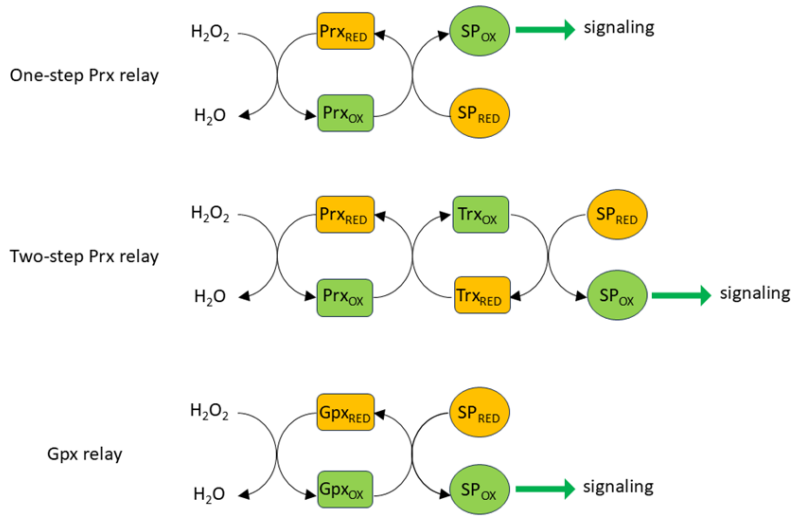

Figure 3. Redox relays. Rather than oxidizing reactive cysteines directly, H_2O_2 generally does so indirectly *via* redox relays which transfer oxidizing equivalents from H_2O_2 (and also peroxynitrite) to signaling proteins (SP). Prxs can oxidize signaling proteins directly or can do so by passing an oxidizing equivalent to Trx, which is generally responsible for restoring several Prx isoforms to their reduced state following their oxidation. Oxidizing equivalents can also be passed from H_2O_2 to signaling proteins *via* glutathione peroxidase[93].

Section 3: Cellular Mechanisms of ROS Production

Mitochondrial complexes together with a microsomal ETC, the Noxs, and other oxidases (e.g., glucose oxidase, xanthine oxidase), peroxisomes, and cyclooxygenases comprise the main biological

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machinery that directly produces or is causally involved in oxidant formation. Of these various sources of ROS, research on HPV has focused only on the potential roles of mitochondria and Nox.

3.1. Mitochondrial Regulation of Cytoplasmic H_2O_2

Although mitochondria can release H_2O_2 into the cytoplasm, e.g. during ischemia-reperfusion [100] and inflammation [101], they can also act as an H_2O_2 sink [58,102–104]. Thus, their net effect on cytoplasmic H_2O_2 levels depends on the balance between these two processes.

3.1.1. Mitochondrial ROS Production

The mechanisms responsible for mitochondrial ROS production have been described by numerous excellent reviews (e.g. [58,105–109]. Here, we will focus mainly on aspects relevant to the possible effects of hypoxia.

3.1.1.1. Oxidative Phosphorylation

Within mitochondria, the energy released by the metabolic oxidation of glucose, fatty acids and amino acids is captured in the form of a highly negative redox potential by the transfer of electrons to NAD^+ and, *via* succinate, to FAD, producing NADH and $FADH_2$, respectively. These electrons are transferred from NADH/$FADH_2$ into the electron transport chain (ETC), consisting of 4 protein complexes with successively more positive redox potentials (Figure 4), and are then used to reduce O_2 to H_2O. The energy released as the electrons flow down this redox potential gradient is used by complexes I,III and IV to pump protons outwardly across the inner mitochondrial membrane (IMM). The energy stored in the resulting transmembrane electrochemical gradient (DP or protonmotive force) is used by ATP synthase to phosphorylate ADP, generating ATP which provides energy for cellular processes. DP has two components: DΨm, the membrane potential across the IMM, and DpH, the pH gradient across the IMM. It has recently been proposed that DΨm is generated, not only by the proton gradient across the IMM, but also by a Na^+ gradient which also exists across this membrane due to the operation of Na^+/H^+ exchange, which is mediated by complex I [110]. The process by which the energy lost by electrons as they are transferred from metabolic substrates to O_2 through the ETC is coupled *via* DP to the production of ATP is termed oxidative phosphorylation.

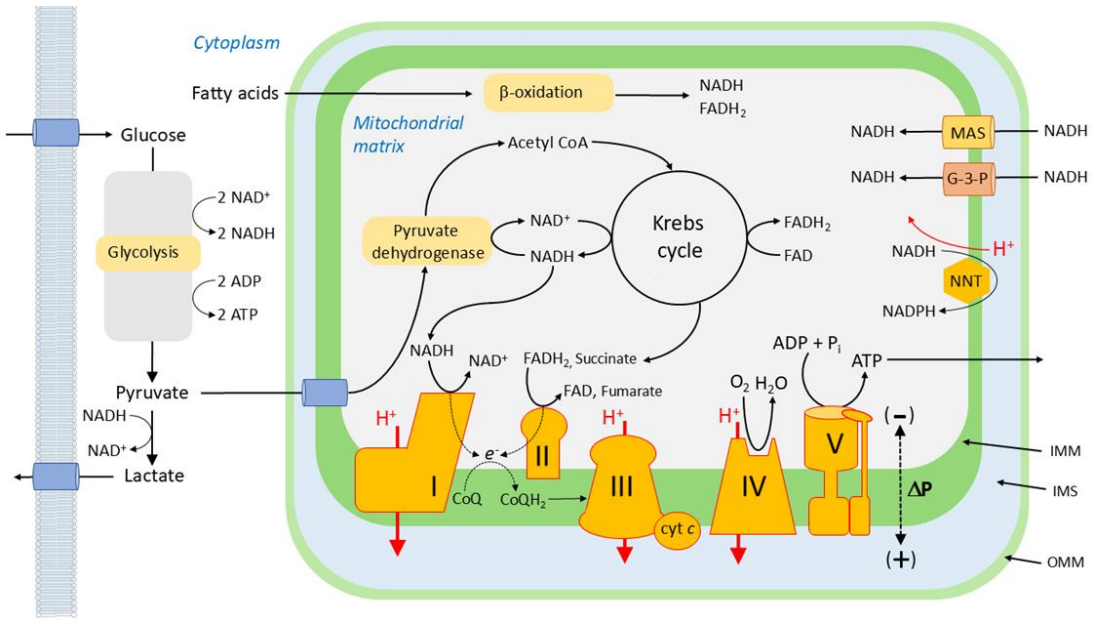

Figure 4. Oxidative phosphorylation. The electron transport chain consists of four protein complexes (I -V). Complex V, the ATP synthase, is not part of the ETC *per se.* Complexes I and II accept electrons (e-) from NADH and $FADH_2$, respectively, which have been reduced by reactions occurring during glycolysis, fatty acid

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metabolism and the Krebs cycle. The electrons from complexes I and II reduce co-enzyme Q (CoQ; ubiquinone) to ubiquinol ($CoQH_2$). Electrons from $CoQH_2$ are then passed to complex III, and then *via* (cyt *c*) to complex IV, where they combine with O2 to form H2O2. This process is driven by the successively less negative redox potentials of the complexes, and the energy lost by the electrons as they fall down this energetic redox gradient is used by complexes 1, 3 and 4 to create an electrochemical proton gradient across the inner mitochondrial wall (DP). The energy stored in DP is used by complex V to phosphorylate ADP, thereby producing ATP which is used by cells to fuel energy-requiring reactions.

3.1.1.2. Electron Flow Through the ETC

Electrons from NADH and $FADH_2$ enter the ETC at complexes I and II, respectively, which use them to reduce coenzyme Q (CoQ; ubiquinone), a small-molecule constituent of the IMM, yielding $CoQH_2$ (ubiquinol). CoQ can also be reduced to $CoQH_2$ by electrons transferred during the oxidation of a number of substrates, including dihydroorotate, proline, fatty acids, glycerol 3- phosphate and H_2S. Each substrate is oxidized by a specific dehydrogenase (e.g. proline dehydrogenase reduces CoQ by oxidizing proline to pyrroline-5-carboxylate).

$CoQH_2$ binds to complex III (aka Q-cytochrome *c* oxidoreductase or cytochrome *bc*1 complex), at the Q_o site, which is close to the outer side of the IMM. One electron from $CoQH_2$ bound at this site is transferred to the nearby Reiske iron-sulfur cluster, (and thence, in succession, to cytochrome *c*1, cytochrome *c*, and complex IV, which utilizes pairs of electrons to reduce O_2 to water). This releases two protons to the intermembrane space (IMS). The other electron is transferred to the b_L (b_{566}) heme group, which is also on the outer side of the IMM. From there, it moves to the b_H (b_{562}) heme group, which is closer to the matrix side of the IMM. This movement is driven by the more positive redox potential of b_H compared to b_L, but is opposed by DΨm; thus a high DΨm will retard this electron transfer. A second ubiquinone binding site, Q_i, is close to b_H, and the electron on b_H is transferred to UQ bound to this site, forming ubisemiquinone ($CoQ^{\cdot-}$). When the next molecule of $CoQH_2$ is oxidized at Q_o, the electron that it donates to b_L and b_H reduces the ubisemiquinone at Q_i to $CoQH_2$, which returns to the $CoQH_2$ pool in the IMM. This causes the uptake of two protons from the matrix. The net result of this process, referred to as the *Q cycle*, is that for each $CoQH_2$ oxidized, two electrons flow *via* cytochrome *c* to complex IV, two protons are taken up from the matrix, and four protons enter the IMS, helping to generate DP [111,112].

3.1.1.3. Factors Governing Mitochondrial ROS Production

There are more than a dozen sites within the mitochondria at which electrons flowing into or through the ETC can react with O_2 to form superoxide; some of the sites can also produce H_2O_2 (see Figure 5, and the legend to Figure for a list of the sites and a definition of their abbreviations) Many of these sites are locations at which dehydrogenases transfer electrons from metabolic substrates to NAD^+ or CoQ. Superoxide produced is rapidly reduced to H_2O_2 by MnSOD (SOD2) in the mitochondrial matrix or Cu/ZnSOD (SOD1) in the IMS. Nevertheless, superoxide evidently exists long enough to exert local effects, for example oxidizing the Fe-S cluster of mitochondrial aconitase in the matrix, leading to its loss of catalytic function [113]. The ratio of mitochondrial superoxide + H_2O_2 production to O_2 consumption is in the range of 0.1 to 0.5% [114].

The rate of superoxide/H202 production at each site is a function of the O_2 concentration ($[O_2]$), the concentration of the site in the reduced state (X^{*-}), and a second order rate constant (k):

$$(dROS/dt) = ([O2])(X^{*-})(k) \quad (1)$$

where X^{*-} is the product of the concentration of the site and the fraction of the site which is in the reduced state [58,106]. Overall mitochondrial ROS production is the sum of that produced at each site. Whereas some sites are incorporated into proteins as prosthetic groups (e.g. flavin mononucleotide in the I_F site on complex I), I_Q in complex I and III_Q in complex III serve as sites within the complexes at which ubisemiquinone is formed and can donate electrons to O_2 to form superoxide or H_2O_2. The mitochondrial ROS-producing sites fall into two isopotential groups defined by the redox couple involved in ROS production. The NADH-linked sites (the a-keto ketoacid

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dehydrogenase complexes and Complex I_F) operate at a redox potential of ~ -280 mV, whereas the coenzyme-Q linked sites (the CoQ linked dehydrogenases, complex I_{CoQ}, complex II_F, and complex III_{CoQ}) [115] operate at a potential of ~+20 mV [109]. The relative amounts of ROS produced by each site vary between cell types and under different conditions [109,114,116]. However, complex I_{CoQ}, complex II_F , KGDH, PDH, OADH and III_{CoQ} are thought to be important mitochondrial ROS sources [58,117], with III_{CoQ} having the highest capacity for ROS production, at least in skeletal muscle [109].

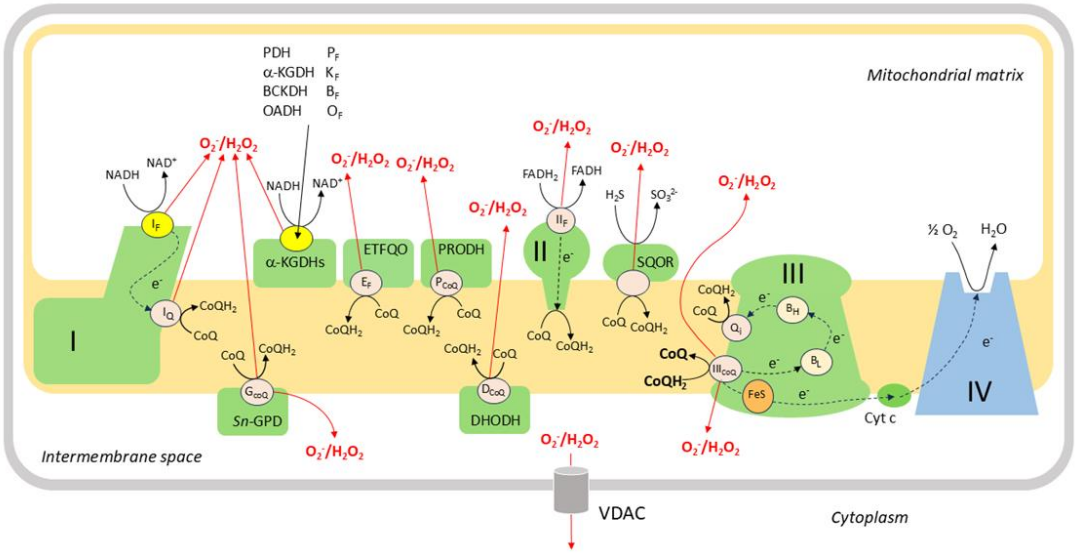

Figure 5. Mitochondrial ROS production. There are more than a dozen sites (e.g. I_Q, G_{CoQ}) at which electrons can 'leak out' of the ETC and react with O_2 to form superoxide and/or H_2O_2. In addition to the classical sites of ROS production at complex I (I_F and I_Q), complex II (II_F) and complex III (II_{CoQ}), ROS are also produced by dehydrogenases associated with the inner mitochondrial membrane during the oxidation of metabolites. Several of these sites (I_F and also P_F, K_F, B_F, and O_F which are at the ROS-producing loci of a-ketoacid dehydrogenase complexes) are in redox equilibrium with the mitochondrial NAD^+: NADH couple. The other sites, which include I_Q (also termed I_{CoQ}), II_F, III_{CoQ} , G_{CoQ}, E_F, D_{CoQ}, P_{CoQ} and the ROS producing site on sulfide quinone oxidoreductase (SQOR) are in redox equilibrium with CoQ: $CoQH_2$ couple in the inner mitochondrial membrane. The G_{CoQ}, E_F, D_{CoQ}, P_{CoQ} sites are on located on the dehydrogenases *sn*-glycerol-3-phosphate (*sn*-GPD), flavoprotein electron-transferring co-enzyme Q oxidoreductase (ETFQO), dihydroorate dehydrogenase (DHODH), and proline dehydrogenase (PRODH). The content of the figure and the names of the cites are based on references [118] and [58].

Whereas all of these sites release ROS into the mitochondrial matrix, a substantial fraction of ROS produced by Complex III_{CoQ} and G_{CoQ} is also released into the IMS [109], from which H_2O_2 is able to diffuse into the cytoplasm through voltage-dependent anion channels (VDAC) in the outer mitochondrial membrane (OMM) [119,120]. H_2O_2 released into the matrix can also access the cytoplasm, as evidenced by observations that ROS released into the matrix by complex I can be detected in the cytoplasm [121] and in the medium around isolated mitochondria [115] and intact cells [122], but is to some extent consumed by intramitochondrial antioxidant mechanisms before it can do so. The ability of Complex III_{CoQ} to release ROS into the IMS, and also its position as the 'bottleneck' through which all electrons from the CoQ pool pass on their way to O_2 [58], has led to suggestions that it is the main site of mitochondrial ROS production involved in regulating the redox balance of the cytoplasm [58,120,123].

The sites proposed to be responsible for hypoxia-induced changes in ROS production in PASMC (and CBCC, the other extensively-studied cell type which responds acutely to physiological hypoxia) include I_{CoQ} [124,125]; II_F [126], and III_{CoQ} [38,127–130]. I_Q can produce ROS during either forward

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electron transport (when the electron is coming from NADH *via* I_F) or during reverse electron transport (RET), which occurs when the Q pool is highly reduced, causing a backflux of electrons from $CoQH_2$ into complex I [131]. The mechanism by which the semiquinone is formed and donates an electron to O_2 at III_{CoQ} is controversial. It is thought that superoxide/H_2O_2 is produced at this site *via* the reduction of O_2 by ubisemiquinone, which exists transiently at III_{CoQ} after $CoQH_2$ has lost one electron to the Reiske complex (referred to as the 'semi-forward reaction'), or due to the backflow of an electron from b_L (the 'semi-reverse reaction') [132–134]. Thus, superoxide production by III_{CoQ} is promoted by an increase in DΨm, which slows the loss of electrons from b_L, [135] and by the drug antimycin, which causes an accumulation of electrons at b_L and b_H by preventing the binding of CoQ to Q_i [133]. Note that III_{CoQ} is located at the site where $CoQH_2$ binds to complex III, which is generally termed Q_o.

Since ROS production by I_{CoQ} and III_{CoQ} is linked to co-enzyme Q, the extent to which the CoQ pool is reduced is a key determinant of mitochondrial ROS production. Importantly, it is thought that ROS production at III_{CoQ} is maximal when the CoQ pool is partially but not fully reduced. [134,136,137].

A number of compounds which affect mitochondrial ROS production by binding to specific sites within the ETC are available. Of these, rotenone, myxothiazol, antimycin, cyanide and sodium azide have been used most widely to study the role of the mitochondria in HPV. Rotenone binds to I_Q, thereby blocking the ETC at this point. Myxothiazol blocks the ETC at complex III by binding to III_{CoQ}. Antimycin binds to Q_i in complex III, stopping electron flow by interrupting the Q cycle. Cyanide and azide, which binds to the Fea3- CuB binuclear center in complex IV [138], block the ETC by preventing the reduction of O_2 to H_2O_2.

3.1.1.4. Mechanisms by Which Hypoxia May Increase Mitochondrial ROS Production

The 'metabolic hypothesis', initially developed to explain O_2 sensing in CBCC [139], proposes that hypoxia, by causing a perturbation of the state of the ETC, generates a signal which engages with effector pathways to initiate appropriate cellular responses. According to the mitochondrial ROS hypothesis, a variant of this proposal, one way in which hypoxia can affect the ETC is that a decreased availability of O_2 tends to slow the rate of its reaction with electrons at complex IV (aka cytochrome *aa*3, cytochrome *c* oxidase or Cox). This reaction, the rate-limiting process in mitochondrial electron transport, comprises a series of steps in which two pairs of electrons transferred from cytochrome *c* are used to reduce an O_2 molecule to two H_2O molecules. This reaction is irreversible, whereas the upstream series of redox reactions which couple the oxidation of NADH and $FADH_2$ to the reduction of cytochrome *c*, are at near-equilibrium with each other. As a result, a decrease of the activity of Cox will cause all parts of the ETC upstream of complex IV, and also the NAD^+:NADH and FAD:$FADH_2$ redox couples, to become more reduced [140]. The consequent reduction of the CoQ pool leads to higher ROS production by Complex III_{CoQ} [132].

In accordance with this concept, levels of physiological hypoxia which initiate HPV in PASMC (and transmitter release by CBCC) cause an immediate increase in cellular NAD(P)H fluorescence which is probably due mainly to a rise in the mitochondrial NADH/NAD ratio. For example, half maximal increases in NAD(P)H fluorescence were observed at PO_2 levels of 21 and 15 Torr in PASMC and CBCC, respectively [141] [142]. This implies that hypoxia is impeding the flow of electrons though the ETC (although as described below, this might not result in a commensurate inhibition of O_2 consumption). Similarly, moderate hypoxia was shown to cause the reduction of cytochrome *c* in CBCC [143] and of CoQ and cytochromes *c* and *aa*3 in PASMC [129,144]. Hypoxia also alters the magnitude of DΨm in both types of cells (although, puzzlingly, in opposite directions [130,145,146]).

Notably, however, the reduction of O_2 at Cox has an apparent P_{50} (i.e. the PO_2 at which the activity of the complex, as reflected by cellular O_2 consumption, is half maximal) of ~0.8 Torr in PASMC [144]. This is ~60 times lower than the PO_2 level which half-maximally stimulates HPV. This raises the question of how mild to moderate levels of hypoxia which cause HPV could perturb the

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ETC, thereby causing a signaling effect (e.g. reduction of the CoQ pool and increased ROS production by Complex III_{CoQ}), even though they are too high to affect its rate-limiting step.

Several explanations have been advanced to explain this apparent discrepancy [3] [34] [141]. One is that the basal mitochondrial PO_2 may be lower than that in the extracellular space (ECS) [147,148] and can therefore fall during moderate hypoxia to a level small enough to significantly limit the activity of Cox. However, the ECS to mitochondria PO_2 gradient in PASMC would have to be very substantial for a fall in PO_2 associated with moderate hypoxia to exert a meaningful effect on the activity of Cox [141].

Gnaiger and colleagues proposed another explanation for this conundrum, based on their finding that the apparent P_{50} for Cox is proportional to its activity [149]. According to their model, oxidase activity is controlled by the rate at which electrons are being fed into it by cytochrome *c*. This in turn depends on the relative expression of Cox compared to that of the upstream complexes which provide cytochrome *c* with electrons; in effect, the individual cytochrome *c* enzymes compete with each other for the pool of reduced cytochrome *c*. Thus, if the expression of Cox relative to that of complexes 1-3 is low, the supply to electrons to each cytochrome *c*, and therefore its activity and the apparent P_{50}, will be higher. Thus, cells with a relatively low expression of Cox will be more sensitive to moderate hypoxia.

The P_{50} is also increased by nitric oxide (NO) which competes with O_2 for binding to Cox [150], especially under hypoxic conditions. The presence of endogenous NOS was shown to be associated with increases in the reduction of cytochromes B_H, *c*1, and *aa*3 and also ROS production under hypoxic conditions In RAW246.7 cells [151]. In line with a role for NO-mediated block of Cox in O_2 sensing, the eNOS antagonist N(ω)-nitro-L-arginine methyl ester (L-NAME) blocked hypoxia-induced ROS production and O_2 sensing in endothelial cells [152–154].

Determining whether an interaction between NO and Cox in PASMC is involved in O_2 sensing during HPV is not straightforward, since NO production by the pulmonary vascular endothelium exerts a powerful vasodilating influence which inhibits HPV by activating the guanylate cyclase/cyclic GMP/protein kinase G pathway. This has been demonstrated by studies in which knockout of eNOS and application of non-selective NOS antagonists increased the amplitude of HPV in studies carried out in perfused lung [155] [16,156] [157,158], and in humans *in vivo* [159]. The fact that HPV persisted [160] or was increased [158] when the synthesis of NO by NOS in PA was prevented seems to militate against the possibility that NO could be playing a role in O_2 sensing in HPV. Even so, there is evidence that at least some types of cells possess NOS-independent pathways for synthesizing NO, and that these are stimulated by hypoxia. For example, hypoxia was shown to induce mitochondria from rat liver to produce NO through a Cox-mediated reduction of nitrite (NO_2^-) [161] . Although the importance of this mechanism has been disputed [162], hypoxia is also thought to activate other heme-dependent pathways within cells which can also generate NO from nitrite [163]. Furthermore, it has been shown that Cox plays an important role in metabolizing NO and that this process is inhibited by hypoxia [162]. This would also help to maintain NO levels during hypoxia, in which case there might be enough present in PASMC during hypoxia to compete with O_2 for binding to Cox. Therefore, although there is currently no evidence that NO is involved in O_2 sensing in HPV, it remains an intriguing possibility.

The mitochondrial concentration of H_2S has also been proposed to increase during hypoxia [164], and since it also inhibits Cox, with a submicromolar K_i for the isolated enzyme [165], it might have a similar effect to that proposed for NO. However, much higher concentrations of H_2S appear to be required to block Cox in intact cells and tissues [166,167]. These concentrations are unlikely to exist under physiological conditions, although this cannot be ruled out [168,169].

Another mechanism by which moderate levels of hypoxia could affect oxidative phosphorylation is described in a study carried out in isolated mitochondria from rat liver by Wilson et al [170], and further supported by work using mitochondria from cardiac myocytes [171]. Wilson and colleagues presented evidence that the *actual* P_{50} for Cox is much higher than its *apparent* value, which is typically derived by plotting the rate of respiration (measured as mitochondrial O_2

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consumption) *vs* PO_2. According to this scheme, even relatively moderate levels of hypoxia (<40 Torr in this study) decrease the activity of Cox. This would tend to slow the flow of electrons from cytochrome *c* to Cox, causing cytochrome *c* to become more reduced. However, this reduction of cytochrome c increases its tendency to 'push' electrons onto Cox. These two tendencies, which have opposing effects on electron flux, cancel each other out. Thus, hypoxia does not alter the rate at which electrons flow into Cox, or on the rate at which it reduces O_2 to water (i.e. the rate of O_2 consumption, which is equivalent to respiration). Instead, the inhibitory effect of moderate hypoxia on Cox is reflected by an increased reduction of cytochrome *c* rather than by a decrease in respiration. Wilson and colleagues observed a reduction of cytochrome *c* which was half-maximal at a PO_2 of 12 Torr, a value which they proposed was a reflection of the *actual* P_{50} for the activity of Cox. This compensatory mechanism would fail as the PO_2 falls towards zero because cytochrome *c* becomes maximally reduced. Thus, with very severe hypoxia, O_2 consumption falls steeply, and the PO_2 - dependency of this decline would give rise to the very low *apparent* P_{50} for the reaction between Cox and O_2 which is observed when measuring O_2 consumption as a function of PO_2. Because the reactions of oxidative phosphorylation upstream of Cox are at near- equilibrium [140,172], the increased reduction of cytochrome *c* at PO_2 levels well above the apparent P_{50} for Cox would 'back up' into complexes 1 - 3 and the co-enzyme Q pool, causing these to become more reduced, and would also raise the $NADH/NAD^+$ ratio. This would elevate the degree of reduction for both CoQ- and NADH- linked sites of ROS production, tending to increase H_2O_2 production.

This mechanism, which would be further promoted if hypoxia also decreased the cell energy state [173], is not necessarily restricted to any particular type of cell. However, it has also been suggested that Cox in PASMC and CBCC is unusually sensitive to hypoxia (i.e. has a higher P_{50}) because they both express high levels of Cox4i2, an atypical subunit of Cox which has been shown to increase its sensitivity to O_2 [174] and is negligibly expressed in other types of cell [175]. If the P_{50} for Cox was raised sufficiently, this would enable physiological levels of hypoxia to decrease the activity of COX and therefore slow the flux of electrons through the ETC and increase the degree of reduction at CoQ- and NADH-linked ROS-producing sites. Supporting this possibility, the P_{50} for the hypoxia-induced rise in NAD(P)H in sympathetic neurons was 0.3 Torr, as compared to 15 Torr in CBCC (*n.b.* the rise in NAD(P)H reflects the inhibition of Cox, since this carries out the rate limiting step of respiration and therefore also of the oxidation of NADH to NAD) [176].

Another factor which may cause a hypoxia-induced increase in mitochondrial ROS production, in this case independently of any effect on Cox, is a rise in the concentration of the Krebs cycle intermediate succinate. Couchani et al [100] showed that ischemia led to the reversal of the reaction carried out by succinate dehydrogenase (complex II), causing an accumulation of succinate in myocardial cells. Cardiac reperfusion then evoked a burst of ROS production due to the oxidation of the accumulated succinate by complex II, and this was blocked by the drug rotenone, which prevents the binding of quinones to complex I. This suggested that the ROS burst was mediated by complex I and was due to RET, which causes a high level of ROS production by complex I, probably at the 1_{CoQ} site [109,177] [178].

The role of succinate and RET in O_2 sensing during HPV is currently unknown. However, based in part on the observation that the complex II blocker dimethyl malonate depressed the response of CBCC to hypoxia, López-Barneo's laboratory [179,180] initially suggested that succinate-linked RET contributes to acute O_2-sensing in these cells by causing ROS production by complex I; a similar finding was reported by Swiderska et al [181] who also used dimethyl malonate. Nevertheless, it has more recently been reported that O_2 sensing in CBCC was not obviously affected in mice in which RET was strongly depressed due to the introduction of a mutation into the ancillary complex I subunit ND6[182]. The involvement of RET in O_2 sensing in CBCC can also be questioned because, whereas ROS generated at complex I due to RET are released into the mitochondrial matrix [109], the matrix redox balance in CBCC was reduced, rather than oxidized, by hypoxia [180]. In addition, the use of dimethyl malonate is problematic because it could potentially depress ROS production at complex III by decreasing succinate-mediated reduction of the CoQ pool [132].

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Hernansanz-Agustin and colleagues [183,184] have proposed a mechanism by which hypoxia can increase complex III-mediated ROS production which is based on evidence that complex I has an intrinsic Na^+/H^+ antiport activity (pumping Na^+ from the IMS into the matrix in exchange for protons) which is greatly increased when it undergoes a hypoxia-induced transition from its normal active state to a 'de-active' conformation in which it loses its usual NADH-ubiquinone oxidoreductase/proton-pumping activity [185] [186]. A deficit of O_2 is proposed to promote this 'A/D transition' by causing a net reduction of the CoQ pool, thus inhibiting electron flow though complex I because there is less oxidized CoQ (ubiquinone) available to accept electrons in quinone binding site [187]. Interestingly, the active/de-active ratio varies over a broad PO_2 range, and deactivation can occur quickly, rendering it a feasible target for moderate hypoxia [188,189].

According to this scheme, A/D transition induced by hypoxia causes matrix acidification by promoting H^+ influx *via* the complex I Na^+/H^+ antiport and by inhibiting the normal Complex I mediated pumping of H^+ from matrix to IMS [183]. This acidification causes the partial solubilization of the calcium phosphate complex which is present at a very high concentration in the [190]. The resulting increase in free $[Ca^{2+}]_{mito}$ promotes Na^+ entry into the matrix *via* the mitochondrial $Na^+/Ca^{2+}/Li^+$ antiporter (NCLX). This causes a rise in matrix $[Na^+]$ which decreases the fluidity of the IMM, especially in the leaflet of the membrane adjacent to the matrix. The authors presented evidence that whereas an increase in $[Na^+]$ did not affect the function of the individual respiratory complexes when they were studied in isolation (i.e. in a mitochondrial membrane preparation), it did decrease the flow of electrons from complex II and G3PD to cytochrome in intact mitochondria. They proposed that the decreased fluidity of the IMM due to the rise in matrix Na^+ induced by hypoxia would retard the mobility of $CoQH_2$ in this membrane, thereby slowing electron flow from complex II and G3PD to complex III. This would slow the Q cycle, thereby increasing ROS production by prolonging the lifetime of ubisemiquinone at the Q_o site. This mechanism is suggested to contribute to O_2 sensing in HPV [183] (see section 5.2.3).

There is also evidence that an increased Ca^{2+} concentration in the mitochondrial matrix, which has been shown to occur in PASMC during hypoxia (see Section 5.2.1) stimulates pyruvate dehydrogenase, a-ketoglutarate dehydrogenase and complex II [191]. This increases the flux of electrons into the CoQ pool, thereby stimulating oxidative phosphorylation and increasing ROS production [192,193]. It has alternatively been proposed that an increased $[Ca^{2+}]_{mito}$ elevates $D\Psi_m$ by reversing a brake on Cox exerted by ongoing ATP-dependent phosphorylation of this complex [194]. Regardless of the mechanism by which $[Ca^{2+}]_{mito}$ or other factors elevate $D\Psi_m$, there is abundant evidence that an increased (i.e. hyperpolarized) mitochondrial membrane potential powerfully stimulates ROS production at complexes 1 and 3 [132,195,135]. Ramzan et al [196] suggested that an increase in $[Ca^{2+}]_{cyto}$, which raises $[Ca^{2+}]_{mito}$ *via* influx through the mitochondrial Ca^{2+} uniporter [192,197], accounts for the rise in mitochondrial ROS production evoked by a range of stressors, including hypoxia, in a variety of cell types. However, in this case, increased mitochondrial ROS production could not be the initial O_2 sensing signal (which would be needed to generate the rise in $[Ca^{2+}]_{cyto}$), although it could act to amplify this signal.

Hajnóczky and colleagues have presented an intriguing proposal that an increase in the Ca^{2+} concentration in the mitochondrial matrix may cause it to swell due to the increased influx of K^+ (and therefore water) into the matrix *via* mitoK_{Ca} channels in the IMM [92]. This then causes a compression of the mitochondrial cristae, which are continuous with the IMS. This would decrease the volume of the cristae/IMS compartment, increasing its $[H_2O_2]$ and thereby promoting the leakage of H_2O_2 into the cytoplasm.

Notably, the concept that an increase in $[Ca^{2+}]_{mito}$ raises mitochondrial ROS production has been vigorously contested [198].

3.1.1.5. Possible Mechanisms by Which Hypoxia Could Decrease Mitochondrial ROS Production

According to Eqn 1, a fall in PO_2 would tend to decrease mitochondrial ROS production by the law of mass action, and most papers which have described hypoxia-induced falls in mitochondrial

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ROS production in cells or isolated mitochondria seem to have implicitly assumed that they occurred for this reason. This idea is consistent with evidence that ROS production by isolated mitochondria is linearly related to the O_2 concentration over a wide PO_2 range [199,200], although others have reported a hyperbolic relationship between the PO_2 and ROS production [115,201], this does not necessarily conflict with the idea that the reactions between O_2 with electrons leaving ROS-producing sites in the ETC are linearly dependent on the [O_2] [137].

Conversely, Archer and colleagues have suggested that a fall in PO_2 also diminishes ROS production by decreasing the rate of electron flow though the ETC, thereby limiting the availability of electrons at the sites in the proximal ETC at which they can react with O_2 [36,202], see also [203]. This would appear to imply that the location at which hypoxia is slowing electron flow must be either at or upstream of the site where the ROS which regulate HPV are being produced, since upstream block of the ETC would be predicted to increase electron occupancy at this site and therefore promote its ROS production. This is consistent with evidence from this laboratory (but not others, see [3]) that the effects of hypoxia on ROS production are mimicked by the I_Q-blocker rotenone [204]. Similarly, these effects have been reported to require the complex I subunit Ndufs2 [125], which forms part of I_{CoQ} and interacts with the N2 Fe-S cluster that is believed to mediate the formation of semiquinone at this site [205] [206] [207] (see Section 5.1.1) . Nevertheless, the nature of the interactions between hypoxia, Ndufs2, and a slowing of electron flow which could result in decreased ROS production by complex I remain to be defined.

3.1.2. Mitochondrial ROS Consumption

Mitochondrial matrix consumption of H_2O_2 depends on the GSH and Trx2 redoxin systems (see Section 2.2), and on peroxiredoxins and catalase [58,104,208]. ROS released into the IMS are subject to degradation by SOD1, GSH and cytochrome C [209]. The antioxidant activities of GSH, TRX2 and peroxiredoxins require that they be returned to the reduced state by NADPH after having been oxidized due to the scavenging of H_2O_2. The maintenance of sufficient matrix NADPH to perform this function depends on the activity of the Krebs cycle enzymes malic enzyme (ME3) and isocitrate dehydrogenase (IDH2), which reduce $NADP^+$, and also on nicotinamide nucleotide transhydrogenase (NNT), an enzyme in the IMM which couples the oxidation of NADH to the reduction of $NADP^+$ within the matrix.

These antioxidant intra-mitochondrial mechanisms can affect the redox balance of the cytoplasm in two ways. Firstly, they limit the extent to which H_2O_2 generated within the matrix reaches the cytoplasm [103,208]. Secondly, the mitochondria can act as a sink for cytoplasmic H_2O_2. A study [104] which examined the effect of knocking out and overexpressing mitochondrial anti-oxidant enzymes on the redox state of the cytoplasm in a H9c2 cells exposed to the organic peroxide tert-butyl hydroperoxide (t-BOOH)(1 mM) reported that knockout of NNT, ME3 or IDH2, and also the mitochondrial anti-oxidant enzyme thioredoxin2, increased the extent to which t-BOOH oxidized the cytoplasm. In contrast, knockout of cytoplasmic antioxidant enzymes did not ameliorate the effects of t-BOOH on the mitochondria. The authors suggested that the mitochondria may function to degrade H_2O_2 which leaks into cells from the ECS. Interestingly, another paper reported that application of 25 and 500 μM extracellular H_2O_2 to EA.hy926 cells caused a rise in cellular H_2O_2 which was larger and occurred more rapidly in the mitochondria compared to the cytoplasm and nucleus [210]. These reports suggest that the mitochondria play a crucial role in the ability of cells to scavenge H_2O_2 leaking in from the extracellular space, although whether this occurs under physiological conditions can be questioned given the antioxidant prowess of cytoplasmic Prxs [211] (see Section 2.2).

Oxidation of the matrix leads to s-glutathionylation of complex I and other proteins associated with ETC ROS production, including pyruvate dehydrogenase (PDH) and a-ketoglutarate dehydrogenase (KGDH) [79]. This depresses oxidative phosphorylation and ROS production, thus stabilizing the redox state of the matrix. Another factor promoting mitochondrial redox homeostasis is that the activity of NNT is crucially dependent on DP, meaning that both the production and

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NADPH-dependent scavenging of ROS within the matrix are enhanced by increases in [NADH] and DP. Nevertheless, Treberg et al [208] demonstrated that the effect of $D\Psi_m$ on mitochondrial ROS production was greater than that on peroxiredoxin-mediated ROS scavenging, suggesting that increasing $D\Psi_m$ would produce a net rise in matrix [H_2O_2].

3.2. ROS Production by NADPH Oxidases

The seven members of the Nox family of oxidoreductases (Noxs 1-5 and Duox 1 and 2) function as adjustable sources of superoxide and/or H_2O_2 which regulate the activities of nearby target proteins. Four Nox isoforms (Nox 1, 2, 4 and 5) are expressed in vascular smooth muscle cells [60]. Nox2 has been shown not to be required for HPV, at least in mice [212], and the role in HPV of Nox 5, which is expressed in primates but not rodents, is unknown. However, Noxes 1 and 4 have been proposed to be involved in HPV (see Sections 5.2.5 and 8). Both of these Nox isoforms are heterodimers comprising a membrane-spanning oxidoreductase subunit, which transfers electrons from NADPH to O_2 *via* flavin adenine dinucleotide (FAD) and two heme groups, and $p22^{phox}$, a smaller transmembrane scaffolding protein. Nevertheless, they differ in important ways.

3.2.1. Nox1

Nox1 is present in the plasmalemma, localized to the caveoli in vascular smooth muscle cells (VSMC) [213]. It releases superoxide into the ECS, from where it can enter cells either *via* plasmalemmal anion channels, or, after being converted to H_2O_2 by EC-SOD, through aquaporins [214]. Nox1 can also be endocytosed upon its activation by certain stimuli, potentially leading to redox signaling by superoxide and/or H_2O_2 both within the endosome and the cytoplasm [215]. Activation of Nox1 is triggered by the binding of the cytoplasmic proteins $p47^{phox}$, $p67^{phox}$, and $p40^{Phox}$. Its recruitment of these proteins is triggered by the phosphorylation of $p47^{phox}$ by protein kinase C (PKC) and/or a Src family tyrosine kinase [60,216]. Alternatively, p47phox and $p67^{phox}$ may be replaced by their homologues NoxO1 and NoxA1, respectively. In this case, NoxO1 is constitutively tethered to $p22^{phox}$, and superoxide production is stimulated by the binding of NoxA1 to NoxO1 [217], which is promoted by PKC [218], and also requires the phosphorylation of threonine 429 on Nox1 (e.g. by protein kinase C β_1; [219]. The combination of $p47^{phox}$ and NoxA1 has also been shown to stimulate Nox in VSMC [220].

Activation of PKC and Src kinases can be initiated by diverse stimuli, including lipid mediators produced by phospholipases A_2, C and D following their activation by the binding of agonists to multiple types of G-protein coupled receptors. PKC and Src family kinases can also be activated by each other, and by ROS [216], thereby enabling positive feedback. Full activation of Nox1 occurs with the subsequent binding of activated monomeric G protein Rac1. This in turn necessitates the stimulation of Rac guanine exchange factors following their tyrosine phosphorylation and binding to phosphatidyl inositol triphosphate, which is generated by transactivation of the epidermal growth factor receptor and the stimulation of phosphatidyl inositol-3 kinase [60].

3.2.2. Nox4

Nox4 is thought to be the most highly expressed isoform in VSMC, in which it is localized to the plasma membrane in association with focal adhesions [213]. It is also expressed in the sarcoplasmic reticulum [221], mitochondria [222], and nucleus [223]. Nox4 primarily produces H_2O_2, which presumably gains access to the cytoplasm *via* aquaporins in the plasmalemma and intracellular membranes. The Nox4/$p22^{phox}$ dimer is constitutively active, suggesting that it can produce appreciable levels of H_2O_2 under basal conditions, and that its ability to do so may regulated by its level of expression [224], and, acutely, by the availability of NADPH and also O_2 [59]. H_2O_2 production by plasmalemmal Nox4 is increased by its association with polymerase delta interacting protein 2 (Poldip2) [225] and in VSMC can be enhanced acutely by G protein coupled vasoconstrictors

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through mechanisms that are not well understood. Little is known about the regulation of intracellular Nox4 [60].

One important property of Nox4 which makes it well suited to act as an sensor for acute changes in the PO_2 is that its K_m (O_2) (the PO_2 at which its production of H_2O_2 is half maximal) has been reported to be ~136 Torr, such that its activity would be decreased by even mild hypoxia. The K_m (O_2) for Nox1 seems not to have been measured, although that for Nox2 is ~25 Torr [59].

4. Intra- and Extracellular H_2O_2 Concentrations

Recent reviews state that the basal intracellular [H_2O_2] is in the range of 1-5 nM [32,226,227], citing as evidence an elegant study by Oshino et al [228] which took advantage of the fact that the enzyme catalase reacts with H_2O_2 to form the intermediate Compound 1 (which can be monitored spectrophotometrically) to calculate that the intracellular cellular concentration of H_2O_2 in rat liver under resting conditions was on the order of 10^{-9} M [228,229] , see also [230].

A similar estimate was reported by Lyublinskaya & Antunes [231], who, using an approach based on measuring the kinetics of oxidation of the H_2O_2 sensor HyPer , determined that the basal cytoplasmic $[H_2O_2]_{cyt}$ in K562 cells is in the low nanomolar range [231]. Their study was designed to determine the involvement of extracellular H_2O_2 in setting the basal cytoplasmic [H_2O_2]. The concept that extracellular [H_2O_2] ($[H_2O_2]_{EC}$) is an important determinant of the cytoplasmic [H_2O_2] had emerged from studies showing that a bolus of H_2O_2 added to the solution bathing cells in culture is taken up (*via* aquaporins [72] [232]) and then rapidly scavenged by intracellular antioxidant systems [233–235]. Because the removal of H_2O_2 by cellular antioxidants is much faster than its uptake, a large H_2O_2 concentration gradient across the cell membrane develops immediately upon its extracellular application [236]. The magnitude of this gradient is maintained at a constant level as the H_2O_2 in the bath is taken up by the cells, such that intra-and extracellular [H_2O_2] undergo a parallel exponential decline [237].

The steady-state transmembrane gradient can be assessed by keeping the cell number low enough to ensure that the uptake of H_2O_2 has a negligible effect on its concentration in the bath, or by using an H_2O_2 - regulating system such as glucose/glucose oxidase/catalase to stabilize the [H_2O_2] in the bathing solution [238,239]. Using the former approach, Lyublinskaya & Antunes reported that the cytoplasmic [H_2O_2], which was 2.2 nM when no H_2O_2 was added to the bath, rose to 3.5 and 8.9 nM, respectively, when 1 and 2.5 μM H_2O_2 were applied to the solution. The authors calculated that an average trans-plasmalemmal H_2O_2 concentration gradient of ~390-fold was present over this range of extracellular [H_2O_2].

The existence of even larger (~1000-fold) [H_2O_2] gradients between extracellular and cytoplasmic [H_2O_2] had previously been predicted by mathematical models developed by Adimora et al [237] and Lim et al [240], which took into account information about the rates of transmembrane H_2O_2 flux, cellular production, and scavenging e.g. [235,241]. Importantly, whereas cellular H_2O_2 was initially thought to be scavenged by catalase and glutathione peroxidase [234], yielding calculated transmembrane gradients of which were small (e.g. ~7 in Jurkat T cells [233]), these models took into account more recent evidence that Prxs, which are present in micromolar concentrations in cells and can have a very high affinity for H_2O_2 (e.g. Prx2), are predominantly responsible for removing it from the cytoplasm [88]. Importantly, Lim et al [240] went on to show that the scavenging of H_2O_2 following its entry into the cell would also set up a significant peroxide concentration gradient *within* the cytoplasm which would develop within milliseconds and remain stable if the $[H_2O_2]_{EC}$ was constant. Thus, they calculated that [H_2O_2] at the inner face of the plasmalemma in a spherical cell with a radius of 10 microns would be ~0.3% of that in the extracellular medium, falling to ~0.08% and 0.02% at 2 and 4 microns into the cell, respectively. These studies imply that the basal intracellular [H_2O_2] in the subplasmalemmal region is strongly influenced by the $[H_2O_2]_{EC}$.

The physiological implications of these findings are difficult to parse, firstly because the [H_2O_2] in solutions bathing biological preparations *in vitro* is typically not controlled or measured during investigations of its intracellular dynamics or effects, and secondly because little is known about the

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$[H_2O_2]_{EC}$ *in vivo*. Although basal $[H_2O_2]_{EC}$ has been suggested to be 1-5 μM [32,226,227], this seems to have been measured only by one laboratory, which reported mean *in vivo* values of 3 and 5 μM for $[H_2O_2]_{EC}$ in rat ventrolateral medulla and hippocampus, respectively [242–244]. Additional indirect evidence supporting the possibility that cells experience $[H_2O_2]_{EC}$ in this range came from an investigation in which the $[H_2O_2]_{EC}$ immediately adjacent to the extracellular surface of the plasmalemma in A549 (tumor-derived) cells in culture was measured using a membrane-tethered sensor [245]. This showed that average $[H_2O_2]_{EC}$ close to the membrane was ~2 μM, and also revealed the presence of discrete H_2O_2 hotspots, presumably associated with the presence of a higher local expression of Nox in that area of the membrane, with concentrations of up to ~12 μM. [246]. The authors suggested that these areas were likely to give rise to corresponding H_2O_2 hotspots in the subplasmalemmal cytoplasm, although they did not confirm this.

Parenthetically, basal plasma $[H_2O_2]$ is also generally thought to be 1-5 μM [247]. However, Sousa et al [248] contend that the basal plasma $[H_2O_2]$ has been overestimated, and that due to its uptake by erythrocytes, is in the range of 10 nM. They note, for example, that taking into account measurements of the permeability of erythrocytes to H_2O_2, Prx2, which should be heavily oxidized when the plasma $[H_2O_2]$ is ≥ 80 nM, is instead found to be almost completely reduced under basal conditions [249].

Although the evidence is very limited, the agreement between the measured values of intra- and extracellular $[H_2O_2]$ and the ~1000-fold $[H_2O_2]$ gradient predicted by mathematical models makes a neat story. Nevertheless, it has also been suggested that the basal cytoplasmic $[H_2O_2]$ may be in the sub-nanomolar range [250]. Lim et al [211] concluded on theoretical grounds that, absent H_2O_2 influx from the extracellular space, cellular H_2O_2 production and scavenging should set the basal cytoplasmic $[H_2O_2]$ at ~80 pM. Since according to their later model [240] the influence of $[H_2O_2]_{EC}$ decreases steeply moving away from the cell membrane, $[H_2O_2]$ would be predicted to approach this level in the core of large cells. Moreover, using HEK293-MSR cells expressing the novel high-sensitivity H_2O_2 indicator Apex2 [251], Eid et al [252] detected a sustained increase in intracellular $[H_2O_2]$ upon the application of $[H_2O_2]_{EC}$ down to 25 nM in HEK293-MSR cells. Sommer et al [130] found that application of 124 nM H_2O_2 caused membrane depolarization and inhibited the K_V current in PASMC, and this laboratory has recently reported that the applying as little as 10 nM H_2O_2 inhibited the current through $K_V1.5$ channels co-expressed with $K_V\beta1.4$ ancillary subunits in oocytes [253]. This implies that the basal $[H_2O_2]$ even adjacent to the cell membrane could have been in the subnanomolar range, although whether this applies to cells *in vivo* would presumably depend on the balance between the rates of H_2O_2 influx from the ECS and its intracellular scavenging.

Another implication of the presence of powerful antioxidant mechanisms present in the cytoplasm is that increases in $[H_2O_2]$ emanating from intracellular sites are highly localized. This was demonstrated by Mishina et al [254], using cells expressing HyPer fused to growth factor receptors in the plasmalemma and protein tyrosine phosphatase 1B (PTP1B) in the endoplasmic reticulum (ER) membrane. They found, for example, that applying epidermal growth factor (EGF) to HeLa cells expressing an EGF receptor – HyPer fusion construct led to the internalization of a fraction of the EGF receptor into endosomes, while the rest remained in the plasmalemma. The endosomal fraction reported an increase in $[H_2O_2]$ due to Nox activity associated with receptor activation, while the portion in the plasmalemma did not. This demonstrated that only internalized EGF receptor was activated, and also that H_2O_2 was not able to diffuse from far enough from the endosomes to reach the plasmalemma. Likewise, H_2O_2 generated by Nox in response to platelet derived growth factor (PDGF) was sensed by PTP1B-HyPer in the ER membrane but not by PDGF receptor-HyPer in the endosomal membrane. In a subsequent study in HeLa cells, this group demonstrated the presence of a steep cytoplasmic H_2O_2 concentration gradient when a H_2O_2 generating system (D-amino acid oxidase/alanine) was localized to the nucleus [255].

However, an investigation utilizing the highly sensitive indicator HyPer7 failed to detect cytoplasmic H_2O_2 gradients, or indeed any rise in the global cytoplasmic $[H_2O_2]$, associated with an increase in mitochondrial ROS production induced by D-amino acid oxidase/alanine in K562 cells

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[256]. On the other hand, by applying antimycin to cells fed with galactose, which enhances mitochondrial ROS production, Hoehne et al [121] were able to demonstrate using HyPer7 that an increase in mitochondrial ROS production at complex III did cause a small rise in $[H_2O_2]_{cyt}$ in HEK293 cells, although this was not observed in cells fed with glucose. Both papers concluded that H_2O_2 was being released by the mitochondria, but that it (and any HyPer7 molecules it may have oxidized [257]) were being reduced before they could diffuse far enough to generate a measurable signal in the overall cytoplasm. Notably, recent experiments using HyPer7 also detected an increase in cytoplasmic H_2O_2 elicited by mitochondrial D-amino acid oxidase/alanine in HeLa cells, leading the authors to suggest that these cells may have less effective antioxidant mechanisms compared to K562 cells. Interestingly, using the novel H_2O_2 indicator HyPerFLEX, which is more sensitive than HyPer7, they also observed an accompanying increase in H_2O_2 in the nucleus and endoplasmic reticulum [258].

Notably, studies by Hajnoczky and colleagues have shown that the close apposition of mitochondria to the endo/sarcoplasmic reticulum (ER) means that mitochondrial ROS release into the nanodomain between these organelles can oxidize a group of cysteines in the cytoplasmic-facing suppressor domain of the IP_3R, inducing Ca^{2+} release [92,259,260]. Using a compartmentally-targeted redox sensor (Grx1-Ro-GFP2), they demonstrated in HEPG2 cells [259] that the oxidative signal due to mitochondrial ROS release was restricted to the mitochondria/ER nanodomain, and did not result in a general cytoplasmic oxidation. Intriguingly, they also presented evidence that the ER-mitochondrial interface nanodomain was more oxidized than the bulk cytoplasm even under basal conditions, suggesting the existence of a standing intracellular redox gradient which may function to enable ER Ca^{2+} release.

Section 5: Models of O_2 Sensing in HPV

Although they have opposed views on the effects of hypoxia on cytoplasmic oxidant signaling in PASMC, the Redox and Mitochondrial ROS theories both propose that the mitochondria are the primary O_2 sensors responsible for causing HPV. This concept is supported by evidence that 1. blockers of the proximal ETC abolish HPV (see Section 7.1), 2. cultured PASMC depleted of their mitochondria by ethidium bromide treatment [261] do not demonstrate hypoxia-induced rises in $[Ca^{2+}]$cyt [38], and 3. mitochondria in PASMC are distributed more peripherally compared to those in mesenteric artery SMC (MASMC), so are better placed to influence effectors located in the plasmalemma [262].

We will first describe the experiments leading to development of each theory separately in Sections 5.1 and 5.2 Because these proposals share a focus on mitochondrial ROS production, the studies carried out by their proponents have often used similar approaches (e.g. assessing the effects of ETC blockers or pro- and anti-oxidants, measurement of ROS), and have therefore yielded information relevant to both. We will therefore consider this evidence as a whole and discuss its implications in Section 7.

5.1. The Redox Theory

The Redox theory of hypoxia of HPV, proposed by Kenneth Weir and Stephen Archer in the mid 1990's [36,263], is based on the concept that HPV is due to the removal by hypoxia of a tonic PASMC vasorelaxation [264] which is maintained under normoxic conditions by a level of cytoplasmic ROS which is higher than that present in systemic artery VSMC. Recent work by Archer's laboratory ascribes this difference to the higher expression of the complex I subunit Ndufs2 in PASMC [125].

According to this hypothesis (Figure 6), the relatively high ongoing PASMC production of H_2O_2 by mitochondria under normoxic conditions leads to a basal activation of voltage-gated K^+ (K_V) channels which maintains the cells in a relaxed state by ensuring that the membrane potential (E_m) is negative enough to suppress the activity of L-type voltage-gated Ca^{2+} channels, thereby minimizing Ca^{2+} influx. By decreasing the flux of electrons through the ETC, hypoxia diminishes the production of mitochondrial superoxide/H_2O_2 [33]. The resulting fall in the cytoplasmic $[H_2O_2]$ depresses K_V

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channel activity, causing membrane depolarization and an increased Ca^{2+} influx which evokes HPV. The mechanism by which ROS activate the Kv channels is unknown but could involve oxidation of Kv channel protein thiol-containing residues. ROS are seen as exerting a similar effect on Kv channels in systemic artery VSMC, although this influence is smaller under normoxic conditions because mitochondrial ROS production is lower. In contrast to PA, systemic arteries dilate to hypoxia, and this is proposed to be due, at least in part, to increased mitochondrial ROS production and the activation of Kv channels [145]. Thus, the opposite responses of pulmonary and systemic arteries to hypoxia are seen as being due to its contrary effect on mitochondrial ROS production, rather than to fundamental differences between the hypoxia-sensitive effector pathways controlling VSMC force development in these arteries and/or their responsiveness to ROS [33].

Although the proponents of the Redox theory have generally stressed the effect of hypoxia on ROS, they also view hypoxia-induced reduction of cellular redox couples (e.g. NAD^+/NADH, NADP/NADPH, GSSG/GSH) as potentially contributing to HPV [36,125]. This could occur, for example, through an action of NAD(P)H on Kv channel b-subunits, which can modulate channel gating by virtue of their activity as aldehyde dehydrogenases [265]. However, taking into account that the cytoplasmic redox potential of the $NADP^+$/NADPH couple in the cytoplasm is approximately -390 mV [266], this couple is already almost entirely reduced under normoxic conditions. This suggests that a fall in [ROS] in and of itself is unlikely to increase the free [NADPH], making it a poor candidate for mediating hypoxia-induced changes in Kv channel activity associated with decreased ROS production. On the other hand, the NAD^+/NADH couple, which has a cytoplasmic redox potential of ~-240, is highly oxidized [252, 266]. This results in a low free cytoplasmic [NADH] [266,267] which is in the appropriate range for regulating Kv channels [268]. Given its involvement in controlling the oxidation of cellular thiol switches, the GSSG/GSH redox couple is of particular interest with regard to signaling. Importantly, the GSSG/GSH ratio has been shown to be altered by the application of low concentrations of extracellular [H_2O_2], and also by hypoxia [269,270] (see Section 6.4 for a further discussion of the role of these redox couples in HPV).

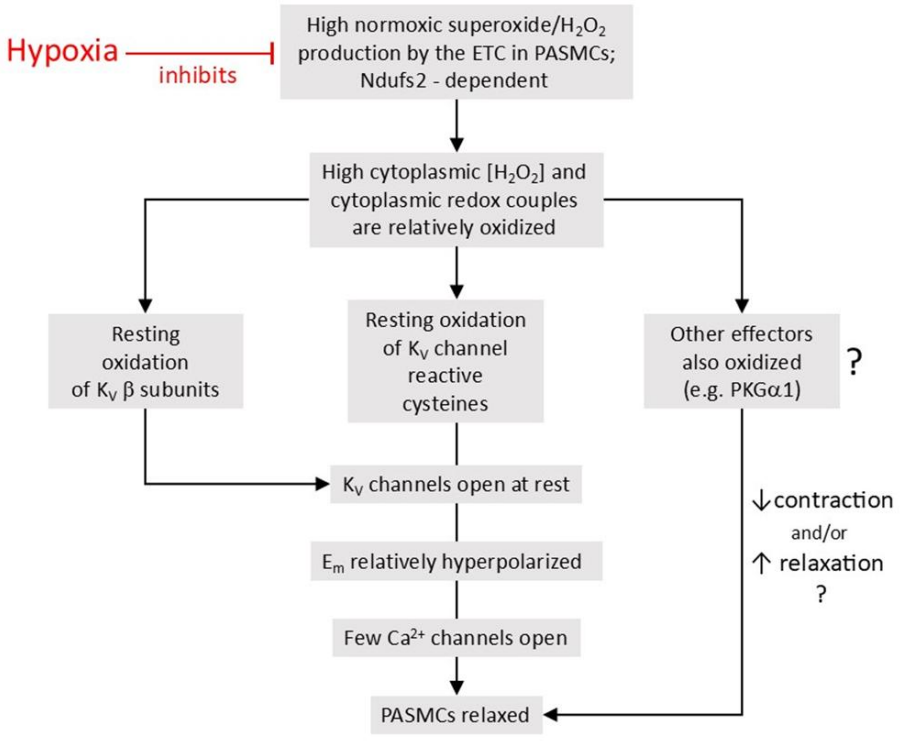

Figure 6. The Redox theory of HPV. This is based on the concept that under normoxic conditions mitochondria in PASMCs are generating a higher level of ROS than in systemic VSMCs. The resulting higher level of H_2O_2

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and/or oxidation of cytoplasmic redox couples activates K_V channels, causing a relatively hyperpolarized Em which limits Ca^{2+} influx through voltage-gated Ca^{2+} channels, thereby contributing to a tonic vasodilation. Other as yet unidentified redox-regulated vasodilating pathways might also be similarly recruited by cytoplasmic oxidation. By diminishing mitochondrial ROS production, hypoxia decreases this vasodilating influence, causing an increase in vascular tone. See the text for further details.

Similarly, although they have emphasized the role of K_V channels as the crucial ROS-sensitive effectors, the involvement of other redox-sensitive downstream pathways which could also respond to a fall in ROS by to promote contraction (or inhibit relaxation) are not ruled out [271]. There is, for example, evidence from Michael Wolin's laboratory that a decrease in H_2O_2 production contributes to HPV by suppressing basal vasodilation due to activity of soluble guanylate cyclase (sGC) and protein kinase G (PKGa1). However, the model for HPV developed by Wolin and colleagues differs from the Redox theory in that it is Nox rather than the mitochondria which is seen as being responsible for basal ROS production, and, unlike the Redox theory, it also proposes an important role for the pentose phosphate pathway in HPV (see Section 8).

5.1.1. Evidence for a Fall in Mitochondrial ROS Production and PASMC Reduction as the O_2 Sensor in HPV

A series of studies in the 1980's, carried out mainly by Weir, Archer and colleagues, investigated the effects of oxidizing and reducing agents on HPV. Weir et al [272] examined the effect of IV injection of the oxidizing agent diamide on HPV recorded in anesthetized dogs when the inspired O_2 level was reduced to 12%, and on the pressor response to PGF_{2a}. Diamide markedly diminished the increases in PAP and PVR to elicited by both hypoxia and PGF_{2a} without affecting cardiac output or systemic vascular resistance. Burghuber et al [273] examined the effect of H_2O_2, generated by adding glucose/glucose oxidase to the perfusate, on HPV in isolated perfused rat lung, finding that it did not alter PAP under normoxic conditions, but abolished HPV and halved the amplitude of the pressor response to angiotensin 2. These responses were prevented if catalase was added to the solution and were mimicked by perfusing the lungs with physiological saline solution (PSS) containing ~10^{-6} M H_2O_2. Although glucose/glucose oxidase caused the lungs to release PGF_{2a} and TXA_2, these were unlikely to have mediated its effects on pulmonary vasoactivity, which persisted when their synthesis was blocked using indomethacin.

Subsequent studies from Weir and colleagues were also performed in isolated perfused rat lungs in which HPV was evoked by reducing the PO_2 in the perfusate to ~40 Torr. Weir et al [274] found that diamide applied during hypoxia also reversed HPV in this preparation, and that adding xanthine/xanthine oxidase (X/XO) to the solution to generate ROS had a similar effect which was prevented if the X/XO blocker allopurinol was present. They suggested that X/XO might be acting by oxidizing cellular sulfhydryl groups, and soon afterwards proposed that hypoxia was inducing PA constriction by causing a similar effect [275]. Archer et al [276] confirmed this effect of X/XO on HPV and found that it was attenuated if SOD and catalase were added to the perfusate. Application of SOD and CAT which had been incorporated into liposomes to facilitate their entry into PA cells similarly suppressed the effect of X/XO. If added in the absence of X/XO, these liposomes increased baseline PAP and enhanced both HPV and the pressor response to angiotensin 2. These results supported the concept that reduction of redox couples in PASMC tended to promote PA constriction, both under normoxic and hypoxic conditions. Both diamide and X/XO suppressed HPV, although it is difficult to interpret this effect because the response to angiotensin 2, which was used as a pre-tone agent to amplify HPV [277] was similarly inhibited by X/XO, and the effect of diamide on the angiotensin 2 response was not described.

Archer et al [278] performed simultaneous measurements of PAP and pulmonary ROS production in isolated and ventilated rat lungs while changing the PO_2 in the inspired gas. Luminol or lucigenin were included in the perfusion solution as ROS indicators, and the PO_2 was varied from the normoxic baseline (20%) to 0, 1, 2.5, 10, or 95%. The key result of this paper was that lowering

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the PO_2 to ≤ 2.5% evoked a fall in luminescence with both indicators that paralleled the decrease in PO_2 and preceded the onset of HPV, which was sustained with 1% and 2.5% O_2, but transient with 0% O_2. Including catalase in the perfusate did not affect lucigenin luminescence under normoxic conditions, whereas this was strongly diminished by SOD. SOD similarly depressed luminol luminescence, which was however somewhat increased by catalase. SOD slightly raised basal PAP, and strongly enhanced HPV and the pressor response to angiotensin 2, whereas catalase had no effect on PAP under any condition. Based on their observations, the authors suggested that hypoxia was decreasing pulmonary superoxide levels. The ability of SOD to increase basal and stimulated vascular tone echoed earlier findings and was seen to support the idea that the oxidation of cell redox couples exerts a tonic vasodilating influence on PA. However, from a contemporary perspective, which views H_2O_2 rather than superoxide as mediating most ROS-dependent intracellular signaling [32], the observations that SOD caused contraction and enhanced HPV appear to be inconsistent with the Redox theory.

A crucial step in the development of the Redox theory came with the observation, which emerged from a collaboration between the Stephen Archer and Joseph Hume's laboratory [279], that hypoxia inhibited the K^+ current in pulmonary (but not renal) artery SMC. This raised the question of whether hypoxia was inhibiting K^+ channels by virtue of its effects on cell redox. Based on the observation that blockers of the ETC cause PA contraction [280] and would be predicted to mimic hypoxia by suppressing mitochondrial ROS production and reducing cell redox couples such as NADH/NAD^+ and GSH/GSSG, Archer et al [263] investigated the effects of several ETC blockers on PAP and ROS production in their rat lung model, and on the K^+ current in freshly isolated rat PASMC. Rotenone, antimycin or cyanide, which act at complexes 1, 3 and 4, respectively, were applied as a bolus injection into the perfusate inflow, and at the doses used all produced an increase in PAP similar to that evoked by hypoxia (ventilation with 2.5% O_2). Pulmonary ROS production, assessed using luminol and lucigenin, was decreased by both rotenone and antimycin. Both blockers also abolished HPV. The fall in lung ROS production caused by hypoxia was strongly attenuated by pre-treatment with antimycin, but was unaffected by rotenone (*n.b.* this appears to be inconsistent with later evidence from this laboratory [125] that HPV is due to decreased ROS production by complex I). Cyanide caused a dose-dependent increase in PAP comparable to that caused by rotenone and antimycin, but unlike these blockers had no effect on HPV and caused a transient increase in lucigenin luminescence. Both of these responses were depressed by SOD, suggesting that they were superoxide-dependent. The K^+ current they recorded in PASMC was strongly blocked by both TEA and 4-AP and was attenuated by hypoxia. The current was also reversibly suppressed by rotenone and antimycin but was not significantly affected by this concentration of cyanide.

In discussing their findings, the authors noted that whereas Rounds & McMurtry [280] had previously highlighted the resemblance between the effects of hypoxia and ETC blockers on pulmonary artery contraction and had speculated that hypoxia might causing a pressor response in PA by depleting ATP, this seemed unlikely in light of evidence that high energy phosphate levels in PASMC are little changed by hypoxia [281–283]. They proposed instead that rotenone and antimycin were causing PA contraction by suppressing mitochondrial ROS production and pointed out that effects of these drugs on PAP, ROS production, and the PASMC K^+ current resembled those of hypoxia. The ability of both ETC blockers to abolish HPV also suggested that they were acting *via* the same mechanism as hypoxia. The observation that cyanide, which did not diminish ROS, had no effect on either HPV or the K^+ current, also aligned with the idea that a fall in ROS was linked to K^+ channel inhibition and contraction. They therefore concluded that that hypoxia was causing HPV by decreasing mitochondrial ROS production (or causing a reducing shift in cell redox couples) which led to a decreased opening of voltage-dependent K^+ channels. These papers established the basic outline of the Redox theory, which was then set forth in detail in a review by Weir and Archer in 1995 [36].

Observations that PA constrict in response to hypoxia, whereas systemic arteries dilate (e.g. [160]) could potentially be explained by differences in either the O_2 sensor or the mediators/effectors

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to which it is coupled. Michelakis et al [145] therefore examined whether the hypoxia-induced fall in ROS which had been observed in PA also occurred in small renal arteries, and whether mitochondria from the SMC of these responded differently to hypoxia.

They observed that both hypoxia (pO_2 ~40Torr) and rotenone inhibited the whole cell K^+ current in rat PASMC, whereas they increased its amplitude in renal artery smooth muscle cells (RASMC). Likewise, both stimuli increased PAP in isolated lungs, but reduced the arterial pressure in perfused kidneys. Lucigenin-enhanced luminescence was >20 times higher in isolated PA compared to RA under normoxic conditions. Hypoxia decreased luminescence by ~80% in PA but increased it about 5-fold in RA, and rotenone had a similar effect. Utilizing Amplex Red or 2',7'-dichlorodihydrofluorescein (DCFH) as indicators, they found that the level of H_2O_2 under normoxic conditions was higher in PA than in RA. The H_2O_2 signal was decreased in PA by hypoxia and antimycin, although it was not increased by either hypoxia or rotenone in RA. Cyanide had no effect on the DCFH signal in either artery.

In further experiments, they characterized mitochondria isolated from homogenates of whole lung (LM) and kidney (KM), using glutamate and succinate to support respiration. In line with their observations in the arteries, ROS production, assessed using lucigenin, was higher in LM vs KM. ROS production was inhibited by antimycin but not cyanide in LM, and neither drug had an effect in the KM.

Using Western blotting, they found higher expression of complexes I and 3 in RA compared to PA, and pointed out that this was consistent with their additional observations that O_2 consumption, indicative of respiration, was greater in KM compared to LM, and that the mitochondrial membrane potential ($\Delta\psi_m$), assessed using JC-1 and TMRM, was more hyperpolarized in RASMC than in PASMC. Moreover, hypoxia increased $D\psi_m$ in PASMC but had the opposite effect in RASMC. Based on their findings that glutathione levels and Mn SOD protein expression were substantially higher in the PA compared to RA, which was consistent with an adaptation of PASMC to the relatively O_2-rich pulmonary milieu, the authors also proposed that PASMC are more oxidized than RASMC, although this conclusion is questionable because the concentrations of oxidized and reduced glutathione, which reflect the cytoplasmic redox potential, were not reported.

The differential effect of hypoxia on the lucigenin vs DCFH/Amplex Red signals in RASMC led the authors to suggest that hypoxia increases superoxide, but not H_2O_2, in these cells, possibly due to the low expression of MnSOD in RASMC. However, using the genetically encoded H_2O_2 indicator HyPer this laboratory subsequently observed that hypoxia increased cytoplasmic H_2O_2 in these cells [125], suggesting perhaps that Amplex Red and DCFH were sensing ROS other than H_2O_2 in their experiments.

Notably, the physiological significance of these results is uncertain, since whereas the physoxic PO_2 for mesenteric arteries is much lower than it is for pulmonary arteries, there is no indication in the paper that this was taken into account in designing the experiments.

Wu et al [284] used dihydroethidium (DHE) in the experiments (although the 'normoxic' PO_2 was not stated, a hypoxic PO_2 of 40 Torr was used in preparations from both arteries) to compare the effect of hypoxia (pO_2 ~30 Torr) on ROS production by cultured human smooth muscle cells from PA and coronary artery (CASMC). In accord with the Redox theory, they found that hypoxia decreased superoxide levels in PASMC. However, hypoxia also caused a similar fall in DHE oxidation in CASMC. The authors pointed out that coronary arteries dilate to hypoxia, and that the fall in superoxide in both types of cells took several minutes to occur whereas HPV in lungs develops within seconds. This led them to speculate that ROS may not be involved in HPV, or that the opposite contractile response of pulmonary and coronary arteries to hypoxia might be due to differences in the effector mechanisms coupled to ROS. Similar observations were made by Mehta et al [285], who found that moderate hypoxia (5% O_2) significantly decreased ROS production in both primary cultured human PASMC and coronary artery SMC. This effect was seen with three indicators (DCFH, DHE and Amplex Red). The fall in ROS showed a (non-significant) trend to be more substantial in the PASMC with each indicator.

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In 2019, Dunham-Snary and colleagues [125] published a study of the role of ROS in HPV in rats and mice which included a further comparison of the properties of pulmonary and renal artery mitochondria. This paper presents the most recent and comprehensive evidence currently available to support the Redox theory and its results relating to the acute effects of hypoxia will therefore be considered in detail. The authors incubated isolated LM and KM at a concentration of ~2.5 mg protein/ml in physiological medium for 15 minutes under normoxic or hypoxic conditions. Using Amplex Red, they measured the H_2O_2 concentration in the conditioned media, finding that under normoxic conditions this was much higher in medium conditioned with LM (~1 μM) compared to KM (<0.2 μM). These results were consistent with their earlier findings [145].

Micropolarimetry experiments were carried out to compare the O_2 consumption rate in cultured PASMC and RASMC, finding that it was about twice as high in the former. Since mitochondria contribute the vast bulk of cellular O_2 consumption, this appears to contradict their earlier observation that the rate of respiration in isolated LM was about a third of that in KM [145]. The authors did not discuss possible reasons for these divergent results, but it is possible that they may have been due to cellular influences on mitochondrial function which would be absent in isolated mitochondria. If so, this suggests that effects of hypoxia on isolated mitochondria cannot simply be extrapolated to more intact preparations [53,286].

Isolated PSS- perfused rat lung was used to examine the effect of oxidants on HPV evoked using perfusate gassed with 2.5% O_2. Injection of 0.1 mL of LM-conditioned medium into the perfusion inflow caused a rapid and marked reversal of HPV (by 56%), whereas injection of KM-conditioned medium, or medium conditioned with LM in the presence of catalase, had no effect on HPV. Perfusion with PSS containing 100 μM t-BOOH also reversed HPV (by 75%), although 10 μM t-BOOH had a negligible effect.

Cultured rat PASMC and RASMC were transfected with HyPer-dMito or HyPer-dCyto to monitor H_2O_2 levels in the mitochondria and cytoplasm, respectively [287]. In PASMC, hypoxia (pO_2 ~20 Torr) decreased H_2O_2 levels in both the mitochondria and cytoplasm. In contrast, hypoxia had no effect on mitochondrial ROS and increased cytoplasmic ROS in RASMC. Hypoxia also increased the $[Ca^{2+}]_{cyt}$ in PASMC. This rise in $[Ca^{2+}]_{cyt}$ was completely reversed by 1 mM t-BOOH and subsequently restored by application of catalase. The effects of hypoxia on $[Ca^{2+}]_{cyt}$ and mitochondrial [ROS] were mimicked by rotenone, which also diminished both the concentration of H_2O_2 in PASMC-conditioned medium and the reversal of HPV induced by this medium. Taken together, these results support the idea that mitochondria in PASMC produce more ROS than those in RASMC under normoxic conditions, that hypoxia causes a fall in ROS production which increases PASMC $[Ca^{2+}]_i$, and that reducing and oxidizing stimuli cause changes in $[Ca^{2+}]_{cyt}$ in the cells which are consistent with the Redox theory, as does block of the proximal ETC with rotenone.

Ndufs2 is a subunit of complex I which plays an important role in the binding and reduction of ubiquinone by complex I [288,289,290,291]. It had been demonstrated [179] that conditional knockout of Ndufs2 in CBCC prevented activation of the carotid body by hypoxia, leading Dunham-Snary et al [125] to investigate its involvement in HPV. They found that the mRNA expression of Ndufs2 was higher in PASMC than in RASMC, as was its protein expression (normalized to TOMM20, another mitochondrial protein). Moreover, complex I in PASMC was enriched in Nduf2s compared to RASMC.

siRNA knockdown of Ndufs2 in PASMC inhibited respiration and increased cellular levels of NADH, consistent with its role in the enabling of electron flow in the ETC. Ndufs2 knockout was not associated with a fall in the quantity of complex I, but its activity, assessed using protein immunocaptured from cell homogenates, was depressed. Knockdown of Ndufs2 also abolished the hypoxia-induced rise in $[Ca^{2+}]_{cyt}$ and decreased the release of H_2O_2 by LM. Importantly, the increase in both mitochondrial and cytoplasmic ROS evoked in PASMC by hypoxia was also decreased. In contrast, siRNA knockdown of Uqcrfs1, the protein containing the Rieske Fe-S cluster (RISP) in complex III, or of COX4i2, a Cox subunit proposed to contribute to O_2 sensing [130] did not prevent these effects. Knockdown of Ndufs1, a protein in the N-domain of complex I, was also without effect.

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In further experiments, siRNA directed against Ndufs2 or control siRNA was administered once to rats *via* airway nebulization. After 48 hours, compared to the controls, the si-Ndufs2 rats demonstrated diminished levels of Ndufs2 mRNA in lung homogenates and exhibited a profound fall in Ndufs2 levels co-localizing with smooth muscle actin, as revealed by immunofluorescence studies of small PA in lung sections. The animals were anesthetized and PAP pressure was measured *in vivo* under normoxic and hypoxic (10% O_2) conditions. PAP under normoxic conditions was not different in the two groups, but hypoxia caused a much smaller increase in PAP in the si-Ndufs2 rats compared to the controls. Similarly, treatment of mice with Ndufs2 siRNA over a period of more than a week had no effect on basal PAP, but profoundly depressed HPV, compared to animals treated with control siRNA. Likewise, the increase in PAP evoked by rotenone in the controls was almost absent in the si-Ndufs2 rats, whereas the response to phenylephrine was enhanced in the latter group. This may have been due to the down-regulation of $K_V1.5$ which also occurred in the si-Ndufs2 animals. They also found that exposing mouse lungs *in vitro* to hypoxia for 30 minutes increased the ratio of reduced to total Ndufs2. 289Hypoxia also inhibited complex I activity, an effect mimicked by treatment with the reducing agent dithiothreitol (DTT). They speculated that this might be due to the reduction of the mitochondrial milieu caused by hypoxia.

Based on the specific inhibitory effect of effect of Nduf2s knockdown on H_2O_2 production and the rise in $[Ca^{2+}]_{cyt}$ evoked by hypoxia, as well as their evidence that hypoxia caused a fall in cytoplasmic H_2O_2 and that HPV was reversed by oxidants, the authors proposed that HPV was triggered by a fall in ROS production which they suggested was due to inhibition of the production of uncoupled electrons (i.e. electrons leaking from the ETC) at Ndufs2.

In accordance with the concept that Ndufs2 is involved in producing ROS in an PO_2-dependent manner, a subsequent paper from this laboratory [292] reported that the I_Q site, in which Ndufs2 plays a vital role in the transfer of electrons from complex I to ubiquinone [289], is responsible for the increase in ROS production associated with a rise in PO_2 in the ductus arteriosus.

A letter by Hüttemann et al [293] criticized the conclusion that COX4i2 was not involved in HPV on the basis that its knockdown in the Dunham-Snary study, which amounted to ~55%, was insufficient to significantly affect its role in O_2 sensing. Their contention was based on their unpublished finding that HPV was largely intact in $COX4i2^{+/-}$ mice, whereas it was abolished in homozygous knockouts [130].

In a response letter, Dunham-Snary & Archer [294] expressed their belief in the validity of their findings, and criticized the study by Hüttemann and colleagues [130] based on its use of a very low pO_2 to elicit HPV and on the small amplitude of the HPV they obtained. They also noted that their conclusion that Ndufs2 was crucial in O_2 sensing in PASMC was consistent with the earlier demonstration by Fernandez-Aguera et al [179] that it was the O_2 sensor in CBCC, supporting the concept that common O_2 sensing mechanisms are operative in various oxygen sensing tissues [271]. However, they failed to mention that the proposed role of Ndufs2 in O_2 sensing in CBCC [179] was opposite to theirs, since its knockdown prevented a hypoxia-induced increase in mitochondrial ROS production, and the resulting suppression the K^+ current, in these cells. Also, K^+ currents were inhibited by oxidants (H_2O_2 and diamide) and increased by the putative antioxidant N-acetyl cysteine. Based on subsequent work [180], it was proposed that hypoxia increases ROS production in CBCC by causing reduction of the ubiquinone pool, thereby lengthening the dwell-time of electrons at the Ndufs2 quinone binding site and increasing the probability that they will react with O_2 at this point. It has also been reported that an increase in ROS production dependent on NDUF2.1, the *C. elegans* ortholog of Ndufs2, is responsible for the locomotor response to hypoxia in these organisms [295]. Similarly, Hernansanz- Agustin et al [184] presented evidence that whereas hypoxia caused a marked increase in cytoplasmic $[H_2O_2]$, which they measured using HyPer, in bovine aortic endothelial cells transfected with scrambled siRNA, the opposite effect occurred if Ndufs2 was knocked out. They also found that Ndufs2 knockout increased the basal (normoxic) cytoplasmic $[H_2O_2]$.

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In considering the results reported by Dunham-Snary et al [294], it is perplexing that reversal of HPV by the LM-conditioned medium was of similar in magnitude to that exerted by much higher concentrations of the membrane permeable H_2O_2 t-BOOH. In these experiments. 0.1 mL of the conditioned medium, which had a H_2O_2 concentration of ~1 μM, was injected into the lung inflow line of the perfusion system, which had a volume of 40 ml and was being continually recirculated at a flow rate of ~0.25 mL/s. The conditioned medium would have been diluted rapidly upon injection and the concentration of H_2O_2 in the perfusate would have very quickly fallen into the low nM range during the 5 minute period over which HPV was progressively reversed. In comparison, application of concentrations of the membrane permeable H_2O_2 analog t-BOOH which were ≥4 orders of magnitude higher were required to cause a comparable reversal of HPV (EC_{50} > 50 μM). In contrast, in a study the which directly compared the vasorelaxing effects of H_2O_2 and t-BOOH (carried out using PGF_{2a} – constricted rat aorta) these oxidants evoked vasorelaxation with very similar concentration-dependencies; with an EC_{50} for both of ≈ 20 μM, [296]. Similarly, 1 μM t-BOOH and H_2O_2 caused identical levels of oxidation when applied to BY4742 yeast cells [297].

The results and implications of the experiments in which protein components of the mitochondrial complexes were knocked down can also be questioned. Ndufs2 knockdown depressed respiration, indicative of a decrease in the activity of the ETC, and also has been shown to cause additional and widespread effects on mitochondrial function, including the depolarization of DΨm [298]. This may occur because the reduction of CoQ, which is thought to initiate proton translocation by complex I, requires electrons donated by an aspartate-histidine pair in the b_1-b_2 loop of Ndufs2, [299]. A change in the configuration of Ndufs2 may also play a role in the active to de-active transition of complex I which was proposed [183] to mediate the increase in ROS responsible for HPV [207]. It is therefore difficult to interpret the effect of Ndufs2 knockdown on O_2 sensing in PASMC.

It is also curious that the knockdown of Ndufs1 and Uqcrfs1 apparently had no effect on the response to hypoxia in PASMC. Ndufs 1 is one of the 14 catalytic-core subunits required for complex I to function, and contains three (N1b, N4, N5) of the seven Fe-S clusters which form a 'wire' carrying electrons from the NADH binding pocket to the ubiquinone binding site [300]. Since Ndufs1 is upstream of N2, the Fe-S cluster with which Ndufs2 interacts, the lack of effect of its knockdown on the response to hypoxia would also appear to rule out a role for Ndufs2 in regulating ROS production at either I_Q or I_F. More generally, knockout of either Ndufs1 or Uqcrfs1, which is required for electron flow from Q_o to cytochrome *c* and thence to Cox and O_2, should suppress mitochondrial respiration and depolarize DΨm, thereby presumably causing the dysfunction of any redox-mediated mitochondrial O_2 sensing mechanism [301,302].

5.1.2. The Involvement of ROS in PASMC K^+ Channel Inhibition During Hypoxia

The Redox theory posits that membrane depolarization due to the hypoxia-induced closure of K_V channels, particularly those incorporating the K_V1.5 subunit, is a primary effector mechanism for HPV, and that this inhibition is mediated by a fall in [H_2O_2] and/or an increase in [NAD(P)H] in the cytoplasm.

The concept that K_V channel closure contributes to HPV is supported by abundant evidence [17,279,303–313], although other processes, including intracellular Ca^{2+} release, store-operated Ca^{2+} entry (SOCE), and Ca^{2+} sensitization are also important [3,10,12,14,305], and additional depolarizing mechanisms (e.g. TASK-1; non-selective cation channels) [314–316] have been identified.

On the other hand, the proposal that this inhibition of K_V channels during HPV requires a reduction of the cytoplasmic redox balance remains controversial. In keeping with the focus of this review on the O_2 sensing rather than effector mechanisms of HPV, and because the question of how K_V channels in PASMC are regulated by redox mechanisms has been critiqued extensively [3,317–319], but seems not to have moved any closer to being resolved over the past decade, we will consider this issue only briefly.

A number of laboratories have investigated the redox regulation of K_V channels in PASMC by monitoring whole cell or membrane patch currents while applying oxidants, reductants, or ETC

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blockers, which were used to decrease mitochondrial ROS production. Both rotenone and antimycin (10mM) were shown to block the K^+ current in PASMC [145,263]. The effect of rotenone is in accordance with Redox theory, although that of antimycin is difficult to interpret, since it is generally thought to increase mitochondrial ROS production [109]. A more detailed study [320] showed that although antimycin, myxothiazol, and rotenone depressed the Kv current in PASMC at positive potentials, they caused a hyperpolarizing shift in its activation threshold, implying that these blockers should open rather than shut Kv channels in the physiological range of membrane potentials. The authors provided evidence that the ETC blockers were exerting these effects on the Kv current by increasing the cytoplasmic cellular [Mg^{2+}]. This could be due to mitochondrial depolarization, an effect exerted by all ETC inhibitors, which could explain why both rotenone and antimycin inhibited the current at positive potentials. To complicate matters further, both rotenone and antimycin inhibited the K^+ current with IC_{50} values of ~1mM in H146 (small cell lung carcinoma) cells by a mechanism which was independent of the mitochondria [321].

The effects of reductants and oxidants on K^+ currents are summarized in Tables 1 and 2, respectively. The majority of studies produced evidence supporting the viewpoint of the Redox theory with regard to the redox regulation of PASMC Kv channels by hypoxia. However, other investigations have reported observations inconsistent with this view [130,320,322,323] and there is also evidence that hypoxia-induced Kv channel inhibition is mediated by a rise in $[Ca^{2+}]_{cyt}$ due to Ca^{2+} release from the sarcoplasmic reticulum [324,325]. Moreover, LY83583, which causes intracellular superoxide production, increased the Kv current in the physiological range of membrane potentials in PASMC, as predicted by the Redox theory, but caused PA constriction rather than relaxation [326]. This suggests that the contractile response of these cells to alterations in the cytoplasmic redox state might not depend only on changes in Kv channel activity.

Yandjue et al [253] recently showed that the effect of H_2O_2 on the Kv1.5 current expressed in oocytes is strongly dependent, not only on its concentration, but on the potential at which the current is evoked. Application of low concentrations of extracellular [H_2O_2] (0.01 – 100 μM), a range which is likely to cause physiologically relevant variationsin its cytoplasmic concentration (see Section 4), tended to not affect or inhibit the current at -20 mV, whereas it was increased by concentrations above 1 mM. Although the implications of these results for the redox regulation of Kv1.5 during HPV remain unclear, the observations made in this paper highlight the need to study the effect of redox mechanisms on channel activity during using conditions which more realistically simulate the effects of hypoxia on specific redox couples and oxidant concentrations. More generally, little is known at the molecular level about the redox regulation of the Kv1.x and Kv2.X isoforms thought to play a role in HPV, and the results of the few studies which have examined this in more depth [327, 267, 328] suggest that an accurate determination of how redox mechanisms (and other factors such as $[Ca^{2+}]_{cyt}$ or channel phosphorylation [44,324,329] contribute to Kv channel closure during HPV will require a more refined and concerted approach to unravelling the effects of hypoxia and redox agents on these channels than has previously been attempted. It will be particularly important to characterize the effects of hypoxia and perturbations on specific redox couples on Kvb-subunit function, the oxidation of cytoplasmic cysteine residues on the Kv1.5α subunits, and the membrane trafficking of Kv channels (which can change rapidly [327,330]) in native PASMC.

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Table 1a. Effects of oxidants on K^+ currents in PASMC.

Source of PASMC	Oxidant (in μM)	Current	Effect on K^+ current	Comments	Refs.
rabbit intralobar PA	GSSG (5000)	Single channel BK_{Ca}	P_{open} ↑by 450%		[331]
4th & 5th order rat PA	diamide (100)	Whole cell K^+	~120% ↑	Current activated by diamide was very noisy (i.e. mainly BK_{Ca}?)	[332]
rabbit intralobar PA	GSSG (5000)	Single channel BK_{Ca}	P_{open} ↑by 455%		[333]
rabbit intralobar PA	DTNB (1000)	Single channel BK_{Ca}	P_{open} ↑ by 504%		[333]
rabbit intralobar PA	DTNB (200)	Whole cell K_V	↑ ~260% at +20 mV	Effect of DTNB shown to be intracellular	[334]
rabbit fetal resistance PA	DTNB (1000)	Whole cell K_V	↑ ~70% at +70 mV	K^+ current not identified but appearance (low noise) suggests that it was entirely or predominantly K_V	[335]
rat small PA	GSSG (2000)	Whole cell K^+	↑ ~40% at +50 mV in hypoxic cells	Basal and enhanced K^+ currents not identified, but low noise suggests they were K_V	[36]
rat small PA (cultured)	H_2O_2 (50)	Whole cell K_V	↑ by ~55% at +60 mV	BK_{Ca} and K_{ATP} currents blocked to isolate K_V	[336]
rat small PA (cultured)	diamide (100)	Whole cell K_V	↑by ~45% at +60 mV	BK_{Ca} and K_{ATP} currents blocked to isolate K_V	[336]
rat 2nd & 3rd order PA	t-BOOH (5)	Whole cell K_V	↓by 30% at +60 mV	Similar inhibition of K_V with 100μM t-BHP implies that block was maximal at 5μM. $[Ca^{2+}]_i$ strongly buffered to block BK_{Ca}, thereby isolating K_V.	[322]
rat 3rd & 4th order PA	LY83583 (10)	Whole cell K_V	activation shifted ~11mV in hyperpolarizing direction	BK_{Ca} and K_{ATP} currents blocked to isolate K_V. Shift in activation reflects increased K_V current amplitude at physiological membrane potentials.	[326]
rat PA 0.2 - 0.5 mm dia.	t-BOOH (10)	Whole cell K_V	↓ by ~35% at +60 mV	$[Ca^{2+}]_i$ strongly buffered to block BK_{Ca}, thereby isolating K_V.	[323]
Non-passaged cultured PASMC from mouse precapillary PA	H_2O_2 (0.124)	Whole cell K_V	↓ by ~60% at +60 mV	BK_{Ca} and K_{ATP} currents blocked to isolate K_V	[130]

Table 1b. Effects of reductants on K^+ currents in PASMC.

Source of PASMC	Reductant (Conc. in mM)	Current	Effect on K^+ current	Comments	Refs.
rat main and 1st/2nd order PA (cultured)	GSH (10)	Whole cell K_V	~25% decrease in steady-state K^+ current, 16 mV depolarization	10 mM EGTA in pipette solution to suppress BK_{Ca} current, thereby isolating K_V current	[337]
rabbit small intralobar PA	GSH (5)	Single channel BK_{Ca}	P_{open} variably decreased	GSH applied to cytoplasmic face of the membrane	[331]
rat small PA	GSH (2)	Whole cell K^+	~40% ↓ at +70 mV	K^+ current not identified but low noise suggests it was K_V	[36]
rat 4th & 5th order PA	Co-enzyme Q (0.1)	Whole cell K^+	~40% ↓ at +40 mV	K^+ current not identified but low noise suggests it was K_V	[332]
rat 4th & 5th order PA	Duroquinone (0.1)	Whole cell K^+	~40% ↓at +40 mV	K^+ current not identified but low noise suggests it was K_V	[332]
rabbit small intralobar PA	DTT (5)	Single channel BK_{Ca}	19% ↓ in P_{open}	DTT applied to cytoplasmic face of the membrane	[333]
rabbit small intralobar PA	GSH (5)	Single channel BK_{Ca}	34% ↓ in P_{open}	GSH applied to cytoplasmic face of the membrane	[333]
rabbit small intralobar PA	DTT (5)	Whole cell K_V	~30% ↓ at +20 mV	10 mM BAPTA in pipette solution to suppress BK_{Ca} current, thereby isolating K_V current	[334]
rat PA	GSH (2)	Whole cell K^+	~50% ↓ at +70 mV	GSH applied in pipette solution K^+ current not identified but low noise suggests it was K_V. (n=1) no statistical analysis	[338]
rat PA	GSH (5)	Single K^+ channels, not identified	~70 % ↓ in P_{open}	(n=1) no statistical analysis GSH applied in pipette solution	[338]
Rabbit fetal resistance PA	DTT (3)	Whole cell K^+	33% ↓ at +50mV ~20% ↓ at -10mV	K^+ current not identified but low noise suggests it was K_V	[335]
Rabbit adult resistance PA	DTT (3)	Whole cell K^+	↓19% at +50mV	K^+ current not illustrated or ascribed to any specific type(s) of channel	[335]
rat 2nd & 3rd order PA	Catalase (100U/ml)	Whole cell, predominantly or entirely K_V	Hypoxia ↓ the K_V current by 33%. This effect was prevented by including catalase in the pipette solution.	Result provides indirect evidence that hypoxia inhibited the K_V current by raising the cytoplasmic H_2O_2 concentration. 10 mM EGTA in pipette solution to suppress BK_{Ca} current, thereby isolating K_V current	[323]

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n.b. Except where noted, PASMC for these studies were freshly isolated on the day that currents were recorded.

5.2. The Mitochondrial ROS Theory of HPV

The proposal that HPV is caused by a fall in cellular [ROS] was challenged by Marshall et al [339], who reported that hypoxia increased ROS in cultured PASMC from fetal calves, and by Killilea et al [340], who made a similar observation in cultured PASMC from rats. Abdalla & Will [341] had also observed that HPV in isolated guinea pig PA was enhanced by two SOD inhibitors (DETCA and TETA), and suggested that HPV was due to a rise in cellular superoxide levels.

Whereas Marshall et al ascribed their results to an activation of Nox, Paul Schumacker and colleagues proposed in 2001 that hypoxia promotes ROS production by PASMC mitochondria, leading to a rise in cytoplasmic [ROS] that results in the stimulation of redox-sensitive effector pathways which cause HPV [38]. This concept grew out of observations by Schumacker's laboratory that hypoxia increased mitochondrial ROS production in cardiac myocytes, leading to stabilization of HIF1α [342], and in HEP3b cells, stimulating the expression- of erythropoietin, glycolytic enzymes, and vascular endothelial growth factor [343].

This 'Mitochondrial ROS hypothesis' was supported and extended in subsequent papers from this and other laboratories, several of which have presented evidence that the site of ROS production is complex III. It has also been proposed that the increase in mitochondrial ROS leads to additional ROS production by Nox which enhances the pressor response to hypoxia (see Section 5.2.5). A schematic of the mitochondrial ROS hypothesis which has grown out of the investigations described in Section 5.2 is shown in Figure 7.

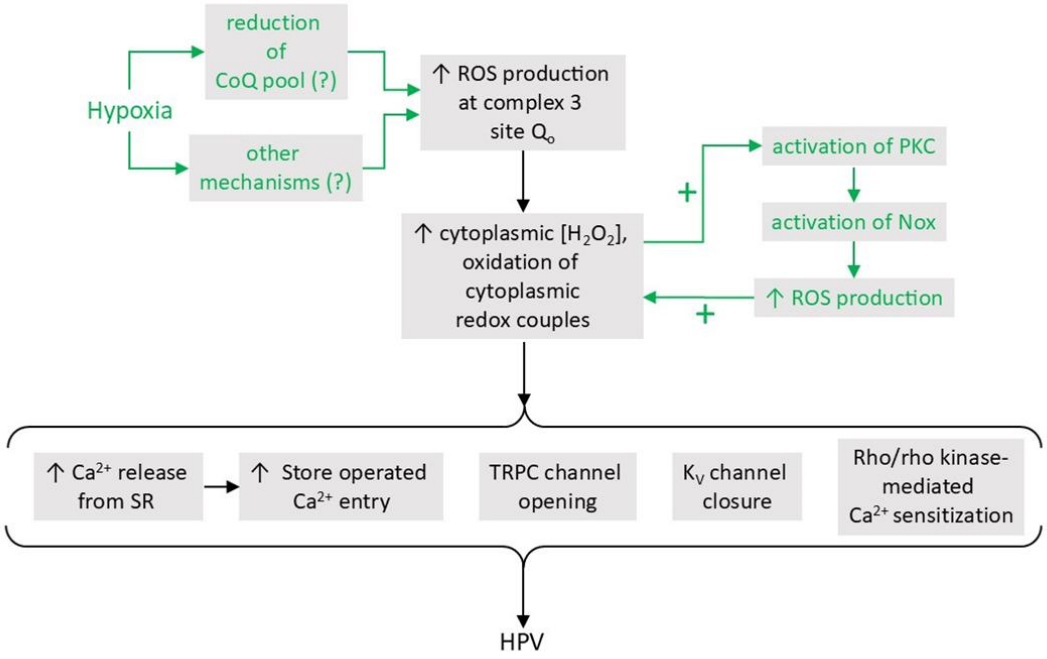

Figure 7. The mitochondrial ROS hypothesis of HPV. According to this proposal, hypoxia causes an increased ROS production in the intermembrane space by complex III. This leads to a cytoplasmic ROS signal which causes contraction by by acting on several HPV effectors to increase SR Ca^{2+} release, Ca^{2+} influx and Ca^{2+} sensitization (see Section 5.2.5 for a brief discussion of these effectors and references supporting their ROS-sensitivity. A comprehensive description of the evidence implicating these effectors in HPV can be found in [3].

Waypa et al [38] studied HPV recorded in isolated ventilated rat lung and microvascular PASMC in primary culture. Hypoxia was imposed by reducing the [O_2] in the ventilating gas from 21 to 2%,

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and angiotensin 2 was used to create pretone. HPV was abolished by rotenone, and myxothiazol, which blocks electron transport in complex III at the ubiquinol oxidation site (Q_o), had a similar effect. Conversely, I ng/mL antimycin, which binds to complex III more distally at the Q_i site where cytochrome *b*562 is oxidized, had no effect on HPV, and cyanide augmented the amplitude of HPV. All of the ETC blockers except myxothiazol caused a transient contraction.

Pyrrolidinedithiolcarbamate (PDTC), an antioxidant said to enhance H_2O_2 clearance by reducing oxidized glutathione, reversibly attenuated HPV by 65% and blocked hypoxic contraction of PASMC. The glutathione peroxidase mimetic ebselen caused a profound and irreversible block of HPV, and diethyldithiocarbamate (DDC), a cytosolic Cu,Zn SOD blocker, inhibited HPV by 68%. Application of DIDS, an anion channel blocker used here to prevent mitochondrial superoxide from entering the cytoplasm through the mitochondrial inner membrane anion channel (IMAC), inhibited HPV to a similar extent, and also blunted the contraction caused by antimycin. At the concentrations used in these experiments, none of these blockers affected the pressor response to the TXA_2 agonist U46619, which was used as a negative control to detect non-specific effects on contraction. In contrast, 3μM apocynin abolished both HPV and the U46619 contraction, whereas it had no effect on either response at a lower concentration (0.3μM). Waypa et al [38] also found that hypoxia caused a largely reversible increase in the release of ROS from PASMC (monitored using DCHF-DA), which was attenuated by myxothiazol. In addition, incubation of PASMC in ethidium bromide for two weeks to deplete cellular mitochondria [261] diminished HPV by 70%.

Based on these results, the authors proposed that hypoxia induces an increase in mitochondrial ROS production at a site upstream of where antimycin binds to complex III, and that this was leads to an increase in the cytoplasmic concentration of H_2O_2 which acts through several effector mechanisms to cause HPV. The authors pointed out that drugs acting distal to ubisemiquinone, including antimycin, tend to increase ROS during normoxia by increasing the reduction of the ubiquinone pool [344], whereas more proximal block would suppress ROS production by oxidizing this pool. The lack of HPV in ρ^o cells, and the inability of apocynin to exert a selective block of HPV supported the idea that ROS produced by the mitochondria rather than Nox were regulating HPV (*n.b.* however, apocynin is not a dependable blocker of Nox; [345]). Based on the block of HPV by DIDS, they also suggested that hypoxia might alternatively increase the entry of superoxide into the cytoplasm by opening IMAC, although in subsequent papers this idea was not pursued.

Leach et al [346] employed a pharmacological approach to evaluate the role of the mitochondrial complexes in HPV using isolated rat intrapulmonary arteries (IPA). They observed that the ETC blockers rotenone, myxothiazol, cyanide and the complex II substrate succinate had no effect on basal tone under normoxic conditions. Rotenone caused an increase in NAD(P)H (assessed by measuring tissue autofluorescence) similar in amplitude to that induced by anoxia. This was not reversed by application of 5 mM succinate, consistent with a complete block of the ETC at complex I by rotenone. Hypoxia (PO_2 of 15-18 Torr) applied to IPA which were slightly pre-constricted with PGF_{2a} caused a biphasic HPV, as typically observed in this preparation under these conditions. Application of either rotenone or myxothiazol abolished both phases of HPV. Importantly, rotenone did not block either phase of HPV if it was co-applied with succinate, whereas succinate did not reverse the inhibition of HPV by myxothiazol. These results indicated that HPV in these arteries required the provision of electrons to the myxothiazol binding site (i.e. Q_o) of complex III, which succinate would be able to restore in the presence of rotenone but not myxothiazol. The authors pointed out that whereas this effect of succinate was consistent with the concept that HPV was caused by an increase in ROS production by the ETC distal to this site, it was difficult to explain if hypoxia was causing HPV by mimicking the effect of rotenone and inducing a decrease in mitochondrial ROS production, as proposed by the Redox theory. The lack of effect of rotenone on basal tension was also inconsistent with the Redox model. Subsequent studies have shown that succinate can also increase ROS production by causing reversed electron transport (RET) through complex I [100,179]. Although this would have been favored by the combination of succinate and myxothiazol, which should highly reduce the ubiquinone pool [177], it seems unlikely that ROS production by RET could explain the

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restoration of HPV by succinate in this study, since this would have been prevented by rotenone [177,347].

Waypa et al [348] investigated the effect of ETC blockers and other agents on the hypoxia-induced rise in $[Ca^{2+}]_{cyt}$ in PASMC using fura 2. Exposure to medium equilibrated with 1.5% O_2 caused a sustained rise in $[Ca^{2+}]_{cyt}$ recorded over 9 minutes. This was attenuated by DPI (here used to block complex I), rotenone and myxothiazol, but was unaffected by antimycin and cyanide; none of these drugs significantly affected the increases in $[Ca^{2+}]_{cyt}$ evoked by angiotensin 2 or 50 μM H_2O_2. In PASMC in which catalase had been overexpressed by recombinant adenovirus transduction, the responses to hypoxia and exogenous H_2O_2, but not angiotensin 2, were diminished compared to those in control cells overexpressing LacZ. Application of cyanide caused a rise in $[Ca^{2+}]_{cyt}$ which was antagonized by myxothiazol and was smaller in PASMC overexpressing catalase, but not lacZ. These results provided further support for the model illustrated in Figure 7. The contrasting effects on HPV of the proximal (rotenone, DPI and myxothiazol) as compared to distal (antimycin and cyanide) ETC blockers led them to propose that mitochondrial ROS production at the ubisemiquinone site in complex III was crucial for triggering the rise in $[Ca^{2+}]_{cyt}$ during HPV. The observation that HPV was blocked by catalase overexpression suggested that H_2O_2 rather than superoxide was the important ROS for HPV, consistent with the idea that H_2O_2 is generated from superoxide by MnSOD in the intermembrane space of the mitochondria, and then crosses the outer membrane to access the cytoplasm.

Weissmann et al [349] carried out a comprehensive study of the effects on HPV of blockers of complexes 1-3, as well as mitochondrial uncouplers which should decrease ROS production. The aim of the investigation was to test the predictions of the Mitochondrial ROS model as set out by Waypa et al [38]. In this and other investigations carried out by this laboratory [144,350–352], HPV was recorded in isolated perfused rabbit lungs which were ventilated *via* the trachea and the PO_2 in the ventilating gas mixture was lowered from 21 to 3% for 10 minutes to reduce the alveolar PO_2 from ~160 to ~23 Torr and evoke the initial transient component of HPV (phase 1). The thromboxane A_2 agonist U46619 was used as a negative control, based on the assumption that the contraction it evokes should be resistant to ETC block, since it was not known to require mitochondrial ROS production. Experiments were repeated in the presence of L-NMMA to determine whether there were any confounding effects of the mitochondrial blockers which were being exerted due to interference with NO release. Whereas some of the results supported the Mitochondrial ROS theory (e.g. blocking complexes 1 and 2 antagonized HPV), others did not (e.g. antimycin did not cause a sustained contraction), allowing the authors to conclude only that mitochondria are somehow involved in phase 1 HPV during a brief hypoxic exposure. Importantly, they also observed that most of the blockers they studied strongly attenuated the response to U46619, and/or were interacting with NO system, suggesting that these drugs have off-target actions on contraction which could obscure the effects they exert on HPV by altering mitochondrial ROS production.

Liu et al [353] used perfusion myography to evaluate the role of ROS in HPV (evoked by exposing the arteries to pO_2 = 29 Torr) in 7th generation branches of porcine PA. HPV was abolished when the solution contained 150 U/ml SOD or SOD + 200 U/ml CAT. They used lucigenin and also EPR spectroscopy with the spin probe DMPO in an attempt to determine the effect of hypoxia on cellular ROS in the arteries, but although both approaches yielded results suggestive of an increase, in neither case were the results significant *(n.b.* the use of DMPO may also have been problematic, [354]). However, hypoxia (pO_2 34 Torr) did cause a rapid and largely reversible increase in DCFH florescence in primary cultured PASMC. This was strongly suppressed by SOD and completely prevented by both CAT and SOD + CAT, leading the authors to conclude that it was due to a rise in cellular oxidative stress rather than hypoxia-induced changes in DCFH leakage or metabolism. The authors confirmed, using FITC labelled SOD and CAT, that both enzymes were entering the cells. Notably, the observation that SOD and SOD + CAT similarly suppressed HPV suggested it was mainly an increase in cellular [superoxide] rather than [H_2O_2] which was causing contraction, a

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finding which appears to be inconsistent with the current view [32] that H_2O_2 rather than superoxide is the crucial ROS for cellular signaling.

Weissmann et al [352] compared the effects of ETC blockers on the 1st and 2nd phases of HPV in rabbit lungs. The complex I blocker MPP^+, applied at a concentration which slightly blocked phase 1, strongly blocked phase 2. Similar semi-selective effects on phase 2 were exerted by rotenone and cyanide. In contrast, 3-NPA (3-nitroproprionic acid; blocks complex III) and antimycin at the concentrations used caused a similar and marked suppression of both phases.

In view of the shortcomings of ROS indicators such as lucigenin, DCFH and luminol (see section 7.4), Guzy et al [355] developed a novel fluorescence resonance energy transfer probe, HSP-FRET, in order to evaluate the role of mitochondrial ROS in hypoxia-induced stabilization of HIF-1a. Their study, carried out in 143B cells, showed that application of exogenous H_2O_2 and hypoxia, but not NO, increased the FRET signal, and that stigmatellin (blocks the ETC at the Q_o site in complex III), suppressed the response to hypoxia. Their results suggested that HSP-FRET was able to detect H_2O_2, and that, at least in these cells, hypoxia increases the production of mitochondrial ROS, which then enters the cytoplasm.

This laboratory [356] then used HSP-FRET to determine whether hypoxia caused an increase in ROS in PASMC. They described HSP-FRET as being predominantly expressed in the cytoplasm, although specific evidence for this was not provided. In PASMC transfected with HSP-FRET, hypoxia (1.5% O_2) caused a gradual increase in ROS which started after ~3 minutes. A simultaneous increase in $[Ca^{2+}]_{cyt}$, assessed using fura 2 also occurred, and similar results were obtained in experiments using YC2.3, a FRET-based Ca^{2+} sensor. Hypoxia also decreased the GSH/GSSG ratio, considered to reflect the general cell redox state [357], from ~50 to ~30. The effects of hypoxia on oxidant signaling and $[Ca^{2+}]_{cyt}$ were suppressed by the antioxidants PDTC and N-acetyl-L-cysteine (NAC), neither of which affected the signal from either probe under basal conditions. Overexpression of glutathione peroxidise in order to deplete cytoplasmic H_2O_2 suppressed the effects of hypoxia on both $[Ca^{2+}]_{cyt}$ and oxidant signaling, as did overexpression of catalase in either the mitochondria or the cytoplasm. Conversely, overexpression of cytosolic Cu, Zn SOD (SOD 1) had no significant effect on the responses of HSP-FRET or YC2.3 to hypoxia, while overexpression of mitochondrial Mn SOD (SOD 2) similarly did not affect oxidant signaling and enhanced the $[Ca^{2+}]_{cyt}$ rise induced by hypoxia. In a subsequent paper from this group, overexpression of Prx5 in the mitochondrial IMS also was found to diminish the rise in $[Ca^{2+}]_{cyt}$ evoked by hypoxia (1.5% O_2 applied for 15 minutes) in these cells [358].

These results supported the concept that hypoxia causes an increase in cytoplasmic H_2O_2 and an oxidizing shift in the redox balance of PASMC resulting from mitochondrial ROS production. The observation that PDTC and NAC blocked the effects of hypoxia but did not themselves cause any response argued against the proposal of the Redox theory that cytoplasmic oxidation under basal conditions maintains vasorelaxation by causing an ongoing stimulation of K_V channels.

A study by Wang et al [359] which examined the role of mitochondrial ROS in the hypoxia-induced rise in $[Ca^{2+}]_{cyt}$ in freshly isolated mouse PASMC yielded results similar to those reported by Waypa and colleagues. They found that hypoxia (1% O_2 for 5 minutes) increased ROS levels (assessed using DCFH and lucigenin) and $[Ca^{2+}]_{cyt}$. Pharmacological blockade of complexes 1, 2 and the Q_o site of complex III did not affect the basal ROS or $[Ca^{2+}]_{cyt}$, signals, but strongly antagonized the effects of hypoxia. In contrast, block of complex III at the Q_i site with antimycin or at complex 4 with NaN_3 had no effect on ROS levels or $[Ca^{2+}]_{cyt}$ under either normoxic or hypoxic conditions. The effects of hypoxia on ROS levels and $[Ca^{2+}]_{cyt}$ in PASMC from mice overexpressing glutathione peroxidase or catalase were smaller than those observed in control mice, whereas they were enhanced in PASMC from glutathione peroxidase knockout mice. They also found that application of 51 μM H_2O_2 caused an increase in the DCFH signal similar to that evoked by hypoxia but resulted in a much smaller increase in $[Ca^{2+}]_{cyt}$. This led them to propose that mitochondrial H_2O_2 production was necessary but not sufficient to evoke the full hypoxia-induced increase in $[Ca^{2+}]_{cyt}$, or alternatively that this response might depend on the mitochondrial compartmentalization of ROS, which would not occur when

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peroxide was applied exogenously. A later investigation [360] similarly reported that hypoxia-induced increases in $[Ca^{2+}]_{cyt}$ in cultured human PASMC were prevented by blocking complexes 1, 2 and Q_o, but were unaffected by block of Q_i or complex IV, and that HPV in small PA from humans showed the same pattern of responsiveness to these drugs.

In order to determine whether hypoxia-induced redox signaling in PASMC was compartmentalized, Waypa et al [361] transfected PASMC with Ro-GFP, another genetically encoded ratiometric redox indicator. Ro-GFP expression was directed to the mitochondrial matrix (Mito-Ro-GFP) or the mitochondrial intermembrane space (IMS-Ro-GFP) by adding polypeptide targeting sequences to its structure; non-targeted Ro-GFP was used to assess the cytoplasmic redox state. Rather than providing a readout of $[H_2O_2]$, Ro-GFP fluorescence is sensitive to the redox state of the GSH/GSSG couple [362]. Because the Ro-GFP signal is ratiometric, it can be calibrated, allowing the degree of its oxidation to be calculated.

Hypoxia (1.5% O_2) caused a progressive increase in Cyto (non-targeted)-RoGTP oxidation from 19 to 35% over 30 minutes. IMS-Ro-GFP had a much higher level of oxidation under normoxic conditions (48%) but was still significantly oxidized (to 62%) by 30 min of hypoxia. In contrast, Mito-Ro-GFP, which was even more highly oxidized under normoxic conditions (63%), became more reduced during hypoxia (to 44%). Overexpression of catalase in the cytoplasm had no effect on the normoxic oxidation level of Ro-GFP but prevented its hypoxia-induced oxidation. Overexpression of catalase in the mitochondrial matrix had no effect on the oxidation of Mito-Ro-GFP under either normoxic or hypoxic conditions. These results imply that changes in H_2O_2 production during hypoxia influence the redox potential in the cytoplasm but not in the mitochondrial matrix. Hypoxia caused the same pattern of compartmentalized changes in Ro-GFP oxidation in renal artery SMC. However, experiments with YC2.3 showed that hypoxia caused an increase in $[Ca^{2+}]_{cyt}$ in PASMC, but not in the renal arteriolar cells. In this case, if mitochondrial ROS are involved in O_2 sensing in both types of SMC, their coupling to effector pathways must differ.

These investigations can be criticized because they were carried out in cultured SMC which may have undergone phenotypic changes. In order to characterize oxidant signaling under more physiological conditions, Desireddi et al. [363] transfected mouse lung slices with Cyto-Ro-GFP, a process which required their incubation in culture medium for two-days. The vascular endothelium was labelled with Cell Tracker Red so the vascular lumen could be imaged and used to measure contraction. The effect of hypoxia on $[Ca^{2+}]_{cyt}$ was assessed in parallel experiments using Fura 2, although this was done after only one day of organ culture, as Ca^{2+} signaling was lost thereafter.

Small PA in lung slices contracted to angiotensin 2, high K^+ PSS and hypoxia after the two day period in organ culture. Hypoxia (1.5% O_2) caused a rapid and sustained increase in Ro-GFP oxidation from 11 to 17%, which reversed upon re-oxygenation. Overexpression of catalase in lung slices had no effect on basal Ro-GFP oxidation but almost abolished the effect of hypoxia. Hypoxia caused an immediate increase in $[Ca^{2+}]_{cyt}$ which was diminished by catalase overexpression, and was abolished by EUK-134, a SOD-catalase mimic [364]. The increase in $[Ca^{2+}]_{cyt}$ was absent in slices superfused with Ca^{2+}- free PSS containing 2.5 mM EGTA, suggesting that it was due to the influx of extracellular Ca^{2+}. However, this concentration of EGTA can deplete releasable intracellular Ca^{2+} stores [365], so these might also have been involved.

These results demonstrated that hypoxia caused a reversible increase in Ro-GFP oxidation (i.e. oxidation of the GSH/GSSG redox couple) in small PA which, although exposed to culture conditions for two days, retained the ability to contract. It is noteworthy, however, that the size of the contractions was small compared to those previously described in freshly prepared mouse lung slices [366,367], and the proportion of arteries demonstrating a contraction to hypoxia was not stated. Also, the loss of Ca^{2+} signaling caused by the two-day incubation period suggests that changes in the PASMC phenotype had occurred.

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5.2.1. Role of Mitochondrial Ca^{2+} and the Rieske Iron-Sulfur Protein in Hypoxia-Induced ROS Production

The Rieske iron-sulfur protein (RISP; also termed ubiquinol-cytochrome c reductase, Rieske iron-sulfur protein 1 and Uqcrfs1), is a subunit of complex III. RISP contains a 2Fe-2S cluster which accepts electrons from the Q_o site and passes them on to cytochrome *c*1, and has been proposed to play a pivotal role in mediating a hypoxia-induced increase in ROS production by complex III [368].

Korde et al [127] examined the effect of RISP knockdown on the response to hypoxia in isolated mouse PA, cultured PASMC, mitochondria isolated from PA homogenates, and complex III obtained from these using immunocapture. PASMC were transfected with the genetically encoded ratiometric H_2O_2 indicator pHyPer-dMito [287] which the authors described as being expressed in the mitochondrial IMS. Hypoxia (PO_2 = 13 Torr) markedly increased the pHyPer-dMito signal and enhanced ROS production (assessed using DCFH) by isolated mitochondria and complex III. Hypoxia increased the activity of isolated complex III but had no effect on O_2 consumption or ATP production by the isolated mitochondria. Mitochondrial H_2O_2 under hypoxic conditions was greatly diminished in PASMC in which RISP expression was strongly depressed by siRNA treatment, as was hypoxia-induced ROS production in isolated mitochondria and Complex III obtained from these cells. RISP knockdown also strongly depressed HPV and the associated rise in $[Ca^{2+}]$ in isolated PA. Overexpression of RISP had opposite effects. Based on these results, the authors proposed that complex III in PASMC is intrinsically sensitive to hypoxia and speculated that this might involve an effect on the transfer of electrons from ubisemiquinone to cytochrome c_1 by RISP.

Based on their observation [369] that hypoxia induces Ca^{2+} release from the SR in PASMC which is dependent on ROS released from the mitochondria, and evidence that Ca^{2+} causes increased ROS production by mitochondria (Section 3.1.1.4), this laboratory investigated whether a hypoxia induces a positive feedback process involving Ca^{2+} and ROS release in PASMC [370]. They found that that SR Ca^{2+} release induced by caffeine (0.2 mM to 2M) or norepinephrine (1 μM – 10 mM) caused increases in mitochondrial $[Ca^{2+}]$ and cytoplasmic [ROS], which was monitored using several ROS probes, including the cytoplasmic H_2O_2 indicator pHyPer-cyto. In addition, raising the $[Ca^{2+}]$ in solution bathing mitochondria isolated from these cells also stimulated their ROS production. Hypoxia also increased mitochondrial $[Ca^{2+}]$ and ROS production by PASMC and isolated mitochondria. These effects were antagonised by RU360, a blocker of the mitochondrial Ca^{2+} uniporter (MCU). RISP knockdown attenuated basal as well as Ca^{2+}- and hypoxia-induced ROS production by PASMC and mitochondria isolated from these cells. These results suggested that hypoxia stimulates complex III-dependent ROS production by increasing mitochondrial $[Ca^{2+}]$, and that this involves Ca^{2+} release from the SR and the opening of the MCU. The authors also reported that SMC cultured from mesenteric arteries had a much lower expression of RISP compared to PASMC and did not increase their ROS production in response to hypoxia, supporting the concept that RISP is important in ROS production and O_2 sensing.

Evidence for a role of RISP in O_2 sensing was also provided by Waypa et al [128], who used compartmentally-targeted Ro-GFP to compare the effects of hypoxia (30 minutes: 1.5% O_2) on oxidant signaling in pulmonary and renal artery SMC from conditional knockout mice in which RISP expression was either normal or decreased by ~60%. In control PASMC, hypoxia caused a pattern of compartmental redox alterations resembling that which had been seen in rat PASMC: the cytoplasm and IMS became more oxidized and the mitochondrial matrix became more reduced [361]. In contrast, Cyto-Ro-GFP in RISP-depleted cells was more oxidized under normoxic conditions and hypoxia caused the cytoplasm to become more reduced. Hypoxia did not alter the redox state of IMS-Ro-GFP in RISP-depleted cells, and the redox state of the mitochondrial matrix, which was more reduced under normoxic conditions compared to controls, was also unaffected by hypoxia. Echoing their earlier observation in rat [361], they found that the pattern of compartmental changes in Ro-GFP oxidation in renal artery SMC induced by hypoxia was similar to that in PASMC, as were the effects of RISP-deletion. In additional *in vivo* experiments, injection of SMC-MHC-Cre/$RISP^{flox/flox}$ mice with tamoxifen was used to knock down smooth muscle RISP expression by ~60%. Measurements of PA

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diameter and $[Ca^{2+}]_{cyt}$ in lung slices revealed that HPV and the associated rise in PASMC $[Ca^{2+}]_{cyt}$ was suppressed in tamoxifen- vs vehicle-treated animals. Moreover, whereas PA wall thickness, RV mass, Fulton index, cardiac function and baseline normoxic PAP were similar in tamoxifen- vs vehicle-treated animals, the increase in right ventricular systolic pressure caused by ventilating the animals with a hypoxic gas mixture (5% O_2/95% N_2) was strongly reduced in the tamoxifen group. The authors proposed that RISP ablation acted at site Q_o to inhibit the formation of ubisemiquinone, which can donate an electron to O_2 to form superoxide. Based on the effects of RISP deletion on the differential compartmental oxidation of Ro-GFP, they suggested that hypoxia might change the configuration of complex III, such that the ROS it produced were directed into the IMS rather than the mitochondrial matrix.

Importantly, however, the depletion of RISP would be predicted to disrupt the Q cycle and the redox state of Q_o, which governs the production of ROS by complex III. Because the flow of electrons through RISP is necessary for the reduction of cytochrome C, its removal has been shown to strongly inhibit respiration [371–373], and can cause mitochondrial depolarization [374,375], and the upregulation of glycolysis, an increase in the NADH/NAD ratio [371,373] and the decreased expression of multiple isoforms of NADPH oxidase [372]. Although there is also countervailing evidence that cells can maintain a normal mitochondrial membrane potential and rate of glycolysis following RISP knockdown [193], its potential for causing disruption of mitochondrial function is such that it the results of these studies should be interpreted with caution.

5.2.2. Mitochondrial Hyperpolarization, Reduction of the Quinone Pool, and Cox4i2

Several studies by Sommer and colleagues [129,130,144] presented evidence that hyperpolarization of the mitochondrial membrane potential ($\Delta\Psi_m$) and reduction of components of the ETC promote hypoxia-induced mitochondrial ROS production in PASMC.

Sommer et al [144] evaluated the effect of hypoxia on the redox state of cytochromes $b_{L/H}$, *c* and *aa*3 in isolated perfused lung and PASMC from rabbit using remission spectrophotometry, a technique in which the redox state of multiple cytochromes is inferred from the wavelength-dependency of light absorbance by cells. They found that HPV was evoked when the pO_2 was lowered to ≤ 75Torr and increased progressively as the level of hypoxia was deepened. A significant change in absorbance was observed at pO_2 ≤ 53Torr, and analysis of the spectra indicated that reduction of cytochromes *c*, *aa*3 and $b_{L/H}$ occurred at pO_2 values of 38, 23 and 8 Torr respectively in the lungs. Based on measurements of the P_{50} for oxygen consumption, they calculated (see [149]) that respiration was significantly inhibited at ambient PO_2 levels of <100 Torr (e.g. by ~2 and ~10% at pO_2 of 38 and 8 Torr, respectively). Experiments in primary cultured microvascular PASMC showed that both $[Ca^{2+}]_{cyt}$ and $\Delta\Psi_m$ were increased at a pO_2 of 23 and 8 Torr. Mitochondrial ROS production (monitored using MitoSox) was also increased at a pO_2 of 8 Torr. Application of either the complex IV blocker cyanide or the complex III/Q_i site blocker HQNQ to isolated lungs caused a reduction of all of the cytochromes and an inhibition of HPV, with both effects developing over the same concentration ranges of each blocker.

The authors concluded that HPV was due to an increase in mitochondrial ROS production by complex III, which was linked to an increased reduction of components of the ETC associated with a subtle but significant inhibition of respiration evoked by hypoxia. They highlighted their observation that cytochrome $b_{L/H}$ remained more oxidized than the other cytochromes, speculating that this might reflect its involvement in ROS production, as suggested previously in a report showing that superoxide production was associated with a similar pattern of hypoxia-induced cytochrome reduction in a monocyte cell line [151].

Cox4 is one of the 10 regulatory subunits of complex IV encoded by nuclear DNA. When phosphorylated, Cox4 functions to inhibit complex IV at a high ATP/ADP ratio [376]. Cox4 exists as two isoforms, Cox4i1 and Cox4i2. The expression of Cox4i1 strongly predominates in most tissues, but Cox4i2 is also highly expressed in lung [175] and CBCC [377,378]. Cox4i2 has been shown to

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promote respiration [353] and therefore potentially increase ΔΨm, a key determinant of mitochondrial ROS production.

Sommer et al [130] therefore explored the relationship between ΔΨm, ROS production and downstream mechanisms causing HPV in Cox4i2 knockout and wildtype mice. They found that hypoxia (1% O_2 for 5 or 10 minutes) caused a hyperpolarization of $\Delta\Psi_m$ in primary cultured PASMC from wildtype mice. This was associated with increased mitochondrial superoxide production, a rise in cellular [H_2O_2], the inhibition of plasmalemmal K_V channels, membrane depolarization, and Ca^{2+} influx. All of these effects of hypoxia were greatly diminished in PASMC from Cox4i2$^{-/-}$ mice. Hypoxia-induced membrane depolarization was also inhibited in human PASMC treated with anti-Cox4i2 siRNA. The results presented in this paper are too extensive to describe in detail; however, the most important evidence is listed in Figure 8.

In essence, both phases of HPV, which this laboratory had first observed in isolated perfused lung from wild type mice [352], were virtually absent in Cox4i2$^{-/-}$ mice. Notably, however, since their studies in PASMC utilized only brief exposures to hypoxia, the specific effector mechanisms shown in Figure 8 are not necessarily involved in phase 2 HPV, although it too was Cox4i2-dependent.

In accordance with their model, the authors found that hypoxia-induced superoxide production was restored by overexpressing wildtype Cox4i2 in PASMC from Cox4i2$^{-/-}$ mice. However, this was not case when Cox4i2 in which cysteine 109 had been replaced by a serine or alanine was overexpressed in these cells. Additional experiments suggested that cysteines 41 and 45 on Cox4i2 were also required for superoxide production. These results suggested that these cysteines might act as thiol switches which would allow Cox4i2 to be redox-regulated during hypoxia in such a way as to increase $\Delta\Psi_m$. However, their original hypothesis that Cox4i2 might be increasing $\Delta\Psi_m$ by enhancing respiration during hypoxia was not borne out by their experiments, and they also found that mitoTEMPO did not prevent hypoxia from increasing $\Delta\Psi_m$, which was not consistent with redox regulation of Cox4i2 being responsible for this effect. Thus, whereas the study presents evidence that the hypoxia-induced increase in $\Delta\Psi_m$ in PASMC was *dependent on* the presence of Cox4i2, it didn't show that a specific effect of hypoxia on Cox4i2 was *causing* the rise in $\Delta\Psi_m$. . It is particularly noteworthy that HPV, recorded as an increase in PAP in isolated perfused mouse lung, was inhibited in a concentration dependent-manner by S3QEL2, which blocks ROS production by Complex III without affecting respiration.

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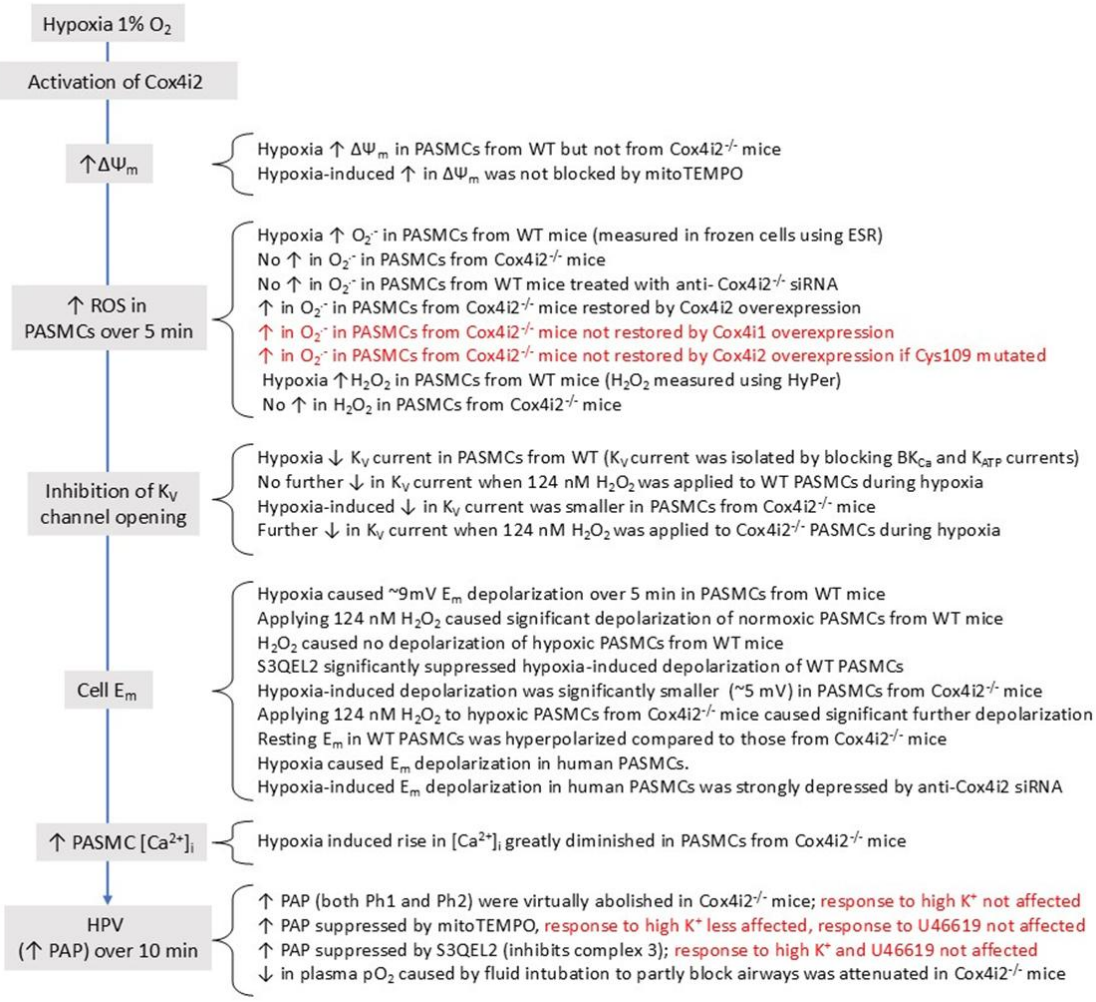

Figure 8. A summary of the experimental evidence reported in Sommer et al [130]. The flow chart on the left shows Text in black shows results related to the characterization of HPV mechanisms, text in red shows the sequence of events proposed to cause HPV. The evidence supporting each step in the sequence is shown to the right in black font, and the red font shows the results of negative control experiments.

A subsequent paper from this group [379], again employed ESR to show that hypoxia (1% O_2) increased superoxide levels in mouse PASMC, and also recorded a rapid increase in cytoplasmic H_2O_2 using $Hyper_{cyto}$. In contrast, hypoxia strongly decreased superoxide levels in whole lung homogenates. A similar effect occurred in cultured lung fibroblasts, leading them to speculate that this might explain the hypoxia-induced fall in ROS in lung homogenate, which had also previously been seen in perfused lungs [278,380]. Using MitoSox, they confirmed their earlier finding [144] that hypoxia increased ROS in the mitochondrial matrix, a finding which however runs counter to measurements made using genetically encoded ROS indicators [125,361]. The authors did not discuss this discrepancy, which could conceivably reflect drawbacks of MitoSox [381].

To further investigate mitochondrial ROS production by the distal ETC, this laboratory [129] utilized AOX mice, which express an alternative oxidase derived from the marine invertebrate *Ciona intestinalis*. AOX becomes active if the flux of electrons through the distal ETC is slowed enough to cause substantial reduction of the quinone pool [382], creating an alternative pathway for electrons to flow from QH_2 to O_2 which bypasses complexes 3 and 4. This prevents over-reduction of the quinone pool while maintaining oxidative phosphorylation, with no apparent deleterious effects on mice [383]. As initially described in tobacco cells, AOX suppresses basal ROS production [384].

They observed that the pressor response to cyanide and both phases of HPV evoked by hypoxia (1% O_2 in the ventilating gas mixture) in isolated perfused lungs from WT mice were almost abolished

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in by AOX expression but were restored by applying the AOX inhibitor n-propyl gallate. Hypoxia-induced PASMC depolarization was decreased by >50% in AOX compared to wildtype mice, and n-propyl gallate caused further depolarization in the PASMC from AOX mice, but not from controls. HPV in isolated PA recorded in control PSS was depressed in the AOX mice, but if arteries were incubated in PSS containing 20 mm K^+ to evoke a degree of resting membrane depolarization, HPV in the WT and AOX mice was of similar amplitude. Perfusion of isolated lungs from AOX with high K^+ PSS also produced some restoration of HPV, although it remained far smaller than that seen in wildtype mouse lung perfused with this solution. They also used ESR to show that the hypoxia-induced increase in superoxide seen in PASMC from wildtype mice was absent in those from AOX mice. Similarly, hypoxia hyperpolarized $\Delta\Psi_m$ in PASMC from WT but not AOX mice.

In further experiments, they transfected rat PASMC with either an AOX-containing or empty expression vector and saw that the presence of AOX slightly enhanced respiration, at very low PO_2. This observation that AOX increased O_2 consumption under hypoxic conditions supported the notion that hypoxia was causing reduction of the quinone pool, since AOX should not be able to donate electrons to O_2 unless this pool is substantially reduced [382]. In accordance with this possibility, and with their earlier observations [144], they found using Raman spectroscopy that hypoxia reduced the quinone pool and cytochrome *c*, but not cytochrome *b*, in WT PASMC. The quinone pool was also reduced in the AOX PASMC, but the redox state of cytochromes *b* and *c* was unchanged, which supported the idea that electron flow though AOX was bypassing complexes 3 and 4.

These investigations by Sommer and colleagues suggest a model in which hypoxia increases ROS production by complex III, possibly *via* the escape of electrons from cytochrome $b_{H/L}$, and that increased reduction of the quinone pool due to a slight inhibition of respiration, as well as mitochondrial hyperpolarization, which had previously been observed by other laboratories [43,145] contribute to this response.

It is noteworthy that Cox4i2A has also been implicated in O_2 sensing in the carotid body [385], reported that the normal hypoxic ventilatory response, which is dependent on O_2 sensing by CBCC, was virtually abolished in Cox4i2 null mice, as was the rise in $[Ca^{2+}]_{cyt}$ in CBCC which underlies the secretory response of these cells to a fall in PO_2. The rise in NADH evoked by hypoxia in CBCC from wildtype controls was also absent in the knockouts. Similar effects were seen when the expression of Cox4i2 was reduced by knocking out *EPas1*, the gene coding for Hif1a. *EPas1* knockout also diminished the expression of two other mitochondrial proteins, COX8b, another regulatory subunit of complex IV, and NDUFA4L2, which interacts with complex IV [376]. These two proteins, together with Cox4i2, have been proposed to contribute to the unusually high $K_m[O_2]$ of CBCC that increases their sensitivity to hypoxia [377,378], although how they would do so is unclear.

The observation that hypoxia had no effect on [NADH] in CBCC from Cox4i2 knockouts, which suggested that it was not inhibiting respiration, is consistent with evidence from a study by Pajuelo-Riguera and colleagues which compared the PO_2 sensitivity of Cox in HEK293 cells exclusively expressing either Cox4i1 or Cox4i2 [174]. Their key finding was that the $K_m[O]_2$ for mitochondrial respiration was almost ~2 fold higher in the cells expressing Cox4i2, indicating that respiration would be more depressed in these cells at low PO_2 levels. The authors pointed out that this should promote reduction of the proximal ETC and of the NAD^+/NADH couple, both factors known to increase mitochondrial ROS production, and was consistent with the loss of HPV seen in PA from Cox4i2 knockout mice.

Notably, however, the effect of Cox4i2 on the $K_m[O_2]$ described in this paper appears to be insufficient to itself cause a meaningful depression of respiration except during severe hypoxia (see Eqn 1 in [149]), implying that its expression in O_2 sensing cells might be necessary but in itself insufficient to promote mitochondrial ROS production at moderate levels of hypoxia. The authors suggested that the expression of COX8b and NDUFA4L2 might also be important for the hypoxia-induced ROS production, possibly through their interaction with Cox4i2. It would therefore be of interest to see if these proteins are expressed in PASMC, and if combined knockout of all three proteins affects HPV at moderate levels of hypoxia.

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5.2.2. HPV and Mitochondrial NCLX

As described in Section 3.1.1.4, hypoxia is proposed by Hernansanz-Agustin and colleagues [183,184] to cause complex I deactivation, leading to acidification of the mitochondrial matrix. Since mitochondria contain a very substantial amount of Ca^{2+} bound to phosphate as a $Ca_3(PO_4)_2$ complex, the dissociation of which is highly pH-sensitive [190], acidification causes an increase in free $[Ca^{2+}]_{mito}$. According to their model, this promotes Na^+ entry into the matrix through the mitochondrial NCLX. The resulting rise in $[Na^+]_{mito}$ decreases the fluidity of the IMM, impeding electron transfer from complex II and G3PD to complex III and thereby increasing ROS production at Complex III_{CoQ} by slowing electron flow within the Q cycle.

Although this scheme was based on data obtained from bovine aortic endothelial cells and mouse embryonic fibroblasts, they presented evidence that this mechanism is involved in O_2 sensing during HPV by showing that hypoxia (1% O_2) strongly increased superoxide production by mouse PASMCs, and that this response was absent in cells in which the mitochondrial NCLX was knocked down using siRNA [183]. Furthermore, the $NCLX_{mito}$ inhibitor CGP-37157 (30 μM) suppressed sustained HPV in isolated rat PA, although the initial peak of the response was unaffected.

However, CGP-37157 at this concentration also blocks L-type VGCC and K_V channels [386,387], both of which contribute importantly to HPV. Also arguing against a role for NCX_{mito} in HPV, Becker and colleagues found that replacement of extracellular Na^+ with Li^+, which should profoundly reduce intracellular $[Na^+]$ [388] and thereby prevent Na^+ influx into the mitochondria through $NCLX_{mito}$, did not inhibit HPV in rat PA. Similarly, treatment of PA with the Na,K – ATPase inhibitor ouabain, which should greatly increase the $[Na^+]$ in the cytoplasm and therefore in the mitochondrial matrix [389] did not inhibit HPV. Thus, HPV persisted in spite of significant perturbations of the intracellular Na^+ concentration which would be predicted to disrupt the Na^+ dependent regulation of the fluidity of the mitochondrial inner membrane proposed to be responsible for the hypoxia – induced rise in mitochondrial ROS production. Nevertheless, the Becker et al study was not designed to evaluate the role of NCLX in HPV and the evidence presented by Hernansanz-Agustin et al that their scheme is involved in O_2 sensing is extensive; thus, a more thorough investigation focusing on the involvement of this pathway in HPV would be desirable.

5.2.3. Hypoxia and Increased ROS Production by Complex II

Paddenberg et al [126] used DCFH-DA to characterize the effect of hypoxia (1% O_2) on ROS levels in PA from mouse lung slices and cultured rabbit PASMC. They observed that three hours of hypoxia increased the number of PASMC and PAECs in PA from the lung slices which exhibited an increase in the DCFH signal above the threshold level they used to indicate ROS production (DCFH-positive cells), and that one hour of hypoxia also significantly increased DCFH fluorescence in rabbit PASMC. The hypoxia-induced increase in DCFH-positive cells was abolished by DPI, rotenone, and the complex II blockers 3-NPA and TTFA. Rotenone decreased the number of DCFH-positive cells under normoxic conditions, as did antimycin, but 3-NPA did not, leading them to propose that hypoxia was acting at complex II to increase ROS production, and also that complex III was contributing to ROS production during both normoxia and hypoxia. Using a histochemical assay, they showed that hypoxia decreased the activity of succinate dehydrogenase in the lung slices. Furthermore, application of exogenous succinate prevented the hypoxia-induced increase in ROS. Based on these results they proposed that hypoxia causes complex II, which normally accepts electrons from succinate to generate fumarate, to switch to using electrons it receives from NADH *via* complex I and the ubiquinol pool to reduce fumarate to succinate, as a result generating ROS.

5.2.4. NAD(P)H Oxidase as a Source of Increased ROS During HPV

In 1996, Marshall et al [339] presented evidence that hypoxia increases Nox-mediated superoxide production in PAs. They employed immunohistochemistry and spectrophotometry, respectively, to detect the presence of gp91-*phox* and the unique Nox cytochrome (b_{-245}) in cultured

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PASMC from fetal calves, and immunoblotting to demonstrate its expression in both PA and systemic arteries. Using lucigenin as a nominal superoxide indicator, they examined the effect of 20 min of hypoxia (~40 Torr) on ROS production by primary cultured SMC from small and large PA, as well as aorta and ear arteries. Hypoxia greatly increased superoxide production in each type of SMC, particularly those from small PA. This effect was absent if SOD (163 units/ml) was present, leading the authors to conclude that lucigenin was detecting an increase in extracellular superoxide. Hypoxia-induced superoxide production was virtually abolished by the Nox antagonist DPI (diphenyleneiodonium) which however has many other effects, including block of complex I[390]) but was unaffected by myxothiazol. HPV in isolated PA from cats was blocked dose-dependently by DPI, with an ID_{50} of ~0.81 μM, whereas the pressor response to norepinephrine was ~15-fold less sensitive to DPI. The authors concluded that hypoxia was causing an activation of Nox, rather than the inhibition which had been described previously [391] (see Section 8.1), and suggested that the K_m of the oxidase for O_2 (10 μM or ~7 Torr) was low enough to ensure that superoxide production would not be limited by the fall in pO_2 associated with moderate hypoxia.

The role of Nox in HPV was subsequently challenged by the observation that HPV was intact in lungs from mice with chronic granulomatous disease, which lack the phagocytic form of gp91-*phox* (Nox2) [198]. Lungs from these mice also exhibited a profound suppression of normoxic ROS production (detected using lucigenin), and in contrast to the observations of Marshall et al [339], ROS production in isolated PA from control mice was strongly depressed by moderate (~40 Torr) hypoxia.

However, Weissmann et al [350] thereafter reported the expression of Mox-1, a different isoform of Nox (now called Nox1), in PASMC from rabbit lung. In order to study its role in HPV while avoiding problems associated with the non-selectivity of the NADPH oxidase blockers diphenyleneiodonium (DPI) and apocynin [392], they used AEBSF, a serine protease inhibitor which had been shown to block NADPH oxidase in phagocytes [393] and inhibited the response to hypoxia in carotid body [394]. AEBSF was also an flawed blocker, as it caused a substantial increase in PAP in isolated perfused rabbit lung. However, this effect was transient and when the PAP had returned to baseline level the pressor response to 10 minutes of hypoxia (O_2 of ~23 Torr in the ventilating gas mixture) was depressed by AESBF in a concentration-dependent manner. The authors also demonstrated that HPV, but not the pressor response to U46619, was attenuated by the SOD inhibitor TETA, which itself did not affect PAP. These results led them to conclude that ROS production by Nox was activated by hypoxia and contributed to HPV.

Weissmann et al [351] reported that HPV in isolated perfused rabbit lung is biphasic, observing that PAP rose to reach a peak ~5 minutes after the imposition of hypoxia (phase 1), then fell by ~50% to reach a nadir at ~15 minutes, and thereafter rose gradually to reach a plateau after 2-3 hours (phase 2). The antioxidant nitro blue tetrazolium (NBT), applied at an initial concentration of 0.9 μM, strongly depressed both phases, as did TETA, applied at an initial concentration of 25 mM. DPI (initial concentration 1.5 μM), applied in the presence of L-NMMA, diminished phase 1 by ~50%, whereas phase 2 was almost abolished. DPI itself evoked a minor increase in baseline PAP, whereas TETA and NBT caused a small fall. However, Weissmann et al 2006 [352] observed that AEBSF (initial concentration 500μM) abolished phase 1 HPV in the rabbit lung, without significantly affecting phase 2. Like Archer and colleagues [212], they found that phase 1 was of similar amplitude in gp91-*phox* knockout mice and wildtype controls. However, phase 1 was significantly depressed in p47-*phox* knockouts, in which ROS production by both Nox1 and Nox2 would be depressed. In contrast, the pressor response to U46619 was similar in all three groups of mice. Baseline normoxic PAP was also not different in the p47-*phox* knockouts, suggesting that basal ROS production by NOX1 or 2 was not contributing to ongoing normoxic vasodilation. These data suggested that ROS production by Nox1 was partly responsible for phase 1 HPV.

In 2008, Rathore et al [39] found that protein for Nox1, Nox4 and the p22-, p47- and p67-*phox* components of Nox was similarly expressed in small endothelium-denuded pulmonary and mesenteric arteries from mice, but saw no expression of Nox2 in either artery. Normoxic arteries or those subjected to 5 minutes of hypoxia (1% O_2) were homogenized and centrifuged to isolate a

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plasma membrane-containing fraction and the activity of Nox was monitored by adding NADPH and measuring the SOD-inhibitable (i.e. superoxide-dependent) reduction of cytochrome C. Nox activity was increased in PA but not MA, presumably reflecting an effect caused by hypoxia which had persisted during the isolation process. This was associated with an increased plasmalemmal expression of p47-*phox*, consistent with Nox activation.

Nox activity during hypoxia was strongly suppressed by the nominal Nox blocker apocynin (1μM) and was smaller in PA from p47-*phox* knockouts compared to wildtype controls. Similar effects were also observed in PA from PKCe knockout mice comparted to wildtype controls and in PA treated with a peptide inhibitor of PKCe translocation (10 μM) but not Gö6796 (0.1 μM), a blocker of conventional PKC isoforms. Although it's not clear that these interventions diminished the effect of hypoxia *per se*, since their effects on Nox activity under normoxic conditions were not described, parallel experiments utilizing DCFH as an indicator showed that in each case hypoxia-induced ROS production was attenuated in freshly isolated PASMC with no effect apparent during normoxia. Cell shortening and rises in $[Ca^{2+}]_{cyt}$ in response to hypoxia were also decreased in PASMC treated with apocynin and in those from p47-*phox* $^{-/-}$ mice, whereas $[Ca^{2+}]_{cyt}$ and the contractile response to caffeine were not affected under normoxic conditions.

The authors also observed that Nox activity during hypoxia was strongly suppressed by rotenone and myxothiazol. They had previously found that hypoxia increased the activity of PKCe in PASMC, that this effect was also sensitive to these ETC blockers, and that the expression of PKCe in PA was much higher than in MA [395]. They therefore proposed that increased mitochondrial ROS production in PASMC due to hypoxia was activating PKCe, causing a stimulation of Nox1 that generated additional superoxide which acted *via* effector pathways to cause HPV. They suggested that the Nox2 expression which had previously been reported in mouse lung might [212] might occur mainly in non-SMC or in pulmonary veins. They also pointed out that the lack of effect of blocking or knocking out p47-*phox* on basal $[Ca^{2+}]_{cyt}$ or the caffeine-induced contraction was not consistent with the concept that that ongoing Nox-mediated ROS production maintains a tonic normoxic vasodilation which can be reversed by hypoxia (see Section 8).

In 2011, Frazziano et al [323] similarly detected a role for both mitochondrial and Nox-derived ROS production in HPV evoked in small rat PA by 10 minutes of hypoxia (22-26 Torr). This group had earlier proposed that HPV is due to the activation of neutral sphingomyelinase 2 (nSMase-2), this causing an increased production of ceramide and a consequent stimulation of PKCζ which acts to depress the K_V current [396,397]. They observed that the increase in cellular ceramide caused by hypoxia was abolished by rotenone, suggesting its dependency on mitochondrial ROS production. Application of exogenous ceramide caused the production of ROS (monitored using DCFH and DHE), and this was blocked by a PKCζ – inhibitory peptide. Hypoxia also caused the phosphorylation of p47-phox and its association with caveolin-1, and this was blocked by the PKCζ – inhibitory peptide [323]. They also found that 300 μM apocynin strongly antagonized HPV. Based on these results, they proposed that nSMase-2 is stimulated by mitochondrial ROS, generating ceramide which activates Nox *via* a PKCζ -mediated phosphorylation of p47phox, leading to the production of ROS which inhibit K_V channels to cause HPV. The evidence that hypoxia induced a PKCζ – dependent phosphorylation of $p47^{phox}$ which was blocked by rotenone supports the model developed by Rathore et al (2008) in that mitochondrial ROS would act through PKC to promote further oxidant signaling by Nox, although the two schemes differ with regard to which PKC isoform causes p47phox activation.

It is notable, however, that the effects of apocynin described in these papers are uninterpretable, since Heumüller et al [345] showed that it blocks Nox only after being converted by myeloperoxidase into a dimer and that this does not occur in VSMC, which lack this enzyme. Apocynin was also found to act as an antioxidant which interacts with both biomolecules and with ROS indicators such as lucigenin.

In contrast to the findings of these papers, Connolly et al (2013) [18], who studied HPV in rat IPA in the absence of pre-tone (under which condition Phase 1 HPV asismall), found that the Nox

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inhibitor VAS2870 (10 μM) had no effect on HPV, although the broad spectrum PKC antagonist Gö6983 (3 μM), which would be expected to block both PKCϵ and PKCζ [398], reduced HPV by ~30%.

Veith and colleagues [399] compared the effect of hypoxia (1% O_2) in perfused lungs from wild-type and Nox4$^{-/-}$ mice, finding that neither phase of HPV was different in these two groups. The basal right ventricular systolic pressure (RSVP), an indicator of the PAP, was also not significantly affected by Nox4 knockout. Murtaza et al [400] subsequently compared the effect of hypoxia on pre-acinar and smaller intra-acinar arterioles in lung slices from these two groups, and saw that wildtype intra-acinar arteries demonstrated a substantial sustained constriction which was halved in the Nox4 knockouts, whereas the pre-acinar arteries showed a smaller response which was similar in the two groups. In view of the results from Veith et al, they suggested that constriction of intra-acinar arteries does not contribute to the overall hypoxia-induced rise in PAP [401] but might be involved in the redistribution of blood flow responsible for ventilation-perfusion matching.

Nagaraj et al [402] recorded HPV (evoked by 1% O_2) in perfused lungs from wild type and p22$^{phox-/-}$ mice, reporting that whereas the basal normoxic PAP and initial transient phase of HPV were similar the two groups, the sustained phase was decreased by ~40%. They also showed that hypoxia increased the activity of RhoK in lung slices from wildtype mice, and that this effect was absent in the p22^{phox} knockouts. Since Noxs 1 and 4 are the isoforms most likely to be involved in HPV, and both require p22^{phox} for their activity, these results suggest that ROS production by Nox1 may contribute to sustained HPV by stimulating Rho kinase-mediated Ca^{2+} sensitization.

The Role of NADPH Oxidase in HPV: Summary and Conclusions

Although Nox2 is not involved in HPV [212], at least in mice, the studies in rodents described above generally support the possibility that HPV depends to some extent on a hypoxia-induced activation of ROS production by Nox1 which is dependent on PKC, a well-characterized activator of Nox1/2 [60]. The possibility that activation of Nox1 by 5 minutes of hypoxia is dependent on mitochondrial ROS production acting *via* PKCe, as evidenced by Rathore and colleagues [39,395], is also is consistent with observations that the amplitude of phase 1 HPV in mice was decreased by ~1/3 when p47-*phox* was knocked out (Weissmann et al, 2006)[352], but was abolished in Cox4i2$^{-/-}$ mice, along with superoxide production [130]. The involvement of a mitochondrial ROS-PKCe -Nox1 axis in the initial phase of HPV is also supported by evidence that the pressor response to a 5 minute hypoxic challenge in isolated perfused rat lung was decreased by ~50% [403] in PKCe knockout mice, and that the rapid increase in $[Ca^{2+}]_{cyt}$ evoked by hypoxia was strongly depressed in PASMC from these knockouts and from wildtype mice treated with PKCe inhibitor peptide [395]. Moreover, the finding that Ro-31-8220, which blocks multiple PKC isoforms including PKCe depressed phase 1 HPV in rat intrapulmonary arteries but had no effect on phase 2 [404] supports the lack of involvement of this pathway in sustained HPV. The investigation by Nagaraj et al [402] also implicates Nox1 in HPV. However, they observed that it was phase 2 rather than phase 1 HPV which was Nox-dependent. The reason for this discrepancy is unknown.

Importantly, with the exception of the initial paper by Marshall et al [339], the investigations described above ignored the question of whether the K_m (O_2) for Nox has an impact on ROS production during hypoxia. The $K_m(O_2)$ for Nox1 has not been reported, but if it is similar to that of Nox2 (~25 torr) [59], its ability to generate ROS would be diminished at the levels of hypoxia used by most laboratories to study its involvement in HPV. This would call into question the plausibility of the findings that ROS production by Nox1 was increased at this level of hypoxia, but at the same time would imply that if these observations are valid, these studies may have greatly underestimated the ability of Nox1 to produce ROS and therefore contribute to HPV at more moderate levels of hypoxia.

This issue is even more salient with regard to the $K_m(O_2)$ for Nox4, which has been shown to be ~136 Torr [59,405]. This implies that using very low levels of PO_2 to impose hypoxia is unsuitable for examining whether increased ROS production by Nox4 contributes to HPV evoked by moderate hypoxia, since severe hypoxia should essentially shut the oxidase down. On the other hand, its high $K_m(O_2)$ means that Nox4 is well suited to act as an O_2 sensor which responds to acute hypoxia by

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decreasing rather than increasing the production of H_2O_2 [59,405]. Indeed, as described in Section 8, it has been suggested that a fall in H_2O_2 production by Nox4 is instrumental in causing HPV in bovine pulmonary arteries.

5.2.5. HPV Effector Mechanisms Coupled to an Increase in PASMC [ROS]

The Redox theory from the outset incorporated K_V channel inhibition as the essential HPV effector mechanism. In contrast, Schumacker's laboratory did not include any specific effector mechanisms in their initial presentation of the Mitochondrial ROS theory [38], although they later suggested [348] that increases in cytoplasmic ROS might be working though activation of phospholipase C [406], which would cause an increase in IP_3 production leading to SR Ca^{2+} release mediated by the IP_3R (see also [407]). Subsequently, it has been demonstrated that SR Ca^{2+} release mediated by the RYR [408] and SOCE mediated by STIM1/TRPC1/Orai1 [409] are both activated in a ROS-dependent manner during HPV. H_2O_2 was also shown to cause an increase in $[Ca^{2+}]_{cyt}$ in PASMC mediated by SR Ca^{2+} release through $IP3_R$ and RyR, as well as Ca^{2+} influx through Ni^{2+}- sensitive cation channels [410].

Other pro-contractile mechanisms mobilized by acute hypoxia in PASMC, including Ca^{2+} influx via TRPC6 channels [11,411,412] and the activation of several protein kinases [413], such as PKC [25,323], Src-family kinases [414,415] [416] and RhoK [416], have also been shown to be stimulated by ROS. It has also been demonstrated that the IP_3R, which may contribute to SR Ca^{2+} release during HPV [3] is activated by ROS released from the mitochondria *via* oxidation of a group of cysteines located in its N-terminal suppressor domain [92,259,260]. Furthermore, as described in Section 5.2.4, Cogolludo and colleagues have provided evidence that an increase in ROS production by the mitochondria and Nox inhibits the opening of $K_V1.5$ channels in PASMCs though a mechanism involving nSMase-2), ceramide and PKCζ [323,396].

6.3. H_2S, ROS and HPV

Olson and colleagues proposed that hypoxia, by inhibiting the O_2-dependent metabolism of H_2S, causes a rise in its cellular concentration which constitutes the PO_2 -sensitive signal triggering HPV. This was based on observations that 1. applying exogenous sulfide caused constricting or dilating effects mimicking those of hypoxia in multiple blood vessels from diverse species 2. blockers of sulfide-producing enzymes inhibit HPV, and 3. sulfide production by lung homogenates and pulmonary vascular preparations is increased at low PO_2 [46]. However, the contention that sulfide as an O_2 sensor in HPV has been challenged [434,435]. Subsequently, Olson suggested that hypoxia might also increase cellular levels of polysulfides and other reactive sulfur species (RSS), which exist in equilibrium with cellular sulfide, but at much higher concentrations [47]. Like ROS, these are feasible HPV mediators since they can regulate protein function by causing oxidative modification of reactive thiols *via* sulfuration [436]. However, evidence that hypoxia can elevate sulfide or polysulfide levels in PASMC quickly enough to trigger HPV is lacking. Indeed, production of sulfide and polysulfides in bovine pulmonary artery SMC was not potentiated during the first 5 hours of a hypoxic (5% O_2) challenge [437].

Nevertheless, the mechanisms of the sustained contractions of rat PA induced by applying exogenous sulfide and hypoxia demonstrate many similarities [18,166]. In both cases, contraction was insensitive to the VGCC antagonist nifedipine, but was inhibited by the antioxidant TEMPOL, and by blockers of the RyR (ryanodine), RhoK (Y-27632), PKC (broad spectrum inhibitor Gö6983), and complex III_{CoQ} (myxothiazol). Moreover, sulfide mimicked hypoxia by causing mitochondrial hyperpolarization [130,144]. Sulfide also increased ROS production by PASMC, an effect that was abolished by the knockdown of sulfide quinone oxidoreductase (SQOR), a protein located in the IMM. SQOR forms part of the 'sulfide oxidation unit' which metabolizes sulfide and in doing so feeds electrons into the CoQ pool and stimulates respiration [167,438]. The contraction of trout gill to sulfide was also antagonized by an antioxidant (DETCA) and blockers of mitochondrial complexes 1, 3 and 4 [439]. In light of the proposal that sulfide stored within the mitochondria as thiosulfide can be

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mobilized by hypoxia [164], as well as the high affinity of SQOR for sulfide [167], it is conceivable that a small hypoxia-induced rise in intramitochondrial [sulfide] could act through SQOR to increase mitochondrial ROS production at complex $III_{Co}Q$, thereby causing HPV.

6.4. Effects of Hypoxia on Redox Couples

Cells contain several important redox couples, each of which regulates different aspects of cellular function. Although the three main redox couples (GSSG/GSH, $NADP^+$/NADPH, NAD^+/NADH) interact in important ways, the flow of electrons between them is regulated enzymatically and by membrane transport systems, so that they are not in thermodynamic equilibrium [266]. The redox potential of each couple also differs between cell compartments.

The GSH/GSSG couple functions as a conduit to transfer electrons from NADPH to cell proteins, causing their reduction, but GSSG is also used for protein glutathionylation, thereby contributing to oxidative redox signaling. Although its involvement in maintaining overall cellular antioxidant defence has been unclear, and has been overshadowed by that of the thioredoxin/peroxiredoxin pathway, a recent investigation suggests that it may it may be more important than has been previously thought, at least in some type of cells [89]. As shown in Table 5, Connolly et al (2013)[18] did not detect a difference between the normoxic redox potentials of the GSH/GSSG couple in rat PA and aorta, which are exposed to a similar normoxic plasma PO_2 *in vivo*, [49]. They also observed that 45 minutes of hypoxia caused the oxidation of this couple in PASMC. Similarly, the GSH/GSSG ratio in cultured rat PASMC was decreased by 2 hours of hypoxia [356]. These observations are predicted by the mitochondrial ROS model and do not support the Redox theory, which posits that the cytoplasm of PASMC is relatively oxidized under normoxic conditions and becomes more reduced during hypoxia, whereas in comparison systemic SMC are more reduced in normoxia and become oxidized during hypoxia. However, the lengthy exposures to hypoxia used in both studies means that their results may not reflect the immediate response to hypoxia.

Measurements of pyridine nucleotide (i.e. NAD(P)H) fluorescence in rat and bovine PA suggest that the NAD^+/NADH and/or the $NADP^+$/NADPH redox couples may become more reduced during hypoxia [141,346,440,441]. However, these fluorescence measurements did not distinguish between NADH and NADPH, or between their free and bound forms. Since it is the concentrations of the free forms of both pyridine nucleotides which determines their redox potentials, and these constitute only a small fraction of their total contents [442], the effects of hypoxia on the individual redox potentials of both couples remain unclear.

The NADPH/$NADP^+$ couple is the ultimate source of electrons which are used by redox relays to reduce oxidized proteins but also provides the electrons which are used by Nox to produce superoxide/H_2O_2. The role of NADPH in determining pulmonary vascular tone during HPV has been investigated in bovine pulmonary artery by Michael Wolin, Sachin Gupte, and colleagues. This work, which is described in Section 8, has shown, somewhat paradoxically, that a hypoxia-induced increase in NADPH may contribute to HPV both by causing the reduction of PKGa1 *via* Trx-1/TrxR-1, and by increasing ROS production *via* Nox, presumably leading to the oxidation of other as yet unidentified effector proteins.

The NADH/NAD^+ couple is mainly involved in controlling respiration by providing reducing equivalents to the ETC, and also acts to reduce mitochondrial $NADP^+$ *via* the NNT, thereby helping to maintain the mitochondrial matrix in a highly reduced state [58,87]. Because its redox potential is much less negative than that of $NADP^+$: NADPH, the NAD^+:NADH couple is not directly involved in regulating thiol oxidation [442]. However, *via* the NNT, it exerts an important indirect influence on the $NADP^+$/NADPH ratio, and therefore thiol signaling, in the mitochondrial matrix. An increase in mitochondrial [NADH] also promotes superoxide production by Complex I, both directly, and indirectly through its effects on KGDH and PDH [87] (Sections 3.1.1.3 and 3.1.1.4).

Although an increase in NADH has been suggested to cause HPV through a ROS-independent pathway (inhibition of cyclic ADPR hydrolase) [443], the impact of changes in the cytoplasmic or mitochondrial NAD^+/NADH ratio on redox signaling during HPV remain unexplored. However,

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Lopez-Barneo's laboratory has proposed that a rise in cytoplasmic [NADH] due to a direct effect of hypoxia makes an important contribution to O_2 sensing in femoral artery myocytes [444] by suppressing voltage-gated Ca^{2+} currents, and in CBCC by inhibiting K_V channel opening [179,445]. A role for NADH in O_2 sensing is supported by evidence that hypoxia can increase the cytoplasmic free [NADH] in coronary artery myocytes [267], and that NADH binds to K_V channel β subunits with a high affinity, thereby affecting channel activity [446]. However, the hypoxia-induced increase in the cytoplasmic [NADH]/[NAD^+] ratio, at least in coronary myocytes, was quite small, and the concentration of NADH used to characterize its effect on the K_V current in CBCC (200 μM) was almost certainly much higher than those present in cells, since the NAD^+/NADH couple in the cytoplasm is almost entirely oxidized (i.e. NAD^+ >> NADH). This is potentially problematic since 1. NADPH also binds to K_V channel β subunits, 2. the cytoplasmic $NADP^+$/NADPH redox couple is almost completely reduced so that NADPH >> $NADP^+$ [266], and 3. the affinity of NADPH for the β subunit is even higher than that of NADH. As a result, it has been questioned whether physiological levels of cytoplasmic free NADH can regulate K_V channel activity, since they may not be sufficient to compete with NADPH for binding to K_V b-subunits [268].

It is noteworthy, however, that because the redox state of the cytoplasmic NAD^+/NADH couple is strongly influenced by the lactate/pyruvate ratio, increases in cellular lactate which can occur under conditions such as systemic hypoxia can lead to a significant rise in cellular [NADH], which may contribute to O_2 sensing in CBCC [447].

7. Critique of the Redox and Mitochondrial ROS Theories

As described in Sections 5.1 and 5.2, the Redox and Mitochondrial ROS theories propose opposite effects of hypoxia on mitochondrial ROS production in PASMCs. The Redox model is based mainly on work by Stephen Archer, Kenneth Weir, Evangelos Michelakis and colleagues, whereas the most salient findings supporting the Mitochondrial ROS theory have come from the labs of Paul Schumacker, Norbert Weissmann and Yong-Xiao Wang. Although the papers from the proponents of these two theories present apparently irreconcilable bodies of evidence, other authors have also published results which can be used to assess the extent to which the literature as a whole supports the predictions of either theory.

Predictions of the Redox theory: The key tenet of the Redox theory is that hypoxia causes contraction by decreasing mitochondrial ROS production. In setting forth the Redox theory [19,33,204], Archer and colleagues have invoked observations in the literature which are in line with the following predictions:

4. Blockers of the ETC which induce a fall in mitochondrial ROS production should mimic hypoxia by causing inhibition of PASMC K+ currents and contraction and should also prevent HPV.
5. Similarly, anti-oxidants should cause a sustained contraction. Their effect on HPV is more difficult to predict, since a small anti-oxidant effect might add to that of hypoxia to enhance HPV, while a large anti-oxidant effect could abolish any further response to hypoxia.
6. Pro-oxidants should prevent and reverse HPV.

Most importantly, hypoxia should diminish mitochondrial production of ROS in PASMC, thus causing a fall in cytoplasmic [ROS] and/or a reduction of cytoplasmic redox couples involved in regulating reactive thiols, particularly on K_V channels.

The Redox theory also predicts that hypoxia, reductants, and blockers of the ETC which decrease mitochondrial ROS production should inhibit PASMC K_V currents, whereas oxidants should have the opposite effects. Whereas it is well established that these currents are indeed suppressed by hypoxia in PASMCs, as described in Section 5.1.2 there is no direct evidence (e.g. changes in reactive thiol oxidation or interactions between K_V a and b subunits in native PASMCs) that this due to redox mechanisms.

Predictions of the mitochondrial ROS model: The Mitochondrial ROS theory is predicated on the concept that hypoxia increases mitochondrial ROS production in PASMC, causing a rise in [ROS] in the mitochondrial IMS and the cytoplasm which induces the oxidation of cytoplasmic redox

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couples which regulate reactive thiols. This effect of hypoxia is seen as not being unique to PASMC, and indeed the Mitochondrial ROS theory grew out of observations that hypoxia increased ROS production in cardiomyocytes [342]. In their reviews, Schumacker and colleagues have focused mainly on evidence that hypoxia increases ROS in PASMC and in other types of cell rather than emphasizing the effects of ETC blockers, anti-oxidants and pro-oxidants on HPV. However, the responses of PASMC to these agents predicted by this hypothesis are reasonably clear:

7. Blockers of the ETC which diminish mitochondrial ROS production should not cause contraction under normoxic conditions but should inhibit HPV.
8. ETC blockers like myxothiazol or rotenone which act at or upstream of the Q_o site of complex III should inhibit HPV. Antimycin, shown by investigators to increase complex III-mediated ROS production in many types of cells, would be expected to cause PASMC to contract during normoxia, but its effects on HPV are difficult to predict.
9. Anti-oxidants should have no effect on PASMC tension in normoxia, but should block HPV.

In accordance with this theory, there is evidence that multiple contractile effector pathways in PASMC are activated by oxidation, although again identification of specific reactive cysteines oxidized in the constituent effector proteins of these pathways in these cells is lacking.

In the next several sections, we critically assess these theories by examining the results from the substantial number of papers which have investigated the effects of ETC blockers, and anti- and pro-oxidants on PASMC contraction and cytoplasmic Ca^{2+} levels under normoxic and hypoxic conditions. We also review the effects of hypoxia on PASMC [H_2O_2], superoxide and the ambient cytoplasmic thiol/disulfide equilibrium which have been obtained from studies employing genetically encoded ROS indicators or ESR. Finally, having considered this evidence, as well as the literature relating to the effects of ETC blockers and pro- and anti-oxidants on PASMC K_V currents, we describe what we feel are the strengths and weaknesses of the Redox and Mitochondrial ROS theories.

7.1. Pulmonary Effects of ETC Blockers

Observations that blockers of the proximal ETC decrease PASMC [ROS] production [145,263], increase PAP in perfused lung or cause PA constriction [145,263,280], block HPV [263], and mimic the effects of hypoxia on K^+ currents in SMC from PA [145,263], renal arteries [145] and ductus arteriosus [448] have consistently been invoked to support the Redox theory [19,33,36,125,263]. It is noteworthy, however, that the meaning of the term 'proximal ETC' used in these papers varied; in some cases it referred to the portion of the ETC upstream of ubisemiquinone [19], whereas in others it included the Q_i site in complex III, since antimycin is described as a blocker of the proximal ETC [33,125]. This ambiguity is worth keeping in mind, since, as described below, it is unlikely that antimycin would produce the same effect on mitochondrial ROS production as drugs such as rotenone which act upstream of ubisemiquinone.

Results of studies of the effects of ETC blockers on PASMC, isolated PA, or lung preparations are summarized in Table 2. Rotenone has been used more than any other ETC blocker to examine the role of mitochondrial ROS production in HPV and was seen to decrease basal ROS levels in most studies where this was examined. Rotenone generally increased basal tension in normoxia and was almost always observed to strongly inhibit HPV or reverse hypoxia-induced rises in $[Ca^{2+}]_{cyt}$. If rotenone was truly decreasing ROS production (which is however questionable [109,117,136,256,445,449,450]) these effects are predicted by the Redox theory. However, as noted previously [3], the generally observed transience of the rotenone-induced contraction does not accord with this model. The ability of rotenone to block HPV is also consistent with the Mitochondrial ROS theory (since it would oxidize the CoQ pool and thereby diminish ROS production by complex III), although the rise in basal tension it caused argues against this scheme.

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Table 2. Effects of ETC blockers on PASMC, PA or perfused lung preparations.

Preparation	Blocker µmol/L	Effect on basal ROS or *the response of basal ROS to hypoxia (RTH)* [ROS indicator]	Effect on basal tension or $[Ca^{2+}]_i$ amplitude compared to that of HPV	Effect on HPV or the hypoxia-induced increase in $[Ca^{2+}]_i$ *(HIIC)*	Ref.
Perfused rat lung	Rot 0.5		transient contraction; ~70% HPV	HPV abolished	[280]
Perfused rat lung	Rot 1.0	↓ 21% basal ROS [luminol]	transient ↑ in PAP; similar to HPV	HPV abolished	[263]
Perfused rat lung	Rot 1.0	↓12% basal ROS [lucigenin]	transient ↑ in PAP; similar to HPV	HPV abolished	[263]
Endo-isolated bovine PA	Rot 50			no effect on HPV	[391]
Perfused mouse lung	Rot 50		contraction; similar size to HPV		[212]
Isolated rat PA	Rot 0.1		no effect on basal tone	HPV abolished	[346]
Perfused rat lung	Rot 10.0		transient contraction; ~60% HPV		[451]
Perfused rat lung	Rot 0.013		transient contraction; ~40% HPV	HPV abolished	[38]
Endo- isolated rat PA[1]	Rot 5.0	↓~40% basal ROS [DCF]			[145]
Endo- isolated rat PA	Rot 5.0	↓~60% basal ROS [lucigenin]	transient contraction; ~60% HPV		[145]
Cultured rat PASMC	Rot 1.3			*~55%↓ in HIIC*	[348]
Perfused rabbit lung	Rot 0.03-0.35		transient ↑ in PAP abolished by eNOS blocker *N*G-monomethyl-L-arginine (L-NMMA)	Selective (*vs* U46619) dose-dependent block of HPV	[349]
Perfused rabbit lung	Rot 0.04			Phase1 HPV↓~40%, Phase2 HPV abolished	[352]
Perfused rat lung	Rot 50	↓~60% basal ROS [LO-12]	↑ in PAP; ~65% HPV		[452]
Mouse PASMC	Rot 1.27	↔ basal ROS , *RTH ↓~60%* [DCF]	no effect on basal $[Ca^{2+}]_i$	~40%↓ in HIIC	[359]
Perfused rabbit lung	Rot 0.35			↓~90%	[144]
Endo- isolated bovine PA	Rot 10	↓~50% basal ROS [lucigenin]	no effect on response to 20 mM K^+	No effect on HPV	[453]
Isolated rat PA	Rot 1.0			HPV ↓~85%	[323]
Isolated rat PA	Rot 1.0		no effect on basal tone		[18]
Perfused rat lung	Rot 50		↑ basal tension; similar to HPV		[125]
Rat PASMC	Rot 0.05		~60% ↑ in basal $[Ca^{2+}]_i$	*~50%↓ in HIIC*	[125]
Perfused rabbit lung	MPP+ 100		↑ in PAP; abolished by L-NMMA	Specific block of HPV (*vs* U46619)	[349]
Mouse PASMC	MPP+ 5.0	↔ basal ROS, *RTH ↓~85%* [DCF]	no effect on basal $[Ca^{2+}]_i$	*~60%↓ in HIIC*	[359]
Cultured human PASMC	MPP+ 5.0	*RTH* ↓ [Redox Red CC1]	no effect on basal $[Ca^{2+}]_i$		[360]

Preparation	Blocker mmol/L	Effect on basal ROS (bROS) or *the response to hypoxia (RTH)* (indicator)	Effect on basal tension or $[Ca^{2+}]_i$ amplitude compared to that of HPV	Effect on HPV or *Hypoxia-induced increase in $[Ca^{2+}]_i$ (HIRiC)*	Ref.
Perfused rat lung	Ant Conc. ??	↓ 60% bROS [LO-12]			[452]
Perfused rabbit lung	Ant 0.002			Phase 1 HPV abolished; Phase 2 ↓~60%	[352]
Mouse PASMC	Ant 5.7	↔ bROS, ↔ *RTH* [DCF]	No effect on basal $[Ca^{2+}]_i$	$[Ca^{2+}]_i$ during hypoxia unchanged	[359]
Endo- isolated bovine PA	Ant 10	↓ 60% bROS [lucigenin]	No effect on contraction to 20 mM K	No effect on HPV	[453]
Isolated rat PA	Ant 18.2		Sustained contraction ~30% of HPV		[18]

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Cultured human PASMC	Ant 5.7	↔ [ROS] during hypoxia [RR-CC1]		$[Ca^{2+}]_i$ during hypoxia unchanged	[360]
Human PA	Ant 5.7			No effect on HPV	[360]
Perfused rabbit lung	HQNQ 2-5		Transient ↑in PAP	Selective block of HPV	[349]
Perfused rabbit lung	HQNQ 50			HPV abolished	[144]
Perfused rat lung	Cyn 1000		Transient ↑ PAP; ~75% HPV		[280]
Perfused pig lung	Cyn 50		Large transient & small sustained ↑ PAP		[283]
Perfused rat lung	Cyn 1.5-15	transient ↑ ~15-36% [luminol]	↑ PAP; similar size as HPV	No effect on HPV	[263]
Perfused rat lung	Cyn 10		Transient ↑ PAP; ~110% HPV	HPV ↑ by ~25%	[38]
Isolated rat PA	Cyn 100		No effect on basal tone	Phase 2 HPV ↑ by ~50%	[346]
Endo-isolated rat PA	Cyn 1.0	No effect [DCF]			[145]
Rat PASMC	Cyn 10		↑ basal $[Ca^{2+}]_i$	No effect on HPV	[348]
Rat PASMC	Cyn 10000		large ↑ basal $[Ca^{2+}]_i$		[455]
Rat PASMC	Cyn 1.0		No effect on basal tone	*Small ↑ in HIIC*	[356]
Perfused rabbit lung	Cyn 50			Phase 1 HPV ↓~25%; Phase 2 abolished	[352]
Perfused rabbit lung	Cyn 10			HPV abolished	[144]
Perfused rabbit lung	Cyn 11			HPV abolished	
Isolated rat PA	Cyn 10		No effect on basal tone		[18]
Perfused rat lung	NaN_3 10000		Transient ↑ PAP; 33 % HPV	HPV abolished	[280]
Cultured mouse PASMC	NaN_3 1000	↔ bROS, ↔ *RTH* [DCF]	No effect on basal $[Ca^{2+}]_i$	*No effect on HIIC*	[359]
Cultured human PASMC	NaN_3 1000	↔ *RTH* [Redox Red CC])		$[Ca^{2+}]_i$ during hypoxia unchanged	[360]
Human PA	NaN_3 1000			No effect on HPV	[360]

Complex I blockers: Rotenone (ROT), 1-methyl-4-phenylpyridinium (MPP^+); Complex II blockers: 3-nitropropionic acid (NPA), 2-tenoyltrifluoroacetone (TTFA); Complex III blockers: myxothiazol (Myx; blocks at Qo), antimycin (Ant; blocks at Qi), 2-n-heptyl-4-hydroxyquinoline-N-oxide (HQNO; blocks at Qi). Complex IV blockers: cyanide (Cyn), sodium azide (NaN_3) [1] Endo - = endothelium-denuded. Abbreviations: RTH = Response of ROS signal to hypoxia; DCF = dichlorofluorescein; HIIC = Hypoxia-induced increased in {Ca^{2+}] signal.

Unlike rotenone, myxothiazol was not observed to alter basal ROS production in any of the three studies in which this was studied. Studies of mitochondria from various tissues show that myothiazol generally has no effect on, or increases, basal mitochondrial ROS production, depending on the cell type and substrates used to fuel oxidative phosphorylation [114,120,344,456,457]. Myxothiazol strongly antagonized the hypoxia-induced increase in ROS recorded in several studies and was consistently seen to depress HPV without itself altering basal tone or $[Ca^{2+}]_{cyt}$. Since myxothiazol blocks ROS production by complex III (although it can increase ROS release upstream of complex III), this combination of effects is generally consistent with the Mitochondrial ROS theory. In light of the observations that myxothiazol did not change basal [ROS], its lack of effect on basal tone also fits with the Redox Theory. However, since the potential effects of myxothiazol on any hypoxia-induced decrease in ROS production by complex I in PASMC are uncertain, the implications for the Redox theory of the block of HPV it exerted are unclear.

Antimycin was seen to lower (6 studies) or not affect (2 studies) ROS levels. These results are perplexing, since there is a widespread consensus, emerging from numerous studies in multiple types of cells, that antimycin increases ROS production by complex III, and can also do so at more proximal sites in the ETC by causing reduction of the CoQ pool [114,121]. If antimycin was indeed causing a paradoxical fall in basal ROS production, the increase in basal contraction it usually (in 4

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of 5 studies) evoked was in accord with the Redox theory. However, although antimycin blocked HPV in 3 studies, as predicted by the Redox theory, it more often (6 studies) had no effect on or increased the response to hypoxia, which does not conform to this model. On the other hand, the normoxic contraction it often caused is consistent with the Mitochondrial ROS theory [38,342], although the apparent fall in ROS production is not. In any case, since no single investigation of antimycin in PA or PASMC evaluated its effects on all of the key parameters, (basal [ROS] and tension development or $[Ca^{2+}]_{cyt}$ in normoxia and hypoxia), it is difficult to draw any meaningful conclusions from its observed effects.

The effect on HPV of the complex IV blockers cyanide and NaN_2, is difficult to predict from either the Redox or Mitochondrial ROS hypotheses. However, since these drugs would be expected to mimic the inhibition of complex IV caused by hypoxia, they can induce reduction of the CoQ pool which might increase ROS production by complex III. Thus, evocation of contraction by these drugs would be consistent with the Mitochondrial ROS hypothesis [348], although this would probably depend on the whether the degree of the block was similar to that caused by hypoxia. Cyanide was observed to cause contraction in most studies in which it was used, this was never seen with azide. The reasons for this difference are not obvious.

In summary, the use of conventional ETC blockers to study HPV has yielded inconsistent results which, when taken together, do not provide convincing support for either the Redox or Mitochondrial ROS models. More importantly, although these drugs, if employed intelligently, can provide important information about how hypoxia influences mitochondrial ROS production, their use has a number of drawbacks which need to be taken into account when interpreting their effects. Firstly, the effects of ETC blockers on overall mitochondrial ROS production are difficult to predict. For example, rotenone inhibits ROS production at complex I_{CoQ} and downstream sites of the ETC, but tends to have the opposite effect on complex I_F, O_F and P_F [109]. Secondly inhibition of respiration caused by ETC blockers has manifold impacts on mitochondrial function which go beyond their direct effects on ROS production [458]. One of these which is likely to have immediate consequences for vascular tone is that ETC blockers depolarize $D\Psi_m$ in isolated mitochondria and intact cells from many cells/tissues, including pulmonary artery and other SMC [459–461]. This inhibits mitochondrial Ca^{2+} uptake, leading to complex effects on Ca^{2+} signaling in SMC which generally tend to inhibit contraction by interfering with the generation of Ca^{2+} waves [461–463] and by causing the opening of BK_{Ca} channels, even though global $[Ca^{2+}]_{cyt}$ may not change [463] or even increase [461]. Also, by depressing O_2 consumption, inhibition of respiration can increase the intracellular O_2 concentration [464–466]. This could potentially decrease the effect of hypoxia on extramitochondrial O_2 sensors, thereby attenuating HPV. In addition, there is evidence from Hernansanz-Agustin et al [183] that rotenone can both mimic and prevent hypoxia-induced complex I deactivation. In addition, rotenone at concentrations of > 0.5 μM as used in many but not all HPV studies, can block K^+ channels [321]. Finally, it is apparent from the comprehensive investigation by Weissmann and colleagues that the observed effects of ETC blockers on PAP in perfused lungs are greatly distorted by the effects of NO release [349].

7.2. Pulmonary Vascular Effects of Antioxidants

As shown in Table 3, antioxidants have often been used to characterize how ROS affect pulmonary vascular tone under normoxic and hypoxic conditions.

The enzymes SOD and catalase have been used more frequently than any other antioxidant agents to study HPV. In most investigations, these enzymes, used either alone or in combination, had no effect on basal tone or HPV. It is unclear whether this reflected a genuine lack of response or was due to their poor entry into cells. However, application of high concentrations of SOD and catalase together to isolated perfused rat lung greatly increased HPV with no effect on basal PAP, and treatment with liposomes containing a low concentration of both enzymes, a procedure designed to increase their entry into cells, increased basal tone and caused a smaller enhancement of HPV [276]. These responses are generally in accord with the Redox theory. Conversely, HPV in pig PA was

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inhibited by the combination of SOD and catalase [353], which supports the mitochondrial ROS hypothesis. Interestingly, in both preparations SOD on its own exerted an effect similar to that of SOD and catalase in combination, which implies that superoxide itself must have been acting as a mediator in HPV.

Most of the studies which employed chemical antioxidants found that they inhibited HPV without affecting basal tone. Nevertheless, an antioxidant – induced increase in basal tension, which would be predicted by the Redox theory, was seen by some investigators. This was observed most often in bovine pulmonary arteries (BPA), in which ebselen, DTT and DHLA raised basal tone (although NBT and Tiron did not). HPV in BPA was inhibited by NBT and ebselen, not affected by Tiron, and increased by DTT and DHLA. It is difficult to fit these results into a coherent pattern, although Wolin and colleagues, who carried out most of these investigations, concluded that a fall in [ROS] contributes to HPV (see Section 8). However, they also found that HPV in BPA is not blocked by rotenone [453], implying that O_2 sensing in BPA is independent of mitochondrial ROS production. Likewise, Olson's laboratory, which reported the effects of DTT and DHLA, ascribed these to an increase in cellular [H_2S] rather than to an effect on mitochondrial [ROS] [164].

Apart from the experiments carried out in BPA, Table 3 shows that studies employing antioxidants have mostly reported that they inhibit HPV but do not affect basal PA tone or $[Ca^{2+}]_{cyt}$ tends to support the Mitochondrial ROS theory.

Nevertheless, although if employed correctly antioxidants can provide information about how ROS regulate cellular processes, their use for this purpose, especially if not supported by parallel measurements of cellular superoxide and H_2O_2, has come under increasing criticism [381]. Each antioxidant has a unique mechanism of action, which is often complex and/or not fully understood (e.g. TEMPOL [467]; TIRON [468] N-acetylcysteine [469]; ebselen [470]). Also, some antioxidants (e.g. N-acetylcysteine, manganese(III)tetrakis(1-methyl-4-pyridyl)porphyrin-- MnTMPyP) can increase the formation of reactive sulfur species [469,471] which could potentially mimic the effect of H_2O_2 on reactive cysteines [48]. In addition, SOD-mimetics such as MnTMPYP would be predicted to cause an increase in cellular H_2O_2 along with the fall in superoxide [472] although this may not be the case for TEMPOL and Tiron, which, although often described as SOD-mimetics, also apparently suppress the production or effect of H_2O_2 [467,473]. This is an important consideration when considering the effects of antioxidants on HPV, as most studies, particularly those using perfused lungs, did not include measurements of ROS (and those that did used ROS indicators which have since been largely discredited).

The overexpression or knockdown of cellular anti-oxidant enzymes allows the manipulation of redox tone in specific cellular compartments, and avoids some of the uncertainties inherent in the use of chemical anti-oxidants or proteins such as SOD and catalase [381]. Investigations using cultured PASMC from mice in which catalase was overexpressed by transfection [348,363] found that this blunted the rise in $[Ca^{2+}]_{cyt}$ evoked by hypoxia in PASMC. Freshly isolated PASMC from mice which had been genetically modified to overexpress catalase also demonstrated a decreased hypoxia-induced increase in $[Ca^{2+}]_{cyt}$ compared to cells from wild-type controls [359]. A similar effect was seen in PASMC from mice in which glutathione peroxidase had been overexpressed in order to enhance the metabolism of H_2O_2, whereas cells from glutathione peroxidase knockouts demonstrated an augmented response of $[Ca^{2+}]_{cyt}$ to hypoxia. It could be argued, in line with the Redox theory, that catalase or glutathione peroxidase overexpression was causing a basal increase in $[Ca^{2+}]_{cyt}$ by reducing cytoplasmic redox couples, thereby preventing a further similar effect of hypoxia. This possibility, however, is not supported by the observation [359] that the basal level of $[Ca^{2+}]_{cyt}$ was not altered in PASMC from any of the genetically altered mice. Therefore, these results consistently agree with the predictions of the Mitochondrial ROS theory.

More recently, Sommer et al [129] reported that HPV was absent in isolated perfused lungs from mice expressing AOX, which has an anti-oxidant effect since it diminishes ROS production by Complex III [383]. Basal superoxide production was similar in the PASMC from AOX mice and wildtype controls, as was the resting membrane potential, indicating that baseline ROS production

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by Complex III was not influencing the cytosolic redox state or K^+ channel activity under normoxic conditions. These results provide strong evidence favoring the Mitochondrial ROS theory.

Table 3. Effects of antioxidants on HPV.

Preparation	Antioxidant	Effect on [ROS]	ROS indicator	Effect on basal tension/PAP or $[Ca^{2+}]_i$	Effect on response to hypoxia	Ref.
in situ blood-perfused rat lung	Catalase 5000 U				No effect on HPV	[474]
Pig PASMC	Catalase 200U/ml	Hypoxia-induced ↑ in ROS abolished	DCFH		No effect on HPV	[353]
in situ perfused rat lung 0% O_2	Catalase 2 x 10^{-3} U/kg body wgt				No effect on HPV	[475]
perfused mouse lung 2% O_2	Catalase 200U/ml					[476]
[1] Endo - Bovine PA	Catalase 1 μM		Luminol/ HRP	[2] No effect on 25mM K contraction	No effect on HPV	[477]
Endo – Bovine PA	Catalase 1 μM	[3]ROS signal virtually abolished	Luminol/ HRP			[453]
Isolated mouse PA	Catalase 1 μM				No significant effect on HPV	[478]
Pig proximal PA	Catalase + PEG-Catalase each 500 U/ml				HPV ↓ by ~85%	[479]
isolated sheep PA	SOD 150 U/ml				No effect on HPV at 6 and 21 minutes	[480]
Perfused rat lung	SOD 100 U/ml			↑ in PAP ~25% of the amplitude of HPV		[263]
perfused rabbit lung	SOD 100μg/ml	~50% ↓ in basal ROS	lucigenin			[454]
in situ blood-perfused rat lung	SOD 2750 U				No effect on HPV	[474]
perfused rabbit lung	SOD 100-2000 U/ml			No effect on PAP	No effect on acute HPV	[481]
Endo + isolated pig PA	SOD 150 U/ml			No effect on basal tension	HPV ↓ by ~75%	[353]
Pig PASMC	SOD 150 U/ml	Hypoxia-induced ↑ in ROS decreased by ~75%	DCFH			[353]
In situ perfused rat lung 0% O_2	SOD 2mg/kg body weight				No effect on HPV	[475]
Endo – bovine PA	SOD 1 μM			No effect on 25mM K contraction	HPV ↓ by ~40%	[477]
Isolated rat 2nd & 3rd order PA	SOD 200 U/ml	No effect on basal ROS]	DHE, MitoSox Red			[415]

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Preparation	Antioxidant	Effect on [ROS]	ROS indicator	Effect on basal tension/PAP or $[Ca^{2+}]_i$	Effect on response to hypoxia	Ref.
Isolated pig proximal PA	SOD 500 U/ml + MnTMPyP 100μM				non-significant ↓ in HPV	[479]
Perfused rat lung	SOD 245 U/ml + CAT 50 U/ml			No effect on basal PAP	No effect on HPV	[276]
Perfused rat lung	SOD 490 U/ml + CAT 100 U/ml			No effect on basal PAP	↑↑ HPV	[276]
Perfused rat lung	SOD + CAT in liposomes low dose			↑ normoxic PAP	↑ HPV	[276]
Perfused rat lung	SOD + CAT in liposomes high dose			↑↑ normoxic PAP	No effect on HPV	[276]
Endo + pig PA	SOD 150 U/ml + CAT 200 U/ml			No effect on basal tension	HPV abolished	[353]
Pig PASMC	SOD 150 U/ml + CAT 200 U/ml			Hypoxia-induced increase in [ROS] abolished		[353]
Perfused rat lung	Ebselen 50 μM			No effect on basal PAP	HPV ↓ by ~85%	[38]
Endo – isolated bovine PA	Ebselen 100 μM	basal ROS (attributed to H_2O_2) ↓~80%	Luminol/ HRP	20mM K contraction (used by this laboratory as a measure of basal tension) ↑ by ~100%	HPV ↓ by ~65%	[477]
Endo - isolated bovine PA	Ebselen 100 μM	basal ROS (attributed to cellular $O2^{-}$) ↓~80%	Lucigenin	25 mM K contraction ↑30-50%	HPV ↓ by ~65%	[453]
Isolated rat 2nd & 3rd order PA	Ebselen 100 μM				No effect on Phase 1 HPV Phase 2 HPV ↓ ~40%	[18]
Perfused rat lung	[4] DETCA 1mM			No effect	HPV ↓ by 70%	[38]
Perfused rabbit lung	DETCA 0.1 – 10 mM				dose-dependent ↓HPV but also of U46619 contraction	[350]
Endo - isolated bovine PA	DETCA 10 mM	'$O_2^{-\cdot}$' ↑ ~220% 'H_2O_2' ↓ ~80%	Lucigenin Luminol/ HRP	20mM K contraction (used by this laboratory as a measure of basal tension) ↑ ~400%	HPV ↓ by 70%	[477]
isolated extra-lobar guinea pig PA	DETCA 5 mM			↑ basal tension; ≤25% of the amplitude of HPV	HPV ↑↑	[341]
Endo - isolated bovine PA	NBT 0.3 mM	basal ROS ↓ ~55%	Lucigenin	No effect on basal tone	HPV ↓ by 90%	[482]
Isolated rat PA	NBT 0.5 μM				Slight ↓ of phase 2 HPV	[483]

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Preparation	Antioxidant	Effect on [ROS]	ROS indicator	Effect on basal tension/PAP or $[Ca^{2+}]_i$	Effect on response to hypoxia	Ref.
Perfused rabbit lung	[5] NBT + L-[6] NMMA 0.2-1.0			No effect on basal tone	Dose-dependent ↓ in HPV	[481]
Perfused rabbit lung	NBT + L-NMMA + acetylsalicylic acid 0.2-1 mM			No effect on basal tone	Dose-dependent ↓ in HPV	[481]
isolated extra-lobar guinea pig PA	[7] TETA 5 mM			sustained contraction; ≤10% of the amplitude of HPV	HPV ↑↑	[341]
Perfused rabbit lung	TETA 1-25 mM			No effect on basal tension	dose-dependent ↓in HPV	[350]
Perfused rabbit lung	TETA +L-NMMA 1-25 mM			No effect on basal tension	dose-dependent ↓in HPV	[350]
Perfused rabbit lung	TETA +L-NMMA Initially 25 mM				↓↓ both phases of HPV	[351]
Endo - isolated bovine PA	Tiron 10 mM	basal ROS ↓ ~55%	Lucigenin	No effect on basal tension	No effect on HPV	[482]
Perfused rabbit lung	Tiron 0.2-0.8 mM	basal ROS (said to be superoxide) ↓ ~90%	Lucigenin			[454]
Perfused rabbit lung	Tiron 0.01-10 mM			No effect on basal tension	No effect on acute HPV	[481]
rabbit PA	Dithiothreitol 3mM			↑ basal tension		[335]
Isolated rabbit PA	Dithiothreitol 3mM			↑ basal $[Ca^{2+}]_i$ and membrane depolarization		[335]
Rat PASMC	Dithiothreitol 3mM			↑↑ basal tension		[335]
Rat PASMC	Dithiothreitol 3mM			↑↑ basal $[Ca^{2+}]_i$		[335]
Isolated rat PA	Dithiothreitol 1mM				Phase 2 HPV completely reversed	[483]
Isolated rat PA	Dithiothreitol 1mM				Phase 2 HPV abolished	[435]
Isolated bovine PA	Dithiothreitol 0.001- 10mM				↑ HPV with ≥ 100 μM DTT	[164]
Isolated bovine PA	[8]DHLA 0.1 mM			↑ basal tension in some arteries	↑HPV in some arteries	[164]
Perfused rat lung	[9]PDTC 0.5 μM			No effect on basal PAP	HPV ↓ by ~65%	[38]
Rat PASMC	PDTC	no basal effect	HSP-FRET	No effect on basal $[Ca^{2+}]_i$	↓hypoxic-induced increase in $[Ca^{2+}]_i$ by ~90%	[356]
Isolated Endo+ and Endo- rat PA	Co-enzyme Q 500μM			small ↑ in basal tension, cell depolarization		[332]

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Preparation	Antioxidant	Effect on [ROS]	ROS indicator	Effect on basal tension/PAP or $[Ca^{2+}]_i$	Effect on response to hypoxia	Ref.
Isolated endo+ and endo- rat PA	Duroquinone 5 -500μM			Large ↑ in basal tension, cell depolarization		[332]
Rat PASMC	N-acetylcysteine 500μM	no basal effect	HSP-FRET		↓hypoxic-induced increase in $[Ca^{2+}]_i$ by ~95%	[356]
Perfused rabbit lung	Trolox 20μM				HPV ↓ by ~47% at 5 min and abolished at 30 min	[484]
Perfused rat lung	TEMPOL ~2.9 mM				HPV ↓ by ~90%	[485]
	TEMPOL ~2.9 mM + L-NAME				HPV abolished	[485]
Isolated 2nd and 3rd order rat PA	TEMPOL 3 mM				No effect on Phase 1 HPV, Phase 2 abolished	[18]
Perfused mouse lung	MitoQ				HPV ↓ by ~50%	[486]
Perfused mouse lung	MnTMPyP			No effect on basal PAP	No effect on HPV	[476]
Mouse lung slices	EUK-34				abolished hypoxia-induced rise in $[Ca^{2+}]_i$	[363]
Isolated sheep PA	N-t-butyl-a-phenylnitrone				No effect on HPV at 6 and 21 minutes	[480]
In situ perfused rat lung	Dimethylthiourea 100mg/kg bw				HPV ↓ by ~40%	[475]
In situ perfused rat lung	Dimethylsulfoxide 1 ml/kg bw				HPV ↓ by ~40%	[475]
In situ perfused rat lung	Allopurinol 30 mg/kg bw				HPV ↓ by ~40%	[475]
In situ blood perfused rat lung	Desferrioxamine 2 mg/kg bw				No effect on HPV	[474]
primary cultured rat microvascular PASMCs	Catalase overexpression				Attenuated hypoxia-induced rise in $[Ca^{2+}]_{cyt}$ by ~40%	[348]
Freshly isolated mouse PASMCs	Catalase overexpression	Basal ROS ↔ , but hypoxia-induced rise attenuated			Attenuated hypoxia-induced rise in $[Ca^{2+}]_{cyt}$ by ~50%	[359]
Mouse lung slices	Catalase overexpression	Abolished hypoxia-induced rise in Ro-GFP oxidation	Ro-GFP		Attenuated hypoxia-induced rise in $[Ca^{2+}]_{cyt}$	[363]
primary cultured rat microvascular PASMCs	Cytosolic catalase overexpression	Abolished hypoxia-induced rise in Ro-GFP oxidation	Ro-GFP			[361]

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Preparation	Antioxidant	Effect on [ROS]	ROS indicator	Effect on basal tension/PAP or $[Ca^{2+}]_i$	Effect on response to hypoxia	Ref.
primary cultured rat microvasc PASMCs	Mitochondrial catalase overexpression	No effect on hypoxia-induced fall in mitochondrial Ro-GFP oxidation				[361]
Primary cultured rat microvascular PASMC	Glutathione peroxidase overexpression	↓Hypoxia-induced rise in HSP-FRET oxidation	HSP-FRET		↓ hypoxia-induced rise in $[Ca^{2+}]_{cyt}$	[356]
Freshly isolated mouse PASMC	Gpx overexpressing mice	↓Hypoxia-induced rise in ROS			↓hypoxia-induced rise in $[Ca^{2+}]_{cyt}$	[359]
Freshly isolated mouse PASMC	Gpx knockout mice	↑ Hypoxia-induced rise in ROS			↑hypoxia-induced rise in $[Ca^{2+}]_{cyt}$	[359]
Mouse PA	AOX expression	↓↓Hypoxia-induced rise in ROS			HPV almost abolished	[129,253]

[1]Endo +/- refer to endothelium-intact and -denuded arteries, respectively. [2] The contraction induced by 25 mM K in this study was used by the authors as an index of basal reactivity. [3] The ROS signal provided by the lucigenin/HRP assay used in this study was considered by the authors to reflect extracellular ROS. [4] diethyldithiocarbamic acid; [5] nitro blue tetrazolium; [6] (N(G)-monomethyl L-arginine); antagonist of endothelial nitric oxide synthase; [7] triethylenetetramine; [8] dihydrolipoic acid; [9] pyrrloidinedithiocarbamate.

7.3. Effects of Oxidants on Basal Tone and HPV

Table 4a,b list the effects of H_2O_2 and other oxidants on pulmonary vascular tone in normoxia and also on HPV and contractions caused by various vasoconstrictors. The most widely studied oxidant, H_2O_2, was used over a wide concentration range (0.5 mM - 10 mM), raising the question of which concentrations are likely to produce physiologically relevant increases in intracellular $[H_2O_2]$.

As discussed in Section 4, the extracellular $[H_2O_2]$ *in vivo* is thought to be 1-5 μM [74], whereas the average cytoplasmic $[H_2O_2]$ is generally said to be in the low nM range [32,226,487] but may be higher in the subplasmalemmal region. According to the mathematical model [240] developed by Sykes and colleagues, and assuming that PASMC have a width of roughly four microns [488], in the presence of an $[H_2O_2]_{ECS}$ of 2mM the basal subplasmalemmal and core $[H_2O_2]$ would be ~6 and ~2 nM, respectively, and applying 10 μM H_2O_2 would increase these H_2O_2 concentrations by ~30 and ~10 nM. It has also been reported [269] [270] that the half-maximal oxidation of the cytoplasmic GSSG/GSH redox couple in HeLa cells and cardiac myocytes was induced by extracellular application of ~10 and ~20 μM H_2O_2, respectively. In both types of cells, the threshold for oxidation of GSH was near ~5 μM $[H_2O_2]$ and oxidation became maximal at ~50 μM $[H_2O_2]$. Interestingly, diamide oxidized GSH over a similar range of concentrations [270]. Since the cellular GSSG/GSH ratio is a key factor in setting the cytoplasmic redox tone [357] and is crucial for maintaining cellular thiol redox balance [104] these considerations suggest that that application of exogenous H_2O_2 or diamide in the concentration range below ~50 mM may be suitable for mimicking physiologically relevant levels of oxidant signaling.

As shown in the tables the results from almost all studies, including those that applied H_2O_2 within or near this concentration range(Table 4a), form a consistent pattern, which was also observed when other oxidants were tested (Table 4b): H_2O_2 caused a sustained contraction/rise in $[Ca^{2+}]_{cyt}$ in PA/PASMC if applied under baseline normoxic conditions, but induced relaxation or reversed rises in $[Ca^{2+}]_{cyt}$ in PA/PASMC which had been constricted/stimulated by hypoxia or vasoconstrictors.

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Table 4a. Effects of H_2O_2 on basal tension/Ca^{2+}, and contractile responses to hypoxia and vasoconstrictors in PASMC, pulmonary artery, and perfused lung.

Preparation	[H_2O_2] μmol/L	Effect of H_2O_2 on basal tension or $[Ca^{2+}]_i$	Effect of H_2O_2 on HPV	Effect of H_2O_2 on response to vasoconstrictor	Ref.
Perfused rat lung	0.5 – 1.0	no effect on basal PAP	HPV abolished	Rise in PAP caused by angiotensin 2 ↓ by 72%	[273]
Bovine PA	0.1 -100			Partial, concentration-dependent inhibition of 5-HT, histamine and high K^+ contractions	[489]
[1] Endo + and – rabbit PA	100 - 1000	large, slow, concentration-dependent contraction			[490]
Rat large PA	200	small biphasic contraction			[491]
Rat PA	30	small contraction	No effect on HPV	No effect on contraction to 5μM $PGF_{2\alpha}$	[492]
Rat PA	500	small contraction	HPV almost abolished	Contraction to $PGF_{2\alpha}$ abolished, high K^+ contraction ↓ >50%	[492]
Rat PA	10,000	small slow contraction			[493]
Porcine intra-parenchymal PA	100-3100	Slow sustained concentration-dependent contraction			[493]
Rat pulmonary vascular pericytes	10 - 1000	concentration-dependent relaxation followed by contraction			[494]
Perfused rat lung	100	contraction; amplitude = ~55% of HPV			[38]
Rat cultured PASMC	50	↑in $[Ca^{2+}]_i \geq$ that caused by hypoxia			[128]
Rat main PA	50	large increase in tension			[495]
Endo – bovine PA	100			No significant effect on contraction to 30K^+	[496]
Endo – bovine PA	1000			↑ 30 mM K^+-induced contraction by ~40%	[496]
Rat PASMC	10-1000	concentration-dependent ↑in $[Ca^{2+}]_i$			[410]
Endo - rat PA	1-100	30 μM H_2O_2 caused sustained contraction with EC_{50} 12 μM, amplitude [2]17% max. Also ↑ in $[Ca^{2+}]_i$		30 μM H_2O_2 caused transient endothelium-dependent 29% relaxation of constriction induced by 10μM $PGF_{2\alpha}$	[419]
Pig PA	100	small transient ↑ in $[Ca^{2+}]_i$ and tension, then large slow ↑ tension			[497]
Endo – bovine PA	1-1000			Conc. - dependent relaxation of contraction to 100nM 5-HT + hypoxia	[498]
Rat PA	10-100	conc.-dependent sustained contraction			[323]
Rat PA	100	small, slowly developing contraction			[407]
Rat PA	110	contraction, 23% max			[499]
Rat PA	30	small transient and sustained contractions (12 & 5% max.)			[416]
Rat cultured PASMC	10	2-fold ↑ in store-operated Ca^{2+} entry			[409]

[1] E^- and E^+ refer to endothelium-denuded and -intact arteries. [2] 'max' refers to the amplitude of the high K^+ solution.

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Table 4b. Effects of other oxidants on basal tension/Ca^{2+}, and contractile responses to hypoxia and vasoconstrictors in PASMC, pulmonary artery, and perfused lung.

Preparation	Oxidant & conc. in μmol/L	Effect on basal tension or $[Ca^{2+}]_i$	Effect on HPV or hypoxia-induced ↑in $[Ca^{2+}]_i$	Effect on response to constrictor	Ref.
perfused rabbit lung	t-BOOH 200	sustained contraction; due to unidentified prostanoid			[273]
Endo - rat PA	t-BOOH 10.0	transient followed by smaller sustained ↑ in tension			[322]
Rat PA	t-BOOH 10-100	concentration-dependent sustained ↑ in tension			[323]
Perfused rat lung	t-BOOH 100		75% reversal of ongoing HPV		[125]
Rat PASMC	t-BOOH 1.0		reversal of hypoxia-induced increase in$[Ca^{2+}]_i$		[125]
In vivo PVR in dogs	diamide 5mg/kg		almost complete reversal of ongoing HPV	Increase in PVR caused by $PGF_{2\alpha}$ was ↓ by >50%	[272]
Perfused rat lung	diamide ~200		transient ~70% ↓ in HPV		[274]
Endo – bovine PA	diamide 1.0	slow ↑ in basal force; due to activation of ERK			[496]
Endo + & - Rat PA	diamide 100			relaxation of contraction to coenzyme Q/duroquinone, E_m hyperpolarization	[332]
Endo + & - Rat PA	diamide 1-1000			relaxed contractions to phenylephrine (IC_{50}= 58μM) & 40mM K^+ (IC_{50}=335μM)	[336]
Perfused rat lung	glu/glu ox	no effect on control PAP; but ↑ PAP after indomethacin	HPV abolished		[273]
Rat main PA	glu/glu ox	large increase in tension			[495]
Endo- Rat small PA	glu/glu ox	rapid-onset rise in $[Ca^{2+}]_i$ (22% of high K^+ response)			[419]
Perfused rat lung	x/xo	no obvious effect	sustained 30-50% ↓ in HPV		[274]
Perfused rat lung	x/xo	no effect	~55% ↓in HPV	contraction to angiotensin 2 ↓ by 58%	[276]
Rat PA	x/xo	sustained dose-dependent contraction (~9% max.)			[415]
Endo + & - Rat PA	LY83583 10.0	contraction of ~15% high K, ↑↑ by 5μM $PGF_{2\alpha}$ pre-tone			[415]
Rat PA	Ly83583 10.0	contracted U46619-preconstricted PA in presence of eNOS blocker L-NAME			[326]
Rat PA	LY83583 10.0	moderate sustained contraction			[407]
Rat PA	Ly83583 0.1 - 10	no contraction, but ↑↑ responses to high K^+ and U46619			[416]
Rat PA	Menadione 10.0	small sustained contraction (~6% max)			[415]
Rat PA	BCNU 100	no effect, but ↑ contractile response to LY83583			[415]
Rabbit fetal resistance PA	DTNB 1000	no effect on basal tone under normoxic conditions	relaxation of PA pre-constricted by hypoxia + phenylephrine		[335]

Abbreviations: t-BOOH = tert-butyl hydroperoxide; glu/glu ox = glucose/glucose oxidase; x/xo = xanthine/xanthine oxidase; BCNU = 1,3-bis(2-dichloroethyl)-1-nitrosourea; DTNB = 5,5-dithio-bis-2-nitrobenzoic acid. .

These opposing actions of H_2O_2 were not necessarily due to the use of different preparations or experimental set-ups by various investigators, since both were recorded when all conditions except the presence or absence of pre-constriction were identical [419]. Notably, a similar pattern has been observed upon application of H_2O_2 to systemic arteries [500,501].

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An investigation of the constricting effects of H_2O_2 in PA [419] found that the EC_{50} for H_2O_2-induced contraction in rat PA was 12μM. 30 μM H_2O_2 caused a rapidly-developing and sustained contraction of small rat PA which was somewhat larger than that evoked by hypoxia (~22-25 Torr) under similar conditions [435]. The H_2O_2-induced contraction was endothelium-independent and associated with a rise in $[Ca^{2+}]_i$ which was reversed by ryanodine but insensitive to the removal of extracellular Ca^{2+}, indicating that it was due to Ca^{2+} release from the SR (see also [410]. The initial phase of the contraction (5-10 minutes) was partly blocked by the RhoK antagonist Y-27632 and was only slightly depressed by the membrane permeable Ca^{2+} chelator BAPTA-AM, suggesting a role for Ca^{2+} sensitization. The sustained contraction was BAPTA-sensitive but not affected by Y-27632. H_2O_2 contracted a-toxin-permeabilized PA; this effect was abolished by blockers of conventional PKC isoforms, which also strongly depressed the contraction in intact arteries, suggesting that it was also partially due to PKC-dependent Ca^{2+} sensitization. It was also reported that application of 10 μM H_2O_2 increased store-operated Ca^{2+} influx in cultured rat PASMC by stimulating the association of STIM1 with both Orai1 and TRPC1 [409].

Although there have been relatively few investigations of how H_2O_2 and other oxidants cause vasorelaxation in PA, the mechanisms which have been characterized in these arteries (opening of K_V channels and activation of protein kinase G [332,336,498,502]) are in line with the evidence available from studies carried out in systemic arteries [37,503–506]. Schach et al [336] also reported that 100 μM diamide inhibited store operated Ca^{2+} entry in rat PA, although a later and more detailed investigation reported that 10 μM H_2O_2 had the opposite effect in rat PASMC [409].

Conclusions drawn on the basis of the effects extracellular H_2O_2 application on PASMC contraction or $[Ca^{2+}]_{cyt}$ should interpreted cautiously, since cellular H_2O_2 signalling is highly localized [92,254,297] whereas external application of H_2O_2 to PASMC increases its global intracellular concentration [255]. A further complication is that adding exogenous H_2O_2 (and diamide) causes oxidation of the mitochondrial matrix [210,270], whereas hypoxia apparently has the opposite effect in PASMC (Section 7.4). Taken together with the evidence that H_2O_2 and other oxidants can activate both constricting and dilating mechanisms in PA, and that which of these dominates depends on the pre-existing level (or type [500]) of contraction, these considerations imply that whereas responses to exogenous oxidants can provide meaningful information about the redox regulation of specific contractile mechanisms if these are studied in isolation, the effect of adding exogenous oxidants on vascular tone or $[Ca^{2+}]_{cyt}$ in PASMC under normoxic or hypoxic conditions may not necessarily reflect the role of endogenous ROS production in HPV.

7.4. Effects of Hypoxia on PASMC ROS Levels

Numerous investigations, many of which are described in Sections 5.1 and 5.2, examined the effects of hypoxia on ROS in PASMC, PA, and lung tissue. As set forth in previous reviews [3,318], studies carried out prior to 2006, which utilized chemical ROS probes such as lucigenin and DCF, presented mixed results: some laboratories found that hypoxia decreased ROS and others found the opposite effect. Possible explanations for these discordant findings include the use of different preparations, levels of hypoxia, and ROS indicators. Indicator signaling arising from extracellular ROS, which could far outweigh that emanating from cells [32], may have obscured effects on cellular ROS in studies carried out in perfused lungs and isolated arteries [3]. Evidence that hypoxia causes a fall in ROS production by PAECs [507,508] and isolated lung fibroblasts [379] also suggests that the effects of hypoxia on overall [ROS] production by lung tissue may not accurately reflect what is happening in PASMC.

The chemical indicators used in these studies, most of which were said to detect superoxide levels, have also been criticized on the basis that they are in reality non-specific [509,510], although with the proper controls they can provide useful qualitative information about cellular oxidant levels [88,381]. However, there are additional difficulties in interpreting the signals arising from these probes which may arise specifically when they are used to measure the effects of changes in PO_2 [88]. For example, DCFH-DA, lucigenin, luminol and L-012 are converted by cellular oxidants (DCFH-

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DA, luminol, L-012) or enzyme reductases (lucigenin) to intermediates which generate a signal when they react with superoxide. Unfortunately, these intermediates also react with O_2 to form superoxide, a process termed 'redox cycling' [510–512]. Lucigenin has been reported to accumulate in the mitochondria and primarily detect intra-mitochondrial superoxide [513,514]. An additional confounding factor with lucigenin is that its redox cycling is promoted by NADH [515], the concentration of which is increased by hypoxia [346]. Apart from the lack of selectivity of both dihydroethidium (DHE) and MitoSox for superoxide due to their non-specific oxidation to ethidium [511], their fluorescence is also affected by their distribution into the mitochondria, which is sensitive to $\Delta\psi_m$ [381,516].

Several of these investigations also utilized the the Amplex Red/horseradish peroxidase (HRP) assay, which measures extracellular [H_2O_2] and can be used to detect changes in cellular [H_2O_2], since these lead to its altered leakage out of cells [517,518]. Amplex Red is considered to provide a high quality, specific and very sensitive measure of overall cellular H_2O_2 production [354]. It is therefore noteworthy that three studies using this assay reported that hypoxia decreased ROS production by PASMC [145,285,452]. However, it is not clear whether this was due to an effect on mitochondrial ROS production, since the activity of Nox is probably curtailed at the PO_2 levels used in most studies of HPV [59], and has been shown to make a substantial contribution to the overall release of H_2O_2 from a range of cell types [519].

In view of the shortcomings of the conventional intracellular ROS indicators , several laboratories have examined the effect of hypoxia in PASMC using genetically-encoded ROS or redox indicators such as Ro-GFP [520], which responds to the ambient thiol/ disulfide equilibrium, and HyPer [287], which reacts with H_2O_2. In addition to responding to specific ROS or redox changes, these can be targeted to specified cell compartments and do not undergo redox cycling, so are therefore seen as greatly superior to chemical ROS indicators [362,381,521,522]. The results of these investigations are summarized in Table 5.

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Table 5. Effects of hypoxia on cell H_2O_2, indices of redox balance, and mitochondrial membrane potential.

Preparation	Level of hypoxia	Indicator/ compartment	Indicator responds to…	Effect of hypoxia	Additional comments	Ref.
Cultured rat PASMC	1.5% O_2	HSP-FRET, mainly in cytoplasm	H_2O_2	↑ oxidation	Hypoxia caused gradual ↑ in HSP-FRET and Ca^{2+} signals with similar timecourses	[356]
Cultured rat PASMC	1.5% O_2	Mito-Ro-GFP mitochondria	GSSG/GSH	↓ oxidation		[361]
		IMS – Ro-GFP IMS		↑ oxidation		
		Cyto – Ro-GFP cytoplasm		↑ oxidation	Hypoxia-induced oxidation of cyto-Ro-GFP abolished by overexpression of cytosolic catalase	
Cultured rat renal artery SMC	1.5% O_2	Mito-Ro-GFP mitochondria	GSSG/GSH	↓ oxidation		[361]
		IMS – Ro-GFP IMS		↑ oxidation		
		Cyto – Ro-GFP cytoplasm		↑ oxidation		
Cultured mouse lung slice	1.5% O_2	Cyto – Ro-GFP cytoplasm	GSSG/GSH	↑ oxidation	Hypoxia-induced oxidation of cyto-Ro-GFP strongly suppressed in lung slices overexpressing cytosolic catalase	[363]
Cultured mouse PASMC	PO_2 = 13 Torr	IMS-targeted Ro-GFP		↑ oxidation		[127]
Cultured mouse PASMC	1.5% O_2	Mito-Ro-GFP mitochondria	GSSG/GSH	↓ oxidation		[128]
		IMS – Ro-GFP		↑ oxidation	Hypoxia-induced oxidation of Cyto- and IMS-Ro-GFP strongly suppressed in RISP-depleted PASMC	
		Cyto – Ro-GFP cytoplasm		↑ oxidation		
Isolated mouse PA	1.5% O_2	Cyto – Ro-GFP cytoplasm	GSSG/GSH	↑ oxidation		[128]
		Mito-Ro-GFP mitochondria		↓ oxidation		
rat renal artery SMC	2.5% O_2	Cyto – Ro-GFP cytoplasm	GSSG/GSH	↑ oxidation	Hypoxia-induced oxidation of Cyto-Ro-GFP strongly suppressed in RISP-depleted renal artery SMC	[128]

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Preparation	Level of hypoxia	Indicator/ compartment	Indicator responds to…	Effect of hypoxia	Additional comments	Ref
rat renal artery SMC	2.5% O_2	Cyto – Ro-GFP cytoplasm	GSSG/GSH	↑ oxidation	Hypoxia-induced oxidation of Cyto-Ro-GFP strongly suppressed in RISP-depleted renal artery SMC	[128]
Perfused mouse lung	1% O_2	HyPer Cyto	H_2O_2	↑ oxidation		[486]
wildtype mouse PASMCs	1% O_2	HyPer expressed in cytoplasm	H_2O_2	↑ oxidation		[130]
Cox4i2 knockout mouse PASMCs	1% O_2	HyPer expressed in cytoplasm	H_2O_2	↓ oxidation		[130]
Rat PASMC	20 Torr	pHyper-dCyto	H_2O_2	↓ oxidation		[125]
		pHyper-dMito	H_2O_2	↓ oxidation		
Rat renal artery SMC	20 Torr	pHyper-dCyto	H_2O_2	↑ oxidation		[125]
		pHyper-dMito		No effect		
Isolated pig PA 100-150μm diameter	4% O_2 PO_2 = 29 TorrO2	Electron spin resonance with DMPO	Superoxide, peroxynitrite See [523]	↑ in 3 of 6 hypoxic PA but in 0 of 6 normoxic PA	Effect of hypoxia was non-significant. The use of nitrone spin probes such as DMPO has been strongly criticized (Dikalov et al 2007)	[353]
Mouse PASMC	1% O_2	Electron spin resonance with CMH	Superoxide, peroxynitrite See [524]	↑ ROS	Signal was due to superoxide, defined by its abolition by SOD	[130]
Mouse PASMC	1,5,10, 15 % O_2	Electron spin resonance with CMH	Superoxide, peroxynitrite	↑ ROS in 1% O_2 but not in ≥ 5% O_2	Signal was due to superoxide, defined by its abolition by SOD	[486]
Cultured wildtype mouse PASMC	PO_2 = 15-22 Torr	Electron spin resonance with CMH	Superoxide, peroxynitrite	↑ ROS	Signal was due to superoxide, defined by its abolition by SOD	[129]
Cultured AOX -expressing mouse PASMC	PO_2 = 15-22 Torr	Electron spin resonance with CMH	Superoxide, peroxynitrite	↔ ROS	AOX expression abolished the ↑ ROS observed in wildtype controls	[129]
Cultured rat microvascular PA	1.5% O_2	GSSG/GSH-412 kit from Oxis Health Products	Mainly cytoplasmic [GSSG[/[GSH]	↑ GSSG/GSH over 2 hrs		[356]
Isolated rat PA	0% O_2 PO_2 = 15-20 Torr	GSH/GSSG See [525]	Mainly cytoplasmic GSSG: GSH redox state	↓ GSH/GSSG redox potential (= oxidation) over 45 mins	GSH/GSSG redox potential fell from -176 to -158	[18]
Isolated rat aorta	0% O_2 PO_2 = 15-20 Torr	GSH/GSSG See [525]	Mainly cytoplasmic GSSG: GSH redox state	↔ GSH/GSSG redox potential (= oxidation) over 45 mins	GSH/GSSG redox potential under normoxic conditions was not significantly different in PA and aorta	[18]
1st passage cultured rat PASMC	PO_2 = 40 Torr	JC-1, TNRM	$\Delta\Psi_m$ (E_m of mitochondria)	$\Delta\Psi_m$ increased (hyperpol) by hypoxia	$\Delta\Psi_m$ was more depolarized in PASMC compared to renal artery SMC under normoxic conditions	[145]

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Preparation	Level of hypoxia	Indicator/ compartment	Indicator responds to…	Effect of hypoxia	Additional comments	Ref.
1st passage cultured rat renal PASMC	PO_2 = 40 Torr	JC-1, TNRM	$\Delta\Psi_m$	$\Delta\Psi_m$ decreased by hypoxia		[145]
Primary cultured human ductus arteriosus cells	PO_2 = 40 Torr	JC-1, TNRM	$\Delta\Psi_m$	$\Delta\Psi_m$ increased by ↑ PO_2 from 45 to 100 Torr		[145]
Cultured rabbit precapillary PASMC	PO_2 = 8, 23 or 38 Torr	JC-1	$\Delta\Psi_m$	↑ $\Delta\Psi_m$ with PO_2 of 8 and 23 but not 38 Torr	Hyperpolarization of $\Delta\Psi_m$ to PO_2 of 8 Torr became significant after ~ 2 minutes	[144]
Cultured rabbit aorta SMC	PO_2 = 8 Torr	JC-1	$\Delta\Psi_m$	↑ $\Delta\Psi_m$		[144]
Cultured rat renal artery SMCs	PO_2 = 8 Torr	JC-1	$\Delta\Psi_m$	↔ $\Delta\Psi_m$		[144]
Isolated mouse precapillary PASMCs	1% O_2	JC-1	$\Delta\Psi_m$	↑ $\Delta\Psi_m$	Progressive hyperpolarization over 8 minute period of hypoxia	[130]
Isolated mouse precapillary PASMCs	PO_2 = 15-22 Torr	JC-1	$\Delta\Psi_m$	↑ $\Delta\Psi_m$	Hyperpolarization of $\Delta\Psi_m$ was not observed in PASMC from Cox4i2 knockout mice	[129]
Isolated mouse PASMC from 2nd and 3rd order PA	6% O_2	Rhodamine 123	$\Delta\Psi_m$	↑ $\Delta\Psi_m$	$\Delta\Psi_m$ hyperpolarized rapidly after 1-2 minutes of hypoxia	[43]

Dunham-Snary et al [125], using HyPer in cultured rat PASMC, found that hypoxia decreased [H_2O_2] in both the mitochondrial matrix and the cytoplasm. They suggested that since a redox mediator produced in the mitochondria must enter the cytoplasm to modulate contractile effector proteins, the fact that they detected similar effects of hypoxia on [H_2O_2] in both compartments supported the validity of their findings. They also speculated that the hypoxia-induced reduction of Ndufs2 thiols which they had observed (Section 5.1) was responsible for inhibiting its production of ROS, implying that the reduction of the mitochondrial matrix might be a key factor in causing HPV.

In contrast, all of the other studies using genetically encoded indicators, including one which used Hyper [130] in mouse PASMC, reported that hypoxia increased [H_2O_2] or oxidized the thiol/disulfide equilibrium in the cytoplasm of mouse and rat PASMC, although, in agreement with Dunham-Snary and colleagues, the mitochondrial matrix became more reduced. Several papers from Schumacker's laboratory also reported that hypoxia increased redox tone in the mitochondrial IMS, consistent with their hypothesis that the increased ROS production by Complex III triggered by hypoxia is directed into the IMS, from which H_2O_2 can enter the cytoplasm. Korde et al [127] reported a similar finding.

Likewise, several papers from Sommer and colleagues reported that hypoxia increased superoxide production in cultured mouse PASMC. Although the spin probe they used (1-hydroxy-3-methoxycarbonyl-2,2,5, 5-tetramethylpyrrolidine; CMH) detects both mitochondrial and extra-mitochondrial superoxide [526], since they (and others) consistently found that the redox poise in the mitochondrial matrix tone was reduced during hypoxia, it seems likely that the increase in superoxide detected by ESR was cytoplasmic. Importantly, Sommer et al [129] also demonstrated using ESR that the hypoxia- induced increase in superoxide production was absent in PASMC from mice expressing AOX, consistent with evidence that mitochondrial ROS production associated with an increased reduction of the ubiquinone pool is depressed in these animals [383,527].

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The generation of ROS by both complexes 1 and 3 is strongly influenced by $\Delta\psi_m$ [58,347,528]. As shown in Table X, hypoxia has been observed to increase $\Delta\psi_m$ in PASMC in each of the three studies in which it has been measured. This is consistent with an increase in mitochondrial ROS production.

In summary, experiments utilizing genetically-encoded ROS indicators and ESR have, with only one exception [125], supported the notion that hypoxia increases ROS production and cytoplasmic oxidant levels in PASMC. The hypoxia induced hyperpolarization of $\Delta\psi_m$ which has been reported consistently also provides indirect support for this idea.

7.5. Concluding Remarks: The Redox and Mitochondrial Models

Overall, compared to the mitochondrial ROS model, the evidence supporting the Redox model is less extensive. It is also generally older, and partly as a consequence of this, is based to a larger extent on results arising from experimental approaches (the use of ETC blockers as hypoxia surrogates, the knockout of mitochondrial complex subunits to characterize their involvement in O_2 sensing) which have been criticized as being unreliable [518,529], or supportive but not definitive (the use of isolated mitochondria, pro- and anti-oxidants, and conventional small molecule ROS indicators[32]).

These considerations also apply to much of the evidence supporting the mitochondrial ROS model and may well account for some of the discrepancies between the results reported by different laboratories. However, placing this type of evidence to the side, the observations that remain predominantly favor the Mitochondrial ROS model. These include, most notably, the inhibition of HPV by S3QEL2 [130] and by the expression of AOX [129], as well of the effects of hypoxia on redox state of the cytoplasm using newer genetically coded indicators and electron spin resonance measurements shown in Table 5.

The Mitochondrial ROS model is also indirectly supported by evidence that Cox4i2, proposed to be crucial for increased hypoxia-induced ROS production during HPV (Section 5.2.2) [130], plays a similar role in the carotid body. In contrast, although the implications of Cox4i2 for the Redox model have not been discussed by its proponents, the evidence that its high expression promotes O_2 sensing in both PASMC and CBCC seems at variance with the idea that hypoxia decreases ROS production, since it is widely accepted that reduction of the CoQ pool is a key stimulus for ROS production. However, whilst the comparatively high expression of Cox4i2 in the two most well-characterized types of cells seen to respond acutely to hypoxia suggests that it could be playing an important role O_2-sensing, possibly by promoting mitochondrial ROS production, it is also the case that hypoxia, at levels similar to those generally used by experimenters to evoke HPV and CBCC activation, induces rapid increases in ROS in many other types of native cells/tissues or cell lines which are not known to express high levels of Cox4i2 [145,179,342,343,355,530–539]. Therefore, it remains to be established that the level of expression of Cox4i2 is a pivotal determinant of hypoxia-induced ROS production.

Despite the evidence for the mitochondrial ROS hypothesis being more compelling at this point, questions remain. For example, the observation that HPV recorded in isolated PA from AOX mice was restored if the arteries were incubated in PSS containing 20 mm K^+ [129] appears to sit uneasily with evidence that an increase in ROS production by complex III is causing HPV by causing SR Ca^{2+} release and Ca^{2+} sensitization (see Sections 5.2.1 and 5.2.5). Since 20 mM K^+, which promotes VSMC contraction by causing depolarization, was able to replace the effect of ROS production by complex III which was absent in the AOX mice, the implication is that ROS produced by complex III contribute to HPV by causing membrane depolarization. If so, the contractile effects over and above those caused by depolarization (i.e. Ca^{2+} release and sensitization) which are evoked by hypoxia are not due to ROS production by complex III. If nothing else, these apparently contradictory observations highlight the fact that the HPV effector mechanisms linked to an increase in ROS remain imperfectly understood.

Another unresolved aspect of the mitochondrial ROS hypothesis is that the nature of oxidizing signal responsible for HPV remains to be convincingly identified. Although H_2O_2 is generally seen as

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being the most important oxidizing species responsible for cell signaling, the evidence that this is the case for HPV is decidedly mixed. Its specific involvement in HPV is supported by evidence from three investigations showing that overexpression of catalase [356,359,363] prevented the hypoxia-induced rise in $[Ca^{2+}]_{cyt}$ in PASMC, and one showing that hypoxia oxidized HyPer [130]. On the other hand, hypoxia was seen to cause a fall in the cytoplasmic concentration or cellular production of H_2O_2 in four other studies using AmplexRed/HRP or HyPer [145,285,294,452], and the mechanisms by which H_2O_2 caused contraction when applied at a concentration which probably caused a quasi-physiological intracellular signal did not closely match those thought to be responsible for HPV[419]. Also arguing against H_2O_2 as the HPV mediator is the carefully conducted study by Liu and colleagues showing that the application of SOD, which they verified led to its penetration into PASMC, strongly suppressed HPV in pig PA [353].

The question of the redox mediator responsible for HPV is particularly relevant in light of recent experiments carried out in HEK 293 cells by Sen et al which suggest that hypoxia may exerting opposite effects on two types of cell oxidant species [518]. This investigation aimed to determine whether the opposing effects of hypoxia on ROS production observed in PASMC and many other types of cells were genuine or might, for example, be due to the use of different indicators with non-identical responses to particular oxidant species.

The effects of hypoxia (1% O_2) in HEK293 cells were recorded using the Amplex UltraRed + SOD assay, both in the presence of HRP to record H_2O_2, and in the absence of HRP, in which case the assay detects other oxidant species instead of H_2O_2. They also used DCF to measure general cell oxidation. They found that hypoxia decreased H_2O_2 release. Importantly, this was associated with a fall in H_2O_2 production by complexes 1 and 3, and also by Nox, plus other sources which were not characterized. This supports the idea that the decrease in H_2O_2 production was caused by a deficiency of O_2 rather than a decrease in the availability of electrons at any specific site. Hypoxia also decreased DCF oxidation. On the other hand, hypoxia increased the release from the cells of (an)other oxidants(s), as sensed by Amplex UltraRed in the absence of HRP. Whereas they did not identify these other oxidant(s), work by Kalyanaranam's laboratory shows that the oxidizing species involved is probably peroxynitrite [511], or, more likely, products of its decomposition [540]. Notably, peroxynitrite (or its products) can also increase the fluorescence of AmplexRed + HRP [540]. Even so, this reaction occurs much more slowly than that with H_2O_2 suggesting that the two assays were genuinely picking up opposing effects of hypoxia on H_2O_2 vs (an)other oxidant species.

These results therefore imply that hypoxia alters the balance between cellular production of H_2O_2 and other oxidizing species, thereby providing a potential explanation for the apparent indicator-dependency of the effect of hypoxia reported in both the HPV literature and by studies carried out in other types of cells and tissues.

If the unidentified oxidant was peroxynitrite, the observations of Sen et al could potentially be explained by the hypothesis, developed by Poyton and colleagues [421], that hypoxia increases the production of both NO and superoxide by the mitochondria, generating a cytoplasmic peroxynitrite signal capable of orchestrating a cellular response. One implication of this model is that since the rate at which NO and SOD react with superoxide is similar [541], they would compete for superoxide released into the IMS by complex III. Thus, an increase in [NO] evoked by hypoxia, whether originating in the mitochondria or elsewhere in the cell [163], could enhance the production of peroxynitrite while at the same time diminishing that of hydrogen peroxide. Furthermore, since Ro-GFP, ESR and HSP-FRET can sense peroxynitrite directly or indirectly through its effects on the ambient redox state, whereas HyPer responds specifically to H_2O_2, some of the apparently contradictory observations which set the Mitochondrial ROS and Redox models apart could in fact represent two facets of the same process.

In any case, we suggest that the role of mitochondrial ROS production in O_2 sensing during HPV remains sufficiently uncertain so as to warrant further studies. For example, the observation by Sommer et al [130] that S3QEL2 inhibits HPV, which we believe is currently the most convincing evidence that it requires ROS production at complex III, has not been confirmed independently, and

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would be strengthened by corresponding evidence that S3QEL2 also suppresses hypoxia-induced cytoplasmic oxidation. It also would be useful if the current evidence for cytoplasmic oxidation could be broadened, for example by using newer and more sensitive indicators such as Ro-GFP2-Tsa2DeltaCRm, which is based on a modified form of peroxiredoxin, [121,297,542]. The possible involvement of NO and peroxynitrite in HPV, would appear to be another issue worth investigation.

In addition, an analysis of the effects of hypoxia on reactive cysteines in putative ROS targets such as IP_3R, RyR and $K_V1.5$ would confirm that any alteration in cytoplasmic redox state is not merely an epiphenomenon. This approach could be implemented using isolated PA or perfused lung, circumventing the use of cultured cells and avoiding attendant complications arising from their altered phenotype and isolation from their normal environment.

8. The Role of the Pentose Phosphate Pathway and the Withdrawal of Normoxic Vasodilation Maintained by Nox4, H_2O_2 and Protein Kinase G in HPV

Michael Wolin and colleagues developed a complex scheme for O_2 sensing in HPV which resembles the Redox theory in that HPV is proposed to be due, at least in part, to hypoxia-induced withdrawal of ongoing ROS-dependent normoxic vasodilation. However, whereas according to the Redox theory this vasodilation is caused by mitochondrial ROS production and a resulting activation of K_V channels, the model developed by Wolin's laboratory envisions that normoxic vasodilation is maintained mainly by H_2O_2-mediated activation of the soluble guanylate cyclase (sGC)/protein kinase G (PKG) pathway, and that Nox4 rather than the mitochondrial ETC is responsible for H_2O_2 production.

Additional studies by Sachin Gupte, mostly carried out in collaboration with Wolin, presented evidence that hypoxia also regulates arterial tone by altering the activity of the pentose phosphate pathway (PPP), thereby leading to changes in the cellular [NADPH]/[$NADP^+$] ratio. This would enable H_2O_2-independent regulation of redox networks, leading to effects on thiol oxidation which alter the function of proteins involved in controlling vascular tone. In PA, for example, hypoxia was shown to stimulate the PPP, thereby increasing the [NADPH]/[$NADP^+$] ratio and as a result decreasing the activity of PKG1a, which is redox-sensitive [37]. Somewhat paradoxically, several of these investigations found that activation of the PPP by hypoxia is due to a rise in cytoplasmic [H_2O_2], which acts through PKCd to stimulate glucose-6-phosphate dehydrogenase (G-6-PD), the initial and rate limiting step in the PPP. This rise in H_2O_2 was also proposed to stimulate contraction through both Ca^{2+}- dependent and -independent pathways.

Although several of these studies investigated HPV in isolated perfused lungs or pulmonary artery rings from rats [441,543] or mice [478], most of this work was carried out using isolated bovine pulmonary artery (BPA) or lung homogenates, sometimes with comparative experiments in bovine coronary arteries (BCA), which relax to hypoxia. Importantly, rotenone, which has universally been found to block HPV in other species (Table 2), had no effect on HPV in BPA [453]. This would imply that mitochondrial ROS production plays no direct role in O_2 sensing in BPA, at least under the conditions used in these studies.

In most of these studies, hypoxia was imposed using PSS gassed with 5%CO_2/95%N_2, resulting in a pO_2 of 8-10 Torr. For studies of HPV, BPA rings were denuded of their endothelium to prevent hypoxic effects which might be mediated by NO and other EDRFs, and were slightly pre-constricted, generally with 20-30 mM K^+ but in some studies with U46619 or 5-HT, in order to enhance HPV. The authors generally adjusted the degree of pre-tone stimulus to maintain a similar amplitude of pre-constriction in the presence and absence of the interventions (e.g. blockers) which were being used to characterize mechanisms engaged by hypoxia or ROS.

8.1. Early Studies: HPV as the Loss of Tonic H_2O_2 and sGC-Mediated Vasorelaxation

A series of papers by Wolin's laboratory published over an approximately 10-year period starting in 1987 developed the concept that PA normally generate a basal level of superoxide (and

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therefore H_2O_2) due to the activity of an NAD(P)H oxidase [391,454,482,489,544–548]. This causes an ongoing stimulation of soluble guanylate cyclase (sGC) and therefore protein kinase G, creating a tonic vasodilating influence. The stimulation of sGC by H_2O_2 was proposed to be mediated by compound 1, a form of catalase which exists during its metabolism of H_2O_2 [489,544]. Vasodilation due to this mechanism is suppressed by hypoxia because there is less O_2 available for the formation of superoxide/H_2O_2, resulting in contraction (i.e. HPV). The development of this scheme is described in a review by Wolin et al [549]. The oxidase responsible for superoxide production in PA was initially seen as utilizing NADH. However, Mohazzab & Wolin [547] pointed out that its properties resembled those of the NADPH-dependent oxidoreductase which had been detected in neutrophils [550] and had been suggested to play a role in O_2 sensing in the carotid body [551]. It was eventually concluded [549] that it corresponded to the phagocytic NADPH oxidase and might be the same as the vascular oxidoreductase which others had shown utilized both NADH and NADPH to generate superoxide [552]. It soon became evident that there was a group of such oxidoreductases, christened the Nox family [553], and that they preferred to use NADPH rather than NAD as a substrate for superoxide production. In later work Wolin and colleagues concentrated on defining the role of NADPH rather than NADH in mediating the effects of hypoxia, stating that the [NADPH] in cells is likely to be 10-20 μM whereas [NADH] is ~1 μM [554].

Most of the subsequent investigations by this group relevant to HPV focused on two interrelated themes. As described in Section 8.2, a series of papers published between 1999 and 2010, spearheaded by Sachin Gupte, examined how hypoxia-induced regulation of the PPP influences the redox state of the NADPH/$NADP^+$ couple in such a way as to cause constriction of PA but relaxation of coronary arteries. Other work, published between 2010 and 2014, further examined how hypoxia, H_2O_2 and NADPH, acting though cellular redox networks, modulate PA tone by regulating the activity of the sGC/PKG axis (Section 8.3). In addition, Wolin's laboratory carried out pioneering experiments to examine the important possibility that HPV is influenced by the presence of extracellular H_2O_2 [477,478]

8.2. PPP Activity as a Determinant of the Effects of Hypoxia on Vascular Tone

An initial investigation of the role of the PPP in regulating vascular contraction [555] was based on the hypothesis that oxidation of the heme iron on sGC would inhibit its activity by preventing the binding of NO, and that NAD(P)H, by reducing the heme iron, might restore the responsiveness of sGC to NO, thereby promoting vasorelaxation. The results of the study provided support for this hypothesis, implying that that the activity of the PPP, which generates cellular NADPH, could potentially regulate vascular tone of BPA *via* this mechanism.

A subsequent paper then considered two possible scenarios by which the activity of the PPP might influence HPV [543]. If NAD(P)H was indeed acting to augment the stimulation of sGC by NO, PPP activation should suppress HPV. Alternatively, based on evidence that $NADP^+$ and GSSG activated the K^+ current in PASMC [331], PPP-mediated reduction of $NADP^+$ and/or GSSG might promote HPV by closing K^+ channels and causing depolarization-induced Ca^{2+} influx. Their experiments showed that antagonists of glucose-6-phosphase dehydrogenase (G-6-PD), which mediates the rate-limiting step of the PPP, suppressed HPV in isolated perfused rat lungs and relaxed contractions evoked by 30 mM K^+ in both PA and aorta. These results supported the second hypothesis, implying that hypoxia-induced activation of the PPP could promote HPV by reducing the cytoplasmic NADPH: $NAD(P)^+$ couple and thereby inhibiting K_V channel opening.

Gupte et al [556] similarly observed that pharmacological blockade of G-6-PD caused relaxation of bovine coronary arteries (BCA) and that this was accompanied by a fall in the tissue contents of NADPH and GSH, whereas total $NAD(P)^+$ and GSSG increased. The reducing agent DTT attenuated this relaxation, suggesting that it was due to an oxidizing effect of some kind. However, they also found that both superoxide and H_2O_2 generation by BCA fell during PPP blockade, implying that this oxidation was not due to an increase in cellular [ROS]. Moreover ebselen, which would be expected to diminish the concentrations of both ROS, did not suppress relaxation. They proposed that the fall

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in ROS production caused by PPP blockade occurred because there was less NADPH available to be used to produce superoxide. Although this study was in coronary rather than pulmonary arteries and didn't look at the effects of hypoxia, it was important for their subsequent studies of HPV because their observations showed that the PPP, presumably by producing NADPH, was able to influence vascular tone through ROS- and sGC-independent mechanisms, and that regulation of Ca^{2+} influx and release was more important in this respect than that of K_V channels.

Wolin's laboratory had previously shown [557] that hypoxia also relaxed BCA *via* a mechanism which appeared to be independent of ROS, since it was not affected by NBT (an antioxidant) and DPI (blocks Nox and the ETC). This suggested that the inhibition of the PPP might also be causing relaxation through this mechanism, and in accordance with this possibility Gupte & Wolin [558] reported that hypoxia increased the [GSSG]/[GSH] and [$NADP^+$]/[NADPH] ratios in these arteries. These effects were associated with a decreased level of glucose-6-phosphate, which is produced by the first step of the PPP, suggesting that hypoxia was inhibiting this pathway. As with PPP blockade, the relaxation to hypoxia was attenuated by DTT pre-treatment. Interestingly, application to BCA of pyruvate (10 mM), which was used to promote the flux of glucose through the PPP by inhibiting phosphofructokinase [559], increased the arterial content of gluscose-6-phosphate and NAD(P)H under both normoxic and hypoxic conditions. Pyruvate also prevented the increase in arterial GSSG and the vasorelaxation evoked by hypoxia. Based on these findings, the authors proposed that, due to a relative lack of G-6-PD in BCA, hypoxia causes a 'metabolic stress' leading to a reduced flux of glucose through the PPP. This causes a rise in the cellular [$NADP^+$]/[NADPH] and [GSSG]/[GSH] ratios which acts through redox networks to cause vasorelaxation by oxidizing thiol switches on a number of proteins controlling contraction (e.g. SERCA) [560].

Gupte et al [554] examined the relationship between G-6-PD and cellular superoxide levels (assessed using 5 μM lucigenin) in BPA and BCA. They reported that mRNA expression of Nox1 was not present in either artery. On the other hand, Nox2 and Nox4 mRNA and protein were similarly expressed in the two types of arteries, although superoxide levels were 40-80% higher in BPA. Both the G-6-PD blocker 6-aminonicotinamide (6-AN) and the drug apocynin, which they used as a Nox antagonist, decreased the lucigenin signal more in BPA than in BCA, suggesting that the higher level of superoxide production in BPA resulted from a higher level of Nox activity due to greater NADPH formation by the PPP. This idea was supported by their observation that the level of NADPH and the expression and activity of G-6-PD was higher in BPA than in BCA. We suggest, however, that this conclusion should be interpreted with caution, since the [NADPH]/[$NADP^+$] ratio was higher in BCA, suggesting that the PPP might be more active in these arteries.

Gupte et al [441] investigated the involvement of the PPP and NADPH in HPV in rats. They found that 5 minutes of hypoxia caused marked increases in the amounts of glucose-6-phosphate and NADPH in the lung. The hypoxia-induced rise in NADPH was abolished by 6-AN and another G-6-PD blocker, epiandrosterone. Both drugs also greatly reduced the amplitude of HPV recorded in isolated perfused lung. Similarly, hypoxia increased the [NADPH]/[$NADP^+$] ratio by about 75% in isolated lobar PA, and this effect, along with HPV, was suppressed by the G-6-PD antagonists. In additional experiments, sGC activity was measured in homogenates prepared from isolated PA which had been subjected to hypoxia in the presence or absence of G-6-PD blockers or the SGC antagonist 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ). It was found that hypoxia strongly increased the activity of sGC. This effect was also prevented by G-6-PD blockade, suggesting it was due to activation of the PPP. ODQ also prevented the increased sGC activity, but, in contrast to PPP blockade, enhanced HPV.

Based on these results, the authors proposed that hypoxia activates the PPP in PA, leading to an increased synthesis of NADPH which is responsible for HPV. At the same time, the increased [NADPH] also stimulates sGC (see also [555] and [561], creating a countervailing vasodilating influence which explained why HPV was potentiated by ODQ. However, this was outweighed by the pro-contractile response, which they speculated was due to K_V channel inhibition.

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In summary, the results of these studies suggested that hypoxia increases and decreases NADPH production by the PPP in BPA and BCA, respectively, and that this difference is responsible for its opposite effects on vascular tone in these arteries. According to the model developed by the authors to explain their observations [562,563], hypoxia *inhibits* the PPP in coronary arteries, leading to vasorelaxation consequent on a fall in cellular NADPH and a resulting oxidation of cellular thiol switches (e.g. on K_V channels and Ca^{2+} channels or pumps). Although ROS production decreases due to the fall in pO_2 and/or the suppression of the PPP, their evidence suggested that this does not exert an important effect on vascular tone. In contrast, PPP activity in PA is higher under basal conditions, and is *increased* by hypoxia. This causes hypoxia-induced contraction (HPV) through two mechanisms. Firstly, the PPP generates sufficient NADPH under normoxic conditions to create an ongoing vasodilating influence dependent on superoxide/H_2O_2 produced by an NADPH oxidoreductase. This vasodilating influence is greater in BPA than in BCA because the former has a higher expression and activity of G-6-PD and therefore has higher basal NADPH levels. This ongoing vasodilation is removed by hypoxia because it attenuates ROS production, causing contraction (i.e. HPV). Secondly, PPP activation by hypoxia, leading to increased NADPH production, promotes force development by causing the reduction of GSSG and/or protein thiol switches in such a way as to suppress relaxation or enhance contraction.

Gupte et al [440] further investigated the role of the PPP in triggering HPV in 4^{th} – 5^{th} order BPA. Contraction mechanisms were examined in intact endothelium-denuded arteries and those which the plasma membrane had been permeabilized with a-toxin from *staphylococcus aureus* to enable the study of Ca^{2+} sensitization. They evaluated the role of G-6-PD in HPV by using PA which had been cultured with anti-G-6-PD or scrambled siRNA for two days. They also compared the responses to hypoxia in a mouse model in which expression of G-6-PD was halved due to a mutation in the 5′ promoter region of its gene, and in mice that were heterozygous for this mutation and had normal levels of G-6-PD expression. They measured G-6-PD activity and the levels of pyridine nucleotides and other metabolic intermediates in homogenates made from frozen BPA.

The authors observed that the activation of the PPP by hypoxia previously detected in rat lung also occurred in BPA. This was shown by their observations that hypoxia increased the activity of G-6-PD as well as the [NADPH]/[$NADP^+$] ratio and the NADPH content of these arteries. They also examined the effect of hypoxia on cellular ROS levels using MitoSox and DHE in arteries which were pre-constricted with PSS containing 30 mM K^+, presumably to mimic the conditions used to study HPV. Hypoxia decreased the MitoSox signal, suggesting a fall in mitochondrial ROS levels, but increased the DHE signal, which they ascribed to cytoplasmic ROS. In light of previous evidence that the activity of G-6-PD is stimulated by H_2O_2, [564] they speculated that an increase in cytoplasmic ROS levels caused by hypoxia was responsible for activating the PPP, a concept which was subsequently supported by the subsequent observation that H_2O_2 could stimulate G-6-PD in BCA through PKCδ [565]. In addition, they found that hypoxia decreased the ATP/ADP ratio and increased the lactate/pyruvate ratio, indicating that glycolysis and oxidative phosphorylation were inhibited. These effects, plus a hypoxia-induced increase in glucose uptake [346], would be expected to increase the intracellular glucose concentration and shunt glucose into the PPP, further increasing its activity.

Curiously, however, they also found that hypoxia strongly increased the NAD^+/NADH ratio. This effect is the opposite to what would be expected when NADH consumption by the ETC is inhibited, and runs counter to the effects of hypoxia on this ratio observed by others in PASMC [346] and CBCC [176]. The implications of this apparent discrepancy were not discussed.

Importantly, they observed that decreasing the expression of G-6-PD strongly suppressed HPV. Furthermore, hypoxia increased contraction by stimulating both Ca^{2+}-dependent mechanisms and Ca^{2+} sensitization, and both responses were almost abolished in arteries treated with anti-G-6-PD siRNA. The former effect was studied by measuring hypoxia-induced increases in contractions of intact PA evoked by 30 mM K^+ PSS and 5-HT in the presence of extracellular Ca^{2+}. The latter effect manifested as a leftward shift of the Ca^{2+} vs contraction curve in permeabilized PA and was also

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suggested by the increase in MLC_{20} phosphorylation which they detected in intact PA under Ca^{2+}-free conditions. Hypoxia also increased the phosphorylation of CPI-17. The hypoxia-induced phosphorylation of MLC_{20} but not CPI-17 was antagonized by 6-AN, suggesting that PPP-dependent Ca^{2+} sensitization was not due to CPI-17. Instead, the authors suggested that hypoxia was causing Ca^{2+} sensitization *via* rho kinase, although no direct evidence for this was presented. The involvement of the PPP in O_2 sensing in BPA proposed by the authors based on these results and those of earlier studies is illustrated in Figure 9.

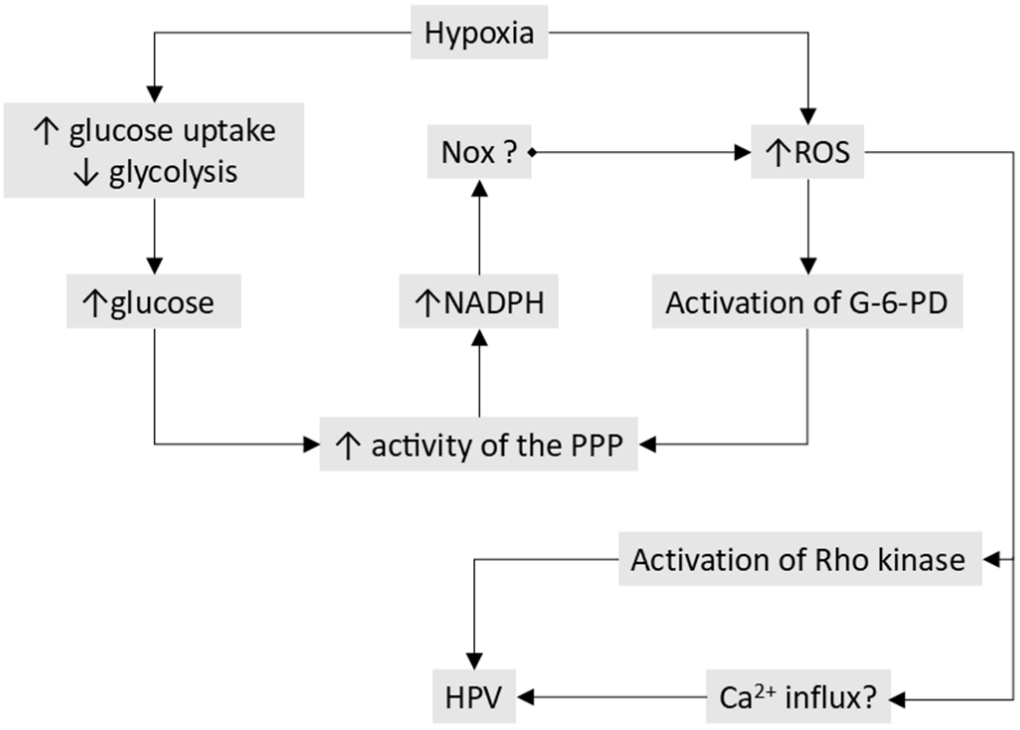

Figure 9. The role of the PPP in HPV as described by Gupte et al [440]. According to their model, hypoxia induces changes in glucose metabolism and cytoplasmic [ROS]. This causes an increased activity of PPP which, acting through NADPH causes a further rise in [ROS]. This activates rho kinase and other pro-contractile pathways, evoking HPV. The figure is based closely on that presented in [440]. See the text for further details.

8.2. Loss of Basal H_2O_2-Induced Stimulation of sGC and PKG as a Mechanism of HPV

8.2.1. Basal H_2O_2 Production and HPV

Ahmad et al [477] tested the hypothesis that the removal by hypoxia of an ongoing H_2O_2-dependent vasorelaxation contributes to HPV by using ebselen (100 μM) to mimic a hypoxia-induced fall in H_2O_2. They found that the contraction of BPA to 20 mM K^+, which they viewed as reflecting basal normoxic contractility, was enhanced by ebselen, while HPV was strongly depressed. Treatment of BPA with the CuZn SOD blocker diethyldithiocarbamic acid (DETCA) exerted similar effects. Both ebselen and DETCA greatly decreased normoxic H_2O_2 levels (detected using a luminol/HRP assay). HPV was also markedly depressed in BPA which had been treated for 24 hours with $CoCl_2$, which increased tissue levels of H_2O_2, putatively by enhancing the expression of extracellular SOD. This suggested that if produced extracellularly in sufficient quantities, H_2O_2 entry into PASMC would increase to the point where hypoxia was no longer able to depress its intracellular concentration enough to evoke HPV. This idea was supported by the observation that applying 1 mM catalase to remove extracellular H_2O_2 restored HPV in the $CoCl_2$ -treated arteries.

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They then [453] examined the involvement of Nox2 and Nox4 in generating the H_2O_2 responsible for maintaining normoxic vasorelaxation in BPA. They found that pharmacological block or siRNA knockdown of Nox2 attenuated superoxide production but did not affect HPV or the contraction to 25mM K^+ under normoxic conditions. Similarly, ETC block using rotenone (10 μM) or antimycin (10 μM) decreased superoxide production but had no effect on either type of contraction. In contrast, knockdown of Nox4, which also depressed superoxide production, halved the amplitude of HPV although it too had no effect on the 25 mM K^+ contraction. Based on these results, the authors concluded that Nox4 was responsible for generating H_2O_2 and maintaining a vasodilating influence which was removed by hypoxia, thereby causing or contributing to HPV. However, this scheme would predict that interventions which diminish intracellular [H_2O_2] even under normoxic conditions should increase contractility, and this conflicted with their finding that siRNA knockdown of Nox4 had no effect on the contraction to 25 mM K^+. To explain this, they suggested that H_2O_2 produced by Nox4 was having both dilating and constricting effects, and that following H_2O_2 depletion the dilating effect was lost immediately whereas the constricting effect wore off more slowly. The immediate loss of the dilating effect would give rise to a rise in tension (e.g. HPV or the contraction caused by the antioxidant ebselen), whereas the eventual loss of the contractile effect would account for the fact that very prolonged H_2O_2 depletion associated with Nox4 knockout did not affect the response to 25 mM K^+. They tested this concept by applying 25 mM K^+ before and after either a short- (35 min) or long-term (several hour) treatment with ebselen (again used to mimic the H_2O_2-lowering effect of hypoxia) and found that short-term ebselen treatment produced the predicted enhancement of the high K^+ response, whereas long-term treatment did not. The authors suggested that the pro-contractile effect of ROS produced by Nox4 might be due to the stimulation of rho kinase.

Together, the results of these two papers supported the hypothesis that HPV is due to the loss of vasorelaxation caused by H_2O_2 produced by Nox4.

8.2.2. HPV and Regulation of sGC and PKG by H_2O_2

The proposal by Wolin's laboratory that a fall in H_2O_2 production caused by hypoxia evokes or promotes HPV by inhibiting the activity of the sGC/PKG pathway grew out of their initial observations suggesting that H_2O_2 stimulates sGC though the action of compound C, a catalase intermediate which forms while it is metabolizing H_2O_2 [489,544,566,567]. The link between this mechanism and HPV was based on evidence [544] that aminotriazole, which inactivates catalase by binding to compound C, caused a contraction and inhibited HPV in BPA. However, it is not clear from the results presented in this paper that the latter effect was statistically significant, and whereas aminotriazole raised basal PAP in perfused rat lung, it did not block HPV [548]. Moreover, in perfused rabbit lung, where aminotriazole did block HPV, it caused an equivalent inhibition of the contractile responses to U46619 and angiotensin 2 [481]. The ability of compound C to block sGC appears not to have been confirmed by any other laboratories.

Wolin's laboratory subsequently explored other mechanisms by which hypoxia could regulate the sGC/PKG axis in BPA, reporting that hypoxia greatly enhanced the stimulation of cGMP production evoked by NO donors [568]. Nevertheless, sGC-mediated vasorelaxation was strongly depressed by hypoxia, although the NO-induced vasorelaxation was maintained by an sGC-independent mechanism, which their experiments suggested was the activation of SERC A. This possibility was later supported by the demonstration that NO can stimulate SERCA in arteries *via* peroxynitrite-mediated S-glutathiolation of Cys674 [569]. The authors did not investigate or discuss how hypoxia was apparently diminishing the ability of cGMP to cause vasodilation. However, since PKG is the only known target of cGMP in vascular smooth muscle, its inhibition by hypoxia is a likely explanation.

They then showed that diamide, an oxidant which mimics the effect of H_2O_2 on thiols, inhibited the activation of sGC by NO donors [561]. Blocking the PPP, which caused a fall in the level of NADPH and should therefore also lead to oxidation of thiols, had the same effect as diamide. This

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would predict that a fall in H_2O_2 and activation of the PPP caused by hypoxia should increase NO-induced stimulation of sGC, which was consistent with their earlier observation.

PKG exists as two isoforms, PKG1 and PKG2, with the former being predominantly expressed in the vasculature. Two splice variants of PKG1, PKG1α and PKG1β, are expressed in vascular smooth muscle. Both are homodimers, with the subunits held together by a leucine zipper interaction and are activated by cGMP. In 2007 Burgoyne et al [37] discovered that PKG1α can also be activated in a cGMP-independent manner by the oxidative formation of a disulfide bond between the Cys42 residues of its two subunits. Although the inactive form of PKG1α is often referred to as a monomer which dimerizes upon the formation of the disulfide bridge [503], this is incorrect; both active and inactive PKG1α are dimers [570]. The monomeric form only exists under the denaturing conditions used for immunoblotting, which cause the dimers without the disulfide bond to come apart to form monomers, whereas dimers with a disulfide bridge remain intact; this allows the proportion of active and inactive forms of PKG1α to be measured. We will use the term 'disulfide dimer complex' (DDC) ' [570] to refer to the form of PKG1a activated by oxidation.

The finding that PKG1α could be directly activated by DDC formation opened up the possibility that decreases in [H_2O_2] caused by hypoxia could act on PKGa1 in PASMC to inhibit its vasodilating effect. This led Wolin's laboratory [498] to investigate the effect of exogenous H_2O_2 on DDC formation and the activity of PKG in BPA. They also examined whether H_2O_2 was still able to cause vasorelaxation in arteries in which the expression of sGC had been greatly diminished by a 48 hour exposure to ODQ, an approach adapted from a protocol described in [571]. They found that application of 100mM H_2 O_2 to BPA significantly increased DDC formation and PKG activity (measured as the phosphorylation of the PKG1α substrate VASP- vasodilator-stimulated phosphoprotein). These effects were suppressed by pre-treating arteries with DTT, suggesting that they were due to PKG1α oxidation. Depletion of sGC by prolonged ODQ treatment, which almost eliminated the dilating response induced by a NO donor, diminished the vasorelaxation evoked by exogenous H_2O_2 by ~50%, indicating that H_2O_2 was activating PKGα1 both directly and indirectly, *via* sGC. Acute ODQ treatment, designed to prevent any effect of NO on sGC, did not block the response to H_2O_2, indicating that this was independent of NO.

Neo et al [502] studied the relative contributions of sGC and PKG1α to HPV. They found that, like H_2O_2, hypoxia decreased the DDC formation and activity of PKGα1. Strikingly, siRNA knockdown of PKG1α mimicked the acute effects of ebselen observed previously [453], increasing the amplitude of the contraction induced by 25 mM K^+ under normoxic conditions but decreasing the amplitude of HPV by ~50%. Additionally, treating BPA with DTT mimicked hypoxia by increasing the contraction to 25mM K^+ and diminishing both DDC formation and VASP phosphorylation. DTT also abolished HPV.

Depletion of sGC by prolonged ODQ treatment also potentiated the contraction to 25mM K^+ under normoxic conditions while reducing the amplitude of HPV by ~60%. siRNA knockdown of the b-subunit of sGC in BPA, which reduced its expression by ~60%, caused similar effects and decreased H_2O_2-induced vasorelaxation and PKG activity. siRNA knock down of PKG1α also suppressed H_2O_2-induced relaxation and VASP phosphorylation of BPA, and attenuated HPV.

These findings supported the hypothesis that in BPA, hypoxia, by diminishing cellular H_2O_2 levels, causes a reduction of regulatory cysteine thiols on both sGC and PKG1a which suppresses their activities. This would attenuate ongoing normoxic vasodilation, thereby causing or augmenting HPV. In contrast to what was observed in BPA, hypoxia caused vasorelaxation in BCA, associated with increases in DDC formation and VASP phosphorylation. This presumably reflected an oxidizing shift in the $NADP^+$/NADPH redox couple which acted through redox networks to oxidize PKG1α, rather than being due to H_2O_2 [556].

Neo et al [572] investigated whether PPP-induced reduction of the $NADP^+$/NADPH couple was influencing the activity of PKG1α by regulating the redox state of antioxidant enzymes such as Trx-1 or Prx1 which could suppress its activation by reducing Cys42. In this case, it would be predicted that experimental interventions which interfered with the function of the PPP or of these enzymes

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would increase PKG1α DDC formation, leading to its activation and a decrease in vascular contraction.

The authors described several observations which supported the idea that the PPP creates a pro-contractile influence in BPA which is exerted *via* NADPH-dependent reduction (i.e. inhibition) of PKG1α. Hypoxia caused an increase in the NADPH/$NADP^+$ ratio, which was antagonised by the PPP blocker 6-AN. PPP blockade acutely relaxed high K^+-pre-constricted PA, and increased PKG1α DDC formation and VASP phosphorylation. In addition, the high K^+ contraction under both normoxic and hypoxia conditions was smaller, and levels of PKG1α DDC formation and VASP phosphorylation were higher, in PA in which G-6-PD had been knocked down by siRNA, compared to controls treated with scrambled siRNA. Similar effects were seen following siRNA knockdown of Trx and TrxR-1. Knockdown of thioredoxin reductase also suppressed 6-AN-induced relaxation, DDC formation and VASP phosphorylation.

In contrast, knockdown of Prx1 had no significant effect on the amplitude of the high K^+ contraction, PKG1α DDC formation, VASP phosphorylation or 6-AN-induced relaxation. They also examined the effect of TrxR-1 and Prx1 knockdown on the responses of PA to the application of 100μM H_2O_2, which caused PKG1α DDC formation and VASP phosphorylation. Knockdown of TrxR-1 enhanced peroxide-induced relaxation, DDC formation and VASP phosphorylation, whereas knockdown of Prx1 had the opposite effects.

The results supported the idea that the PPP exerts an inhibitory effect on the activity of PKG1α, doing so by maintaining a high level of cellular NADPH which acts through Trx-1 and TrxR-1 to reduce PKG1a, thus suppressing its vasorelaxing effect. This inhibitory effect on the underlying vasorelaxing influence exerted by PKG is enhanced during hypoxia because the PPP is activated [440], promoting HPV. This concept was in line with the observation [573] that G6P-D, Trx-1 and PKG co-precipitated, suggesting that they form a complex.

The results also suggested that Prx1 is not involved in O_2 sensing in BPA. This was shown by the observation that Prx1 knockdown had no effect on the high K^+ contraction under normoxic conditions, which would mean that this antioxidant enzyme is not contributing to the normoxic vasorelaxation which is removed by hypoxia. The authors also observed that depletion of sGC using chronic ODQ treatment had no effect on 6-AN mediated relaxation under hypoxic conditions, implying that the PPP and NADPH were not directly regulating cGMP production. The authors therefore concluded that regulation of PKG1a DDC formation by NADPH might contribute to hypoxic responses associated with changes in the redox state of NADPH.

Figure 10 summarizes the proposed involvement of the sGC/PKG axis and hypoxia-induced removal of a tonic normoxic H_2O_2-dependent vasodilation as a mechanism contributing to HPV.

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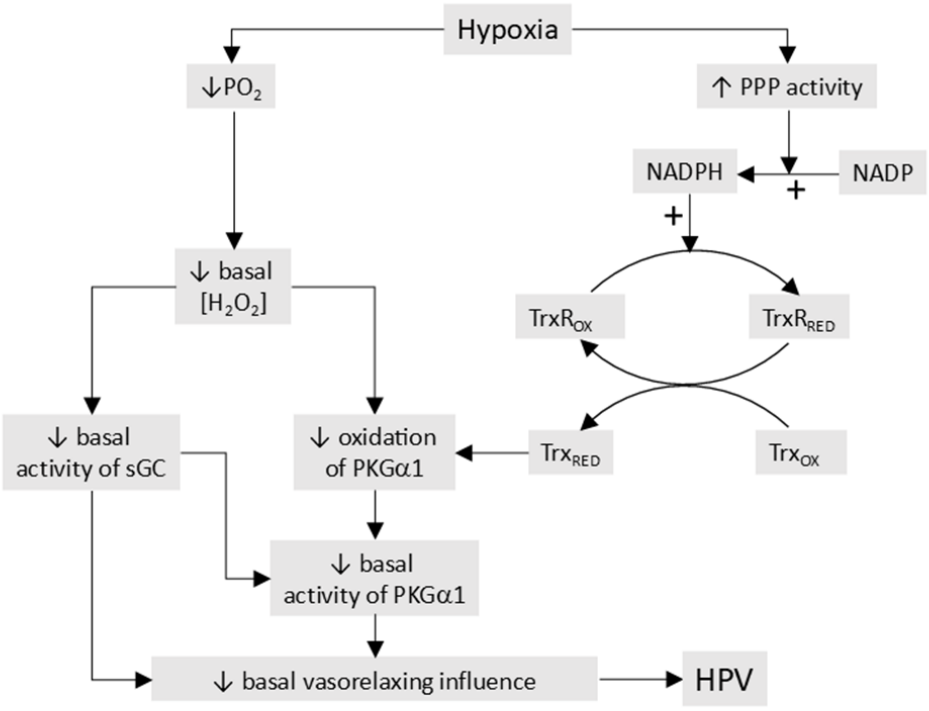

Figure 10. Proposed role of PKGa1 and sGC in HPV. According to this model, HPV is largely due to the removal of a basal vasodilating mechanism which is caused by an ongoing activation of sGC and PKGa1. Hypoxia removes this influence via two pathways. Firstly, by activating the PPP, it increases the NADPH/$NADP^+$ ratio. The increase in NADPH, causes the reduction of TrxR-1 and Trx-1, thereby reversing the resting oxidation and activation of PKGa1, diminishing its vasorelaxing influence. Secondly, the fall in PO_2 decreases the ambient intracellular concentration of H_2O_2, further decreasing the activation of PKGa1 both directly by promoting its and indirectly by inhibiting sGC. This figure is largely based on information present in [572].

8.3. Does the Presence of Extracellular H_2O_2 affect HPV?

As described in Section 4, it is thought that the basal extracellular H_2O_2 concentration is in the range of 1-5 μM, although the evidence for this is meagre. This level of extracellular H_2O_2 is predicted to cause an inward leak which would significantly raise its intracellular concentration, especially adjacent to the cell membrane [240], and it is therefore conceivable that this could influence the effects of changes in mitochondrial H_2O_2 production on redox-sensitive HPV effector targets, especially those in the plasmalemma. Wolin's laboratory examined the possible impact of extracellular H_2O_2 on HPV in two studies, using catalase, which was applied at a low concentration (1μM) said to scavenge only extracellular H_2O_2 These investigations, carried out in isolated PA from cow [477] and mouse [478], found no effect of catalase on HPV under control conditions. However, HPV in both arteries was suppressed by manoeuvres which raised the extracellular [H_2O_2], an effect which was partly reversed by catalase. Based on other results in their paper, Patel et al, [478] proposed that this was due to oxidation-dependent stimulation of PKGa1, presumably exerted by an excessive inward leak of H_2O_2. These results should be interpreted with caution since no evidence was presented that catalase was not penetrating into the cells (which it has been shown to do [353]). However, taken at face value, they support the possibility that the presence of extracellular H_2O_2 might influence HPV.

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8.4. Summary and Critique: Does Activation of the PPP and the Withdrawal of H_2O_2/PKG – Mediated Normoxic Vasodilation Cause HPV?

The picture of the involvement of ROS and cellular redox in HPV which emerges from the papers published on this subject by Wolin, Gupte and colleagues is complex, but incorporates two proposed pathways as shown in Figures 9 and 10:

1. Under normoxic conditions, the production of superoxide/H_2O_2 by Nox4 is greater in PA than in systemic arteries because the PPP, being more active, generates more NADPH. The resulting higher level of H_2O_2 creates an ongoing vasodilating influence by stimulating PKG1a, both directly by oxidizing Cys42 on PKG1a, and indirectly by activating sGC. Hypoxia decreases the production of superoxide/H_2O_2 by Nox4, thereby raising vascular tone by inhibiting this baseline vasorelaxation.
2. Hypoxia also suppresses the activity of PKG1a by stimulating G-6-PD and the PPP, causing a consequent increase in the NADPH/$NADP^+$ ratio. This acts, at least in part, through Trx-1 and TrxR-1, to reduce PKG1a, causing it to become less active, which promotes contraction. The activation of G-6-PD is proposed to result from an increase in cytoplasmic [ROS], which promotes also HPV by stimulating rho kinase and Ca^{2+}-dependent contractile mechanisms.

Below, we look critically at several aspects of these proposals.

8.4.1. Decreased Production of H_2O_2 by Nox as a Cause of HPV

The high $K_m(O_2)$ of Nox4 (Section 3.2.2), which allows its production of H_2O_2 to be sensitive to even small decreases in PO_2, renders it an eminently feasible O_2 sensor for HPV. It has been shown, for example, that H_2O_2 production by Nox4 expressed in HEK293 cells falls by ~50% when the PO_2 is decreased from 150 to 80 Torr [59].

Nonetheless, the knockout of Nox4 had no effect on basal PAP or HPV [399] in mice, indicating that that it does not regulate basal vascular PA tone and is not required for the response to hypoxia, at least in this species. On the other hand, sustained HPV was diminished by the knockout of p^{22phox} [402], suggesting that another Nox isoform, probably Nox1, plays a role in HPV. However, $p22^{phox}$ knockout had no effect on basal PAP. This argues against the possibility that HPV in mice is due to the withdrawal of basal Nox-induced vasodilation, suggesting instead that Nox is contributing to sustained HPV by increasing its production of ROS.

These observations do not rule out the possibility that the loss of basal ROS production by Nox4 is important for HPV in species other than the mouse. Nonetheless, the involvement of decreased ROS production by Nox4 in HPV in cow can be questioned on several fronts. Most importantly, direct evidence that hypoxia decreases ROS levels in BPA is inconsistent. A hypoxia-induced fall in ROS in intact BPA was demonstrated in only a single study [545], which measured H_2O_2 using a high concentration of lucigenin with HRP. Lucigenin at this concentration is subject to redox cycling making it a suboptimal indicator for examining the effect of hypoxia on ROS levels (Section 7.4). Conversely, the investigation employing the intracellular indicator DHE reported that hypoxia increases ROS in BPA [440]. Although DHE also has its drawbacks, its suitability as a detector of cell oxidation has been vigorously defended [574].

Evidence [453] that the withdrawal of normoxic vasodilation caused by Nox4-generated H_2O_2 contributes to HPV in BPA can also be challenged. As described in this paper, siRNA knockout of Nox4 did not affect the contraction to 25 mM K^+, which the authors used an index of basal reactivity. This conflicted with their hypothesis that Nox4 exerts a tonic vasodilating effect on basal tone, which predicts that Nox4 knockout should increase the K^+ contraction. The authors explained this apparent discrepancy by proposing that ROS generation by Nox4 exerts opposing vasodilating and vasoconstricting effects, and that when ROS production is acutely interrupted by hypoxia, the dilating effect is immediately removed, whereas the constricting effect only disappears after a delay of several hours.

However, down-regulation of sGC and PKG1α, the putative targets by which H_2O_2 produced by Nox4 was suggested to suppress basal tone, did increase the contraction to 25 mM K^+ [502]. This

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observations seeme to undermine the authors' explanation for the lack of an effect of Nox4 knockout on basal reactivity, which provides an important underpinning for their proposal that that Nox4-derived H_2O_2 causes normoxic vasodilation. As to the possibility that the attenuation of superoxide/H_2O_2 production by other Nox isoforms by hypoxia might contribute to HPV, this laboratory did not detect the presence of Nox1 in these arteries [554], and demonstrated that although blocking Nox2 with gp91-dsat diminished ROS levels in BPA, it had no effect on either basal reactivity or HPV [453]. We therefore believe that the proposal that a significant component of HPV in BPA is due to the loss of basal vasorelaxation associated with H_2O_2 production by Nox remains speculative.

8.4.2. Does a Fall in H_2O_2 During Hypoxia Cause HPV by Inhibiting sGC?

If HPV is partly caused by the removal of H_2O_2-induced basal oxidative activation of sGC and/or an increase in PPP-derived NADPH [498,502,544,545] it would be predicted that 1. block of sGC or PKG should inhibit HPV and also mimic hypoxia in causing PA constriction, 2. hypoxia should decrease the cGMP content of PA, and 3. the activity of sGC should be increased by H_2O_2 or equivalent oxidant stimuli.

The first prediction is supported by an *in vivo* study of HPV in cats [575] which showed that the sGC blocker methylene blue (MB) increased baseline PAP and abolished the response to hypoxia. MB also increased baseline normoxic PAP in pigs and sheep, although its effect on HPV was not investigated [576]. Conversely, MB [577,578] and ODQ [579] had no effect on baseline normoxic PAP, and augmented HPV in isolated perfused rat lung. In isolated rat PA, MB increased HPV, although this effect was absent in severe hypoxia [580]. Vermeersch and colleagues [581] compared HPV in wildtype mice and those in which the a_1 subunit of $sGCa_1b_1$ isoform, the predominant form of sGC in the vasculature, had been rendered less active by deletion of exon 6 of the a_1 subunit gene. RVSP under normoxic conditions and during a 10-minute hypoxic challenge was not different in the two groups and hypoxia had no effect on the lung cGMP content in the control mice. Also, although ODQ slightly reduced the amplitude of HPV in isolated perfused mouse lung, it did not increase normoxic PAP, going against the idea of sGC-mediated normoxic vasorelaxation [582]. Fouty et al [579] found that the PKG inhibitor Rp-8-pCPT-cGMPS had no effect on basal PAP or HPV in isolated perfused rat lung. They verified that Rp-8-pCPT-cGMPS suppressed PKG activity, by monitoring phosphorylation of the $Ins(1,4,5)P_3$ receptor, a PKG target. The differential effect of Rp-8-pCPT-cGMPS and ODQ on HPV in this study is difficult to explain, since PKG is the only known target of cGMP in vascular smooth muscle. Nevertheless, neither drug affected basal PAP. With regard to the second prediction, contrasting effects of hypoxia on cGMP levels in PA have been reported. In BPA, hypoxia decreased cGMP in endothelium-denuded BPA [544]. In rat PA, however, hypoxia decreased the cGMP content of intact but not endothelium-denuded PA arteries [583]. This suggested that hypoxia was suppressing the activity of sGC, but that this was secondary to a fall in endothelial NO release rather than being due a direct effect of hypoxia on sGC in the smooth muscle. Taken together, the results of these studies argue against a role for sGC in causing basal PA vasorelaxation in rodents, although this might occur in cats in a manner similar to that proposed for BPA.

The proposal that H_2O_2 production under normoxic conditions creates a vasodilating influence in BPA which is in part due to the activation of sGC, and that this is decreased by hypoxia, rests on evidence that 1) exogenous H_2O_2 caused vasorelaxation and increased the cGMP content in endothelium-intact arteries [489] , 2) hypoxia decreased the cGMP content of endothelium-denuded arteries [544], 3) depletion of cellular sGCb1 using prolonged ODQ treatment increased basal reactivity (assessed as the contraction to 25mM K^+ under normoxic conditions), [498], 4) depletion of sGCb1 using long term ODQ treatment or siRNA knockdown inhibited vasorelaxation and activation of PKG (assessed using VASP phosphorylation) by exogenous H_2O_2 under hypoxic conditions [498,502], and 5) depletion of sGC using siRNA knockdown increased basal reactivity but decreased HPV [502].

Importantly, Neo et al [498] found that the vasorelaxation of BPA induced by the H_2O_2 occurred in endothelium-denuded arteries, and whereas it was decreased by downregulating sGC expression,

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it was not affected by acute treatment with ODQ [498]. Since ODQ would block stimulation of sGC due to any remaining NO, they concluded from these results that the activation of sGC by H_2O_2 must be NO-independent (indeed, this laboratory had previously presented evidence that NO-dependent activation of sGC was inhibited by oxidation [561]).

In this case, their model predicts that if hypoxia causes a fall in H_2O_2 production it should decrease the cGMP concentration in endothelium-denuded arteries, an effect they had previously observed in BPA [544]. However, the cGMP content of endothelium-denuded rat PA was not affected by hypoxia [583], and in porcine PA, Ye et al also found that hypoxia did not affect the cyclic GMP level in the presence of either ODQ or the eNOS antagonist nitro-L-arginine [584] and that reducing agents either did not affect (DTT and GSH), or increased (L-cysteine and tris(2-carboxyethyl)phosphine), the cGMP content of nitro-L-arginine-treated arteries. Intriguingly, they also reported that sGC dimerization, which is required for its activation by NO [585], was present under basal conditions, and was decreased by both hypoxia and reductants. Accordingly, NO-dependent vasodilation was depressed by reductants. These observations contradict the evidence in BPA that oxidation depresses NO-dependent and increases NO-independent sGC activity but support the general concept that the reduction of sGC by hypoxia can cause its inhibition. However, since NO release falls during hypoxia [586], the extent to which the redox regulation of NO-dependent sGC activation could influence HPV is uncertain.

8.4.3. Does a Fall in H_2O_2 During Hypoxia Cause HPV by Directly Inhibiting Protein Kinase G?

The proposal that a decrease in H_2O_2 levels and/or an increase in NADPH production by the PPP caused by hypoxia contributes to HPV by reversing oxidation-induced PKG1α activation is consistent with extensive evidence showing that PKG exerts an important vasodilating influence in systemic arteries through its phosphorylation of multiple target proteins which control vascular tone [587]. It is also well established that PKG activity in arteries is increased by the oxidation of Cys42 leading to formation of a disulfide bond PKG1α in mice [37,570,588], and that this creates an ongoing vasodilating influence under baseline conditions, at least in systemic resistance vessels, as evidenced by the observation that blood pressure is raised in mice expressing a 'redox-dead' form of PKGa1 [589].

With regard to the involvement of PKG1α in HPV, this hypothesis is supported by evidence, described in Section 8.2.2, that: 1. exposure of BPA to 0.1 and 1 mM H_2O_2 increased PKG1α DDC formation and phosphorylation of its target VASP and these responses were reversed by DTT [498]. 2. hypoxia caused contraction and decreased PKG DDC formation and VASP phosphorylation of endothelium-denuded BPA, whereas opposite effects on PKG occurred in BCA, which relax to hypoxia, 3. DTT increased force in BPA under both aerobic and hypoxic conditions and diminished PKG1α DDC formation and VASP phosphorylation, 4. siRNA knockdown of PKG1α increased basal reactivity (i.e. the contraction to 25mM K^+ under normoxic conditions) but depressed HPV [502], and 5. block of the PPP, which would reduce the supply of NADPH available to reduce and therefore inactivate PKG1α, diminished the contraction evoked by 25 mM K^+ under both normoxic and hypoxia conditions [572].

However, it is not clear that the effect of the PPP on contraction exerted through the redox regulation of PKG1α [572] is specific to HPV. The authors proposed that the PPP was promoting hypoxic contraction of BPA by increasing the production of NADPH, which would transfer reducing equivalents *via* TrxR-1 and Trx-1 to PKG1α, thereby suppressing its vasodilating activity (Figure 10). In support of this scheme, they found that interventions used to block the influence of the PPP on contraction (i.e. siRNA knockdown of G6PD, Trx-1, TrxR-1) decreased the contraction induced by 25mM K^+ under hypoxic conditions. Importantly, however, these interventions caused a comparable decrease in the high K^+ contraction recorded under normoxic conditions, suggesting that the PPP similarly promotes contraction under both normoxic and hypoxic conditions rather than selectively enhancing HPV. Along similar lines, Chettimada et al, who also showed that that blocking the PPP with 6-AN increased HPV, found that this caused equivalent increases in the activity of PKG under

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normoxic and hypoxic conditions [573]. Rather than demonstrating that the activation of the PPP acting *via* an NAPDH/TrxR/Trx/ PKG1α pathway is a specific cause of HPV, these results suggest that NADPH produced by the PPP may promote contraction by inhibiting PKG1a regardless of the oxygen concentration.

There is currently no information about the role of PKG1α oxidation in acute HPV in other species, although there is evidence [590] that this is increased during chronic hypoxia and tends to ameliorate pulmonary hypertension in mice.

8.4.4. Is Activation of the PPP Important for HPV?

Although we have argued that the evidence that the PPP causes/promotes HPV by inhibiting PKG1a is unconvincing, it is possible that stimulation of the PPP by hypoxia could trigger HPV *via* other contractile mechanisms, e.g. activation of RhoK [440], or inhibition of K_V channels by the interaction of NADPH with their b subunits [265]. Certainly, the evidence that hypoxia stimulates the PPP and causes contraction in BPA whereas it inhibits the PPP and causes relaxation in BCA offers support, albeit indirect, for the concept that it regulates the contractile response to low PO_2. Unfortunately, it is difficult to draw any conclusions about the involvement of the PPP in HPV because no other laboratories seem to have investigated this. Nevertheless, in agreement with the observation that the G-6-PD antagonist 6-AN blocked HPV in BPA, we have found that dehydroepiandrosterone, which also blocks this enzyme, has a similar effect in rat PA (Figure 11).

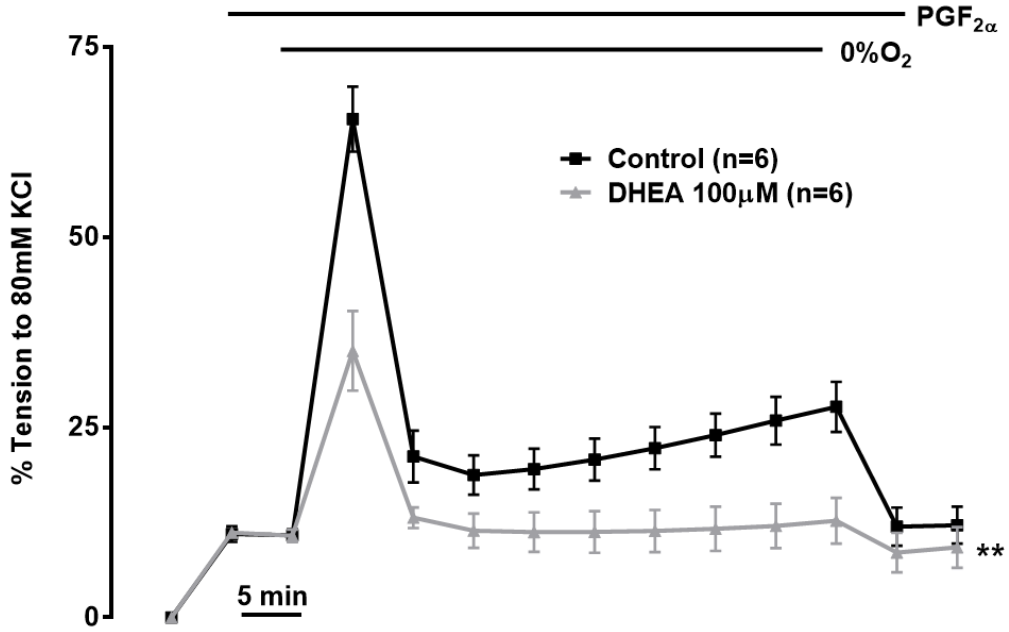

Figure 11. HPV was evoked by gassing small endothelium-intact PA from rats in a conventional myograph with 95% N/5%CO_2/0% O_2 as previously described; the PO_2 during hypoxia was shown to be 15-20 Torr under these conditions[10]. A low concentration of $PGF_{2\alpha}$ was applied before hypoxia to create a small degree of pre-tone; this increases the amplitude of HPV, particularly the transient initial phase (phase 1). Application of DHEA significantly ($p < 0.05$ by 2- way ANOVA and also paired T-test) depressed the amplitude of phase 1 and also of phase 2, which is sustained. In the absence of pre-tone (in which case phase 1 is not evident), DHEA also virtually abolished HPV in 7 arteries (not shown) (taken from Prieto-Lloret et al, unpublished results). .

There is also evidence that the PPP in other types of cells is rapidly stimulated by oxidative stress [591] and several mechanisms by which the oxidation of thiols in glycolytic enzymes (e.g. GAPDH, PKM2) can divert metabolic flux into the PPP have been defined (e.g. [592]. Supporting the involvement of G-6-PD and the PPP in regulating contraction, the vasoconstrictor U46619 caused an

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increase in G-6-PD activity in BCA which was mediated by a ROS-dependent stimulation of PKCd [565]. Also, complexes containing G-6-DP, Trx-1 and PKG can be detected in BPA homogenates [573], and L-type VGCC in BCA and human internal mammary artery SMC were shown to be activated by a direct interaction with G-6-PD in caveoli [593]. Further investigation of the role of PPP activation in HPV, focused on determining the effect of hypoxia on the redox state of the $NADP^+$:NADPH couple [594] and on identifying the effectors through which the PPP could induce HPV, would therefore be of great interest.

9. Conclusion

The proposal that cell redox mechanisms acting on cell thiols are responsible for O_2 sensing in HPV was first mooted by Kenneth Weir and colleagues in a remarkably prescient study published more than 40 years ago [274]. As we have described, experiments in the intervening time have led to the development of three main models designed to describe how hypoxia induces changes in PASMC ROS production and cytoplasmic redox state which engage with specific HPV effector mechanisms. Although many of these studies utilized techniques which were innovative and state-of-the art at the time they were carried out, the rapid pace of conceptual and technical development in the field of redox biology, especially over the past decade, has brought into question the interpretation of many the observations which underpin these models. Largely because it is supported by a larger evidence base, and one which draws more heavily on the use of newer techniques which are at present viewed as being more valid, we believe that the Mitochondrial ROS hypothesis currently provides the most convincing picture of how hypoxia acts through cell redox mechanisms to cause HPV.

Nevertheless, many interesting questions remain. The mechanisms by which hypoxia can increase mitochondrial ROS production remain uncertain, as do the identities of the HPV effectors which are linked to an increase in ROS, and the precise nature of the involvement of Cox4i2 in O_2 sensing in PASMC. The possible involvement of NADH in HPV, possibly *via* the regulation of K_V or voltage-gated Ca^{2+} channels, is a long-standing but unresolved concept which has been given a renewed lease of life by recent observations in CBCC. Indeed, the question as to what extent common mechanisms are used by the various homeostatic sensors of acute hypoxia present in the body remains open. Recent evidence suggesting that hypoxia may exert complex effects on the cytoplasmic redox state (due to spatial and/or temporal compartmentalization?) rather than generating a simple up/down ROS signal is also worthy of note. In addition, concerns about the use of non-physoxic conditions and regarding whether cells were being exposed to O_2 concentrations which differed from those which were intended also suggest that the quest for the O_2 sensing mechanism in HPV should not be regarded as finished.

Happily, progress in the development of increasingly sensitive/specific indicators for cellular H_2O_2 [542], peroxynitrite [595] Han, free NADH ([596]) and NADPH ([89]), as well as probes for intracellular PO_2 ([597]) has made it more possible than ever to explore the involvement of cell redox mechanism as O_2 sensors in PASMC and other cells. It will be interesting to see where the research in this area takes us.

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