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The Role of Redox Changes in Oxygen Sensing

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Abstract

The specialized oxygen-sensing tissues include the carotid body and arterial smooth muscle cells in the pulmonary artery (PA) and ductus arteriosus (DA). We discuss the evidence that changes in oxygen tension are sensed through changes in redox status. “Redox” changes imply the giving or accepting of electrons. This might occur through the direct tunneling of electrons from mitochondria or redox couples to an effector protein (eg. ion channel). Alternatively, the electron might be transferred through reactive oxygen species from mitochondria or an NADPH oxidase isoform. The PA's response to hypoxia and DA's response to normoxia result from reduction or oxidation, respectively. These opposing redox stimuli lead to K⁺ channel inhibition, membrane depolarization and an increase in cytosolic calcium and/or calcium sensitization that causes contraction. In the neuroendocrine cells (the type 1 cell of the carotid body, neuroepithelial body and adrenomedullary cells), the response is secretion. We examine the roles played by superoxide anion, hydrogen peroxide and the anti-oxidant enzymes in the signaling of oxygen tensions.

Keywords

Oxygen sensing; Redox; Hypoxic pulmonary vasoconstriction

1. Introduction

Although all tissues are sensitive to oxygen there are a number of specialized tissues in the body that sense oxygen and serve the function of optimizing oxygen uptake and delivery. These tissues, which can be considered as the Homeostatic Oxygen System, include the carotid body, neuroepithelial bodies in the lungs (NEBs), chromaffin cells of the fetal adrenal medulla and smooth muscle cells in a variety of vessels (the ductus arteriosus and resistance pulmonary, fetoplacental and systemic arteries) (Figure 1). In the case of the carotid body type 1 cells, NEBs or chromaffin cells, hypoxia leads to exocytosis of neurotransmitters. In the vessels, hypoxia changes tone but interestingly in opposite directions in different vessels. Hypoxia causes relaxation in the ductus arteriosus (DA) and most systemic arteries, while stimulating vasoconstriction in the resistance pulmonary and fetoplacental arteries. There are teleologic reasons why these differences are advantageous,

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basically in each case the effect is to enhance O₂ uptake or optimize O₂ delivery, (Weir et al., 2005) but the physiology of these tissues is outside the scope of this review.

The effector mechanisms responsible for causing exocytosis in carotid body type 1 cells during hypoxia involve inhibition of potassium current (IK), depolarization of the cell membrane and calcium entry through the voltage-gated L-type calcium channels. In the pulmonary artery smooth muscle cells (PASMCs), in addition to inhibition of IK, hypoxia also increases calcium and reinforces hypoxic pulmonary vasoconstriction (HPV) by three redox-dependent mechanisms 1) enhancing entry through the L-type calcium channel independent of membrane depolarization (Franco-Obregon & Lopez-Barneo, 1996) 2) causing release of intracellular calcium from the sarcoplasmic reticulum and associated repletion through store-operated channels, and 3) causing calcium sensitization by activating small G proteins, such as RhoA (reviewed in (Weir et al., 2008)). In the DASMCs it is the shift from hypoxia to normoxia (as occurs at birth), rather than from normoxia to hypoxia, which causes inhibition of IK, release of SR calcium and calcium sensitization. If the executive mechanisms are the same, the question remains, what is different about oxygen sensing in the PASMCs and DASMCs that explains their diametrically opposite responses? In response to most stimuli, other than oxygen, the PA and DA have similar vasomotor responses; both contract when stimulated with vasoconstrictors such as phenylephrine, endothelin or potassium chloride. They both relax in response to prostaglandin E₂, prostacyclin and nitric oxide. Thus there is something about P_{O₂} which is interpreted differently by the two tissues. The Redox Hypothesis holds that changes in the reduction/oxidation balance in these specialized cells reflects changes in P_{O₂} and changes the function of redox sensitive targets by altering the oxidation of susceptible amino acids, notably cysteines and methionines. The sensors (such as mitochondria or oxidases) are connected to the effectors (ion channels/SR/small G proteins) by signaling molecules (which include reactive oxygen species, such as hydrogen peroxide and superoxide; redox couples, such as nicotinamide adenine dinucleotide and glutathione and probably redox-sensitive enzymes, such as thioredoxin, tyrosine kinases and phosphatases). This hypothesis which dates from the early 1980s holds that there are nonenergetic signals derived from mitochondria, that ROS are physiologic signaling molecules and that ion channels have a conserved role in oxygen sensing (Archer et al., 1993) (Archer et al., 1986) (Weir & Archer, 1995) (Weir et al., 2005). One early impediment that hindered acceptance of this theory was a misconception of the structure of mitochondria. Modern imaging of mitochondria using mitochondria-targeted green fluorescent protein shows that the mitochondria permeate the PASMC much like electrical wiring and are ideally positioned to control the function of both membrane and cytosolic factors by the release of redox signaling molecules (Figure 2).

2. Redox History

In the fetus, the pulmonary and systemic arteries are exposed to similar pressures and oxygen tensions. Yet, when a pregnant ewe is exposed to hyperbaric oxygen, fetal pulmonary vascular resistance falls dramatically (down 68%), with no change in systemic arterial pressure or resistance (Assali et al., 1968). This observation led to the concept of active, normoxic pulmonary vasodilatation (Weir, 1978). Reactive oxygen species (ROS), such as hydrogen peroxide, are known to be formed in the course of normal cellular metabolism and the rate of production varies with the ambient level of oxygen (Freeman & Crapo, 1981) (Freeman et al., 1982) (Oshino et al., 1975). In the early 1980s we investigated the possibility that these reactive oxygen species (ROS), when produced in low amounts, might serve as signaling molecules; rather than in their classic role as nonspecific mediators of injury. We and others measured ROS and found that they increased in the lung in direct proportion to P_{O₂}. Moreover, these changes in ROS production occurred within seconds of a change in P_{O₂} and preceded alteration of vascular tone. We examined the ability of a panel

of oxidants to mimic oxygen and reverse or inhibit HPV. As predicted, 2-butanone peroxide, t-butyl hydroperoxide, xanthine/xanthine oxidase (generating superoxide anion (O_2^-), hydrogen peroxide and the sulfhydryl oxidant diamide inhibited hypoxic pulmonary vasoconstriction (HPV), in both the isolated perfused rat lung and the intact animal (Weir & Will, 1982) (Weir et al., 1983) (Weir et al., 1985). Both 2-butanone peroxide and t-butyl hydroperoxide markedly reduced HPV but t-butyl hydroperoxide also decreased systemic pressure and resistance. In contrast, 2-butanone peroxide did not affect systemic hemodynamics. Diamide reversed HPV and moderately reduced the pulmonary pressure responses to prostaglandin F₂α and angiotensin II. Pre-incubation of diamide with glutathione (GSH) prevented diamide's ability to reverse HPV. The addition of xanthine/xanthine oxidase moderately reduced HPV in the isolated rat lung (in the absence of pulmonary edema) and this effect was blocked by the xanthine oxidase inhibitor, allopurinol. At that time, the combination of glucose/glucose oxidase, generating hydrogen peroxide, was also reported to prevent HPV (Burghuber et al., 1984). Based on these findings, it was proposed that hypoxia caused pulmonary vasoconstriction by shifting the PASMCs to a more reduced redox status on the basis of decreased ROS and/or increased reduction of redox couples, such as GSH /GSSG and NAD(P)H/NAD(P) (Archer et al., 1986).

Subsequent work confirmed that xanthine/xanthine oxidase reduced HPV in the isolated rat lung (Archer et al., 1989b), although there was a small increase in the wet to dry weight ratio of the lungs (6.4 ± 0.3 v 5.8 ± 0.3 controls). The combination (X/XO) also reduced the constrictor response to angiotensin II (AII) and consequently the effect was not specific to HPV. In fact, this should not be surprising, as an increase in inspired oxygen also reduces the pulmonary vascular response to several vasoconstrictor agents (Tucker et al., 1977). The concept of normoxic pulmonary vasodilatation indicates that oxygen, and the redox mechanism, through which it is proposed to work, should have a general vasodilator effect, opposing endogenous and exogenous vasoconstrictors. The X/XO -induced inhibition of HPV was largely prevented by pretreatment with superoxide dismutase (SOD), with or without exogenous catalase (Archer et al., 1989b). In addition, PEG-SOD + catalase tended to increase HPV (Archer et al., 1995), suggesting that O_2^- or an ROS downstream was having a vasodilator effect. It is important to remember that O_2^- can behave as an oxidizing agent and accept electrons, forming H_2O_2 , or as a reducing agent, for instance donating electrons to potassium ferricyanide, cytochrome C or 5,5'-dithiobis (2 nitrobenzoic acid) (DTNB) (Peterson et al., 1994). The transfer of electrons from ferrous iron to an enzyme or channel protein can be illustrated as follows: $Fe(2+) + O_2 \rightarrow Fe(3+) + O_2^- \rightarrow Fe(3+) + O_2 + \text{reduced electron acceptor}$.

3.1 Redox Controversies

If redox changes are the signal which telegraphs the onset of hypoxia, the relative importance of change in one of the redox couples versus changes in ROS is unclear. Whether ROS go up or down during hypoxia and in which microdomains is still debated. Controversy also remains as to where in the mitochondrial electron transport chain (ETC) signaling the ROS are generated (I and/or III) and which other sources of ROS may be important (Figure 3). There are many reasons why it has been difficult to achieve consensus. Investigators study the effects of hypoxia on isolated organs, isolated vessels, freshly dispersed vascular smooth muscle cells or cultured cells after a varying number of passages. The lungs or vessels may or may not be pre-constricted or prestretched. The conditions vary in terms of severity of hypoxia, sometimes studying oxygen tensions that are unlikely to exist in PASMCs from resistance pulmonary arteries in life (eg <30 mmHg), although they are plausible in systemic tissues. Levels of pH and P_{CO_2} may not be monitored. Some laboratories focus on the first 10–20 minutes of hypoxia and others on a period of hours. The

techniques used to measure ROS include fluorescent dyes (such as luminol, lucigenin, DCF, mitoxox, spin traps, and RoGFP) and may reflect the input of varying radical species from varying compartments of the lung. Different animal species are studied at different developmental stages. ROS such as O_2^- or H_2O_2 may be generated or added outside the cell, when the real question is usually their role within the cell and also it is worth considering that the redox gradient across the cell membrane may be important. The physiologic levels of these ROS is debated and the actions of the endogenous ROS are often not validated by the use of specific SODs or catalase. Measurements are seldom made of glutathione, thioredoxin or peroxiredoxin. These important differences in experimental protocols have to be considered when we try to explain how oxygen levels are sensed and what is different in different tissues (eg PA v DA).

While there is no consensus or clear explanation for the opposing findings, clarity would be enhanced if investigators heeded the physiologic attributes of the hypoxia. For example, putative mechanisms of HPV should operate uniquely in the pulmonary circulation, as compared to systemic arteries or ductus, which dilate to hypoxia. The responses should be rapid in onset (<1 minute for all O_2 -sensitive tissues) and must be reversible with restoration of normoxia. Reductionist models, using cell lines, are popular because of their ease of use. This ignores the fragility of the authentic response of specialized O_2 sensitive tissues. Careful inspection of data often reveals the hypoxic responses are often small, slow to onset or are not reversible, suggesting the preparations are nonphysiological. Passage of cells in culture or exposure of tissue to high oxygen levels rapidly decreases the expression of O_2 -sensitive ion channels and impairs O_2 responses, such as the ability of the DA to constrict to oxygen (Thebaud et al., 2004). In contrast, maintaining the DA for the same length of time at physiologic levels of hypoxia preserves the expression of O_2 -sensitive ion channels and the ability of the ring to constrict to O_2 . It is also optimal to base conclusions about O_2 sensing on fresh tissue and cells that are either freshly dispersed or are at a low passage number. Likewise, measurements should use physiologic conditions (temperature 37C, pH 7.35–7.45, P_{CO_2} 30–40mmHg with a hypoxic P_{CO_2} of 35–50 mmHg and normoxic PO_2 of 100–140mmHg P_{CO_2}). All too often experiments are performed at 25C and without careful buffering and bubbling of the media. Under these conditions most of the homeostatic oxygen system's tissues do not function normally. Finally, many of the ROS probes also measure nitric oxide, eg. DCF. To reduce error, we recommend combining compartment-specific redox probes (roGFP) with the use of several ROS probes, such as Amplex Red (for H_2O_2) and lucigenin and L-012 for superoxide. In general, the further one gets from the in vivo situation, the greater the likelihood of spurious results.

4.1 Redox signaling of hypoxia

In some tissues the redox signaling cascade seems straightforward. In neonatal adrenomedullary chromaffin (AMC) cells, as in PASMCs and carotid body type 1 cells, hypoxia inhibits outward potassium current (IK) causing membrane depolarization and calcium entry through L-type calcium channels (Thompson & Nurse, 1998) (Thompson et al., 2007). This leads to secretion of catecholamines and ATP. The mechanism is likely to involve a reduction in the production of hydrogen peroxide (H_2O_2), because measured ROS levels fall and exogenous H_2O_2 reverses both the inhibitory effect of hypoxia on IK and the associated stimulation of ATP secretion. In addition, the introduction of catalase into the AMC cells through the patch pipette or the external application of the antioxidant N-acetylcysteine (NAC), both mimic hypoxia and prevent (occlude) any further effect of hypoxia. More insight into the source of the redox signal is provided by the observation that the mitochondrial complex I inhibitor, rotenone, also mimics and occludes the effects of hypoxia on IK, ROS and ATP secretion (Thompson et al., 2007). The rotenone inhibition of IK could be reversed by succinate (source of electrons for complex II) and by exogenous

H₂O₂. Thus it appears likely that the hypoxia-and rotenone-sensitive site in the AMC relates to complex I or a very similar electron shuttle. Further confirmation of the involvement of ROS came from the observation that succinate reversal of rotenone inhibition of IK could itself be prevented by the sulfhydryl source, NAC. The importance of a block to electron shuttling at complex I is demonstrated by the finding that succinate, circumventing the block, also reversed the stimulation of ATP secretion from neonatal AMCs induced by either rotenone or hypoxia. A final instructive point is that the oxygen-sensing mechanisms change as the organism matures, such that AMC from neonatal and juvenile rats (14–21 days old) have differences in O₂ responsiveness (Thompson et al., 2007). There appear to be two levels at which oxygen sensing is disconnected with maturation. Hypoxia does not reduce ROS production by juvenile AMCs or cause ATP secretion. Moreover, even when ROS are decreased by NAC in juvenile cells, ATP secretion does not follow. These experiments clearly demonstrate the importance of ROS, generated at a rotenone-sensitive site, in the signaling of changes in oxygen tension. They also suggest that maturation of both sensor (mitochondria etc.) and effector (ion channels etc.) mechanisms occur, as previously reported in DASMIC, where both P_{CO₂}-dependent ROS modulation in DASMIC mitochondria and K⁺ and Ca²⁺ channel O₂-sensitivity are fully developed only at term (explaining the predisposition to patent ductus seen in preterm infants) (Thebaud et al., 2004); (Kajimoto et al., 2007).

A rotenone-sensitive oxygen-sensing site has also been described in the carotid body type 1 cell (Ortega-Saenz et al., 2003). In these experiments the rotenone-induced stimulation could not be bypassed by succinate, raising the possibility that the oxygen-sensing site might be outside the mitochondria. However, it is clear that electron-transport-chain inhibitors, such as rotenone, stimulate the carotid body by inhibiting IK and mimic the effects of hypoxia (Wyatt & Buckler, 2004) (Williams et al., 2004). These studies suggest that normoxia may be signaled by the generation of ROS, particularly H₂O₂, and hypoxia by a decrease in ROS production. Concordant with this concept are other reports that H₂O₂ increases IK in neuroepithelial bodies, H146 cells and AMCs (OKelly et al., 2000) (Wang et al., 1996).

In all these tissues the evidence indicates that hypoxia decreases ROS, probably H₂O₂. This decrease leads to inhibition of potassium channels resulting in membrane depolarization and calcium entry, ultimately causing exocytosis of ATP and neurotransmitters. As mentioned earlier, it has been less clear where the ROS are generated, although the experiments reviewed on AMCs suggested a mitochondrial origin (Thompson et al., 2007). The evidence favoring this source was strengthened by the finding that mitochondria-deficient AMCs (rho 0 cells) do not show inhibition of IK, an increase in cytosolic calcium or catecholamine secretion in response to hypoxia or rotenone (Buttigieg et al., 2008). Entirely opposite observations have been made in rho 0 H146 cells in regard to IK inhibition by hypoxia. In these cells the absence of mitochondria made no difference to the ability of hypoxia to inhibit IK (Searle et al., 2002). These authors made the additional finding that the potencies of rotenone and antimycin A, as ETC inhibitors, are substantially reduced by hypoxia. They postulate that these compounds may contain a reducible quinone group, which consequently decreases their activity during hypoxia. More positive identification of another source of ROS has been made in the NEBs and related H146 tumor cell line (Cutz et al., 2009). In these airway chemoreceptors, the classical NADPH oxidase (NOX2), is coexpressed with oxygen-sensitive potassium channels. In addition, siRNA against NOX2 inhibits the release of serotonin that is normally induced by hypoxia. Consequently, in some cells, NOX2 may be an alternative site of ROS production in normoxia. It is interesting to note that there is considerable interactive redox signaling between NADPH oxidases and mitochondria (Daiber, 2010), so one may influence the release of ROS by the other.

5.1 Oxygen sensing in DA and PA

As mentioned before, hypoxia causes pulmonary vasoconstriction but dilatation of the DA. Both DAMSCs and PSMCs are, in redox terms, more reduced during hypoxia (Michelakis et al., 2002) (Kajimoto et al., 2007). In the hypoxic fetal environment the DA is dilated and in the DAMSCs potassium channels are open. As in the AMCs, rotenone mimics hypoxia, decreasing H_2O_2 production but in this instance, the fall in H_2O_2 increases potassium current and causes relaxation (Michelakis et al., 2002b). At birth, with an increase in oxygen, the DASM is more oxidized, IK is decreased and the cell membrane is more depolarized, leading to contraction and closure of the DA. This sequence can be mimicked by extracellular t-butyl H_2O_2 (Michelakis et al., 2002b) (Reeve et al., 2001b). As might be expected, increasing intracellular catalase through the patch pipette prevents normoxic inhibition of IK, indicating that the inhibition of the current is caused by endogenous H_2O_2 (Reeve et al., 2001b).

The opposite responses of the DA and PA to hypoxia, dilatation and constriction respectively, are mimicked only by electron transport chain inhibitors, such as rotenone, which decrease ROS in both (Archer et al., 1993) (Michelakis et al., 2002b) (Michelakis et al., 2002) (Reeve et al., 2001) and by redox-active reducing agents. In the latter case, a reducing agent, such as dithiothreitol (DTT), increases IK, hyperpolarizes membrane potential, and decreases cytosolic calcium in DASMCS, while doing the opposite in PSMCs (Olschewski et al., 2004). Incidentally, oxidizing agents such as dithionitrobenzoic acid (DTNB) have exactly opposite effects to DTT. Another oxidizing agent, diamide, has been reported to reduce capacitative calcium entry in the PA, again mimicking normoxia (Schach et al., 2007). Diamide, originally described as a glutathione oxidizing agent, itself increases IK in PSMCs (Reeve et al., 1995). In addition, the inclusion of oxidized glutathione (GSSG) in the patch pipette increases IK, while reduced glutathione (GSH) decreases it (Weir & Archer, 1995). It is possible that the redox ratios, GSH/GSSG, NAD(P)H/NAD(P) or reduced/oxidized thioredoxins control the gating of K^+ channels, either directly or by modulating the ROS levels in localized domains. Hypoxia is well documented to increase the ratio of reduced to oxidized redox pairs in the lung. (Chandler et al., 1980) (Shigemori et al., 1996) (Leach et al., 2001) (Reeve et al., 2001), and carotid body (Biscoe & Duchon, 1990). One report demonstrates the redox effect not only of pyridine nucleotides but also the associated Kv-subunits. When Kv 1.5 is expressed in COS-7 cells, the current is not altered by inclusion of the oxidized pyridine nucleotides, NAD or NADP, in the patch pipette. However, if Kv β 1.3 is also transfected into the cell, then there is marked inactivation of the K^+ current, which can be prevented by inclusion of NAD or NADP. In single-channel studies, NAD also increases the mean open time of the Kv 1.5 channel. These reports illustrate how a change in redox status induced by hypoxia might alter K^+ channel gating in PSMCs (Tipparaju et al., 2005).

The relationship of redox changes to alterations in oxygen tension described so far has been fairly consistent. The only unknown being the signaling that permits a more reduced SMC environment to cause HPV in the pulmonary arteries and exocytosis in AMCs and NEBs, while causing relaxation in the DA. However, a problem arises because reports differ as to whether ROS go down with hypoxia in the pulmonary vasculature or up. The evidence has been reviewed in detail in a Point: Counterpoint debate (Ward, 2006) (Weir & Archer, 2006). Additional papers have provided arguments on both sides (Bonnet et al., 2006) (Waypa et al., 2006) (Wang et al., 2007) (Archer et al., 2008) (Waypa & Schumacker, 2008). An elegant paper describes the use of the redox-sensitive protein, RoGFP to examine ROS changes (Desireddi et al., 2010). Superfusion of mouse lung slices with hypoxic (1.5% O_2) media caused a rapid increase in oxidation of PASM Cytosolic RoGFP and an increase in intracellular calcium, both of which were prevented by overexpression of catalase,

suggesting that H_2O_2 was responsible. These data are persuasive although the level of hypoxia was apparently severe and the vessels were not pressurized or stretched. The importance of basal tone in the vascular production of ROS has recently been emphasized (Cui et al., 2008). It should be noted that RoGFP is not itself specific for H_2O_2 (Rhee et al., 2010) but specificity was provided by the use of catalase. It has also been reported that, in cultured PASMCs, the same level of hypoxia (1.5% O_2) led to a “slight” increase in oxidation in the cytoplasm and mitochondrial intermembrane space, while the mitochondrial matrix became more reduced (Waypa et al., 2010). Very similar observations in respect to redox changes were made in renal artery SMCs (RASMCs). The cytoplasmic oxidation of RoGFP in PASMCs during hypoxia could be prevented by the prior overexpression of cytosolic catalase. Hypoxia was shown to increase cytosolic calcium levels in PASMCs but not in RASMCs. Consequently, the signaling in this model, which determines that calcium only rises in the PASMCs in response to hypoxia seems to be downstream from the rise in cytoplasmic ROS, which occurred in both cells. Catalase was not used with the RASMCs, but there is no reason to think that a different ROS oxidized RoGFP in the cytoplasm of these cells than the H_2O_2 in the PASMCs. One would assume that the signaling, which is opposite in the PA and DA, is at least likely to be the same or similar within each vessel for all the effector mechanisms; gating of the K^+ channels, L-type Ca^{2+} channels, SR Ca^{2+} release and Rho activation. It follows from this group of experiments that ROS elevation might be necessary for oxygen sensing in this model but is not sufficient to explain differences between vessels. We have previously found that the mitochondrial function varies between tissues. In a comparison between pulmonary and renal artery for example, only the PASMC had mitochondria that showed significant oxygen sensing capacity (Michelakis et al., 2002).

The effects of hypoxia and the ETC inhibitor, rotenone, were compared in rat isolated arteries, vascular SMCs and perfused organs. Hypoxia and rotenone decreased ROS, inhibited IK and constricted PAs, while increasing IK and dilating RAs. Depending on the technique employed, hypoxia and rotenone tended to increase ROS or have no effect in the RAs. These observations could reflect differences in the ion channels between PAs and RAs or proximal signaling but we consider that mitochondrial diversity is just as likely to be involved. PA mitochondria had lower respiratory rates and higher rates of ROS and H_2O_2 production versus RAs. This reflected the lower expression of proximal ETC components and greater expression of SOD2, the major endogenous source of H_2O_2 , in PAs versus RAs. We concluded, “Differential regulation of a tonically produced, mitochondria-derived, vasodilating factor, possibly H_2O_2 , can explain the opposing effects of hypoxia on the PAs versus RAs”. Thus it appears, whether due to genetic diversity or acquired responses to local P_{O_2} environments, the PA and RA have different mitochondria and this is relevant to O_2 sensing (Michelakis et al., 2002).

Further complexity is added to the ROS signaling concept by a report that ROS go down in PASMCs in the first 5–10 minutes of hypoxia but are elevated by 48 hours (Wu et al., 2007). In the case of coronary arterial SMCs ROS went down and stayed down during hypoxia. The same group has found that, at least in regard to the “oxygen-sensitive” $\text{Kv } 1.5$ channel, the oxygen sensitivity is determined proximal to the channel protein (Platoshyn et al., 2006). They overexpressed the gene for this channel in rat PASMCs and mesenteric artery SMCs. The potassium current was markedly increased in both during normoxia. However, it was only in the PASMCs that hypoxia decreased the current. Consequently, there is another component in the signaling cascade besides the channel protein that behaves differently in these two vessels.

A final experiment which may shed light on the role of ROS in HPV is described in the fawn-hooded rat (FHR) (Bonnet et al., 2006). In this rat the normal filamentous structure of

the mitochondria is disrupted, there is loss of mitochondrial electron transport chain complexes, especially complex I, decreased ROS production, less expression of Kv 1.5 and reduced IK. HPV in the isolated perfused lung of the FHR is markedly diminished compared to that of Sprague-Dawley (S-D) lungs. These changes mimic those seen in lungs taken from S-D rats which have been exposed to chronic hypoxia. These lungs also have reduced ROS production and diminished HPV. The concept is that the FHR behaves as if it were chronically hypoxic, even in normoxia, presumably on the basis of diminished ROS production. As a result, it is thought that the PASMCMC is already reduced, and IK is diminished during normoxia. As a consequence acute hypoxia has less effect. Supporting this concept is the observation that adding H₂O₂ to FHR PASMCMCs restores Kv 1.5 expression. In the fawn-hooded rats decreased expression of MnSOD and mitochondrial abnormalities are detectable by 12 weeks of age and after 20 weeks HIF1 α activation, Kv suppression and pulmonary hypertension are also demonstrable. Recently it has been discovered that the loss of O₂ sensing and the reduced cytosolic state of the FHR's PASMCMC relates to downregulation of superoxide dismutase 2 (SOD2). SOD2 is silenced by methylation of CpG islands at 2 key sites in SOD2 and this accounts for loss of production of H₂O₂ and an associated activation of the hypoxic transcription factor, HIF1 α . This work suggests that derangement of normal O₂-sensing (in this case by suppression of H₂O₂ production) can eventually lead to excessive cell proliferation, impaired apoptosis and pulmonary hypertension, as well as altered HPV.

6.1 Lessons from heart failure

In type 1 cells taken from the carotid body of rabbits in which heart failure (CHF) has been induced, IK is reduced (Li & Schultz, 2006). Interestingly, the inhibition of IK and the depolarization caused by hypoxia is greater in the CHF cells than in control type 1 cells. Two observations have followed. One is that angiotensin II signaling increases the sensitivity of the Kv channels to hypoxia. This reminds the reader of the priming of HPV seen with angiotensin II and other vasoconstrictors. The other observation is that in the carotid bodies taken from CHF rabbits O₂⁻ levels are elevated and the levels of both Cu/Zn superoxide dismutase (SOD) and manganese (Mn) SOD protein expression are diminished (Ding et al., 2009) (Ding et al., 2010). Carotid sinus nerve activity in response to hypoxia is increased in the CHF rabbits. Transfection of either of the SODs to the carotid body of the intact CHF rabbits decreased the carotid sinus nerve response to hypoxia, which is otherwise elevated in CHF. Transfection also decreased O₂⁻ in the carotid body as expected and increased the diminished IK. These findings indicate that O₂⁻, or an ROS downstream, mediates or modulates oxygen sensing in the carotid body. The effect of the increased O₂⁻, or decreased H₂O₂, is to increase hypoxic signaling.

How might this observation apply in the pulmonary vasculature? MnSOD (SOD2) is reduced in the PASMCMCs of patients with idiopathic pulmonary arterial hypertension and in the fawn-hooded rats, that spontaneously develop pulmonary hypertension (Bonnet et al., 2006). If MnSOD in normal PASMCMCs is reduced by the use of siRNA, H₂O₂ generation and Kv 1.5 expression are decreased (Archer et al., 2010). Endogenous H₂O₂ is a pulmonary vasodilator, as catalase increases pulmonary vascular resistance (Francis et al., 2010) (Burke-Wolin & Wolin, 1989). One hypothesis would be that SOD controls the watershed between O₂⁻, peroxynitrite and hydroxyl radicals on one side and H₂O₂ on the other (Weir et al., 2010). As expected, SOD increases H₂O₂ generation in an "in vitro" experiment (Archer et al., 1989a). It is known that oxidative damage occurs in the mitochondria of heterozygous MnSOD knockout mice but not in the cytoplasmic proteins, at least in the liver (Williams et al., 1998). If SOD might determine the balance between vasoconstriction/proliferation and vasodilatation/apoptosis, what is the evidence that the administration of SOD alters HPV or chronic hypoxic pulmonary hypertension? The use of an SOD mimetic,

tempol (decreasing O_2 - and increasing H_2O_2), abolishes HPV in the isolated rat lung (Hoddy et al., 2007) (Figure 4) and normalizes right ventricular systolic pressure in rats exposed to chronic hypoxia (Elmedal et al., 2004). In the FHR with PAH, MnTBAP therapy is also beneficial in reducing PH and improving functional capacity. The authors attribute the benefit to restoration of physiologic H_2O_2 production; rather than a general “antioxidant effect” or the enhancement of the biological activity of nitric oxide which occurs when superoxide anion is scavenged and the half-life of nitric oxide is prolonged.

Chronic hypoxia has been found to increase O_2^- in the intrapulmonary arteries of wild-type mice but not in NADPH oxidase (gp91phox) knockout mice. The knockout mice do not develop pulmonary hypertension or medial thickening of the small pulmonary arteries on exposure to three weeks of hypoxia (Liu et al., 2006). This experiment not only indicates a role for O_2^- but suggests that specific isoforms of NADPH oxidase may be important. In contrast, we observed acute HPV to be intact in the gp91phox knockout mouse (Archer, 1999). Other workers find that NADPH oxidase (p47 phox) is active in the mechanism of acute HPV (Weissmann et al., 2006). Another report suggests that O_2^- generated by xanthine oxidase and inhibitable by allopurinol is involved in the mechanism of chronic hypoxic pulmonary hypertension (Hoshikawa et al., 2001). Finally, the over-expression of extracellular (EC)SOD in mice decreases chronic hypoxic pulmonary hypertension and muscularization of small pulmonary arteries (Nozik-Grayck et al., 2008). It is clear that SOD plays a critical role in O_2 sensing. The experiments cited in the CHF rabbits indicate that both CuZnSOD and MnSOD are involved. The over-expression of EC-SOD study above highlights the role of that isoform. This conclusion is strengthened by the observation that increasing EC-SOD activity decreases HPV in bovine PA rings (Ahmad et al., 2009).

It might be asked how an extracellular enzyme would alter oxygen sensing. However, most of the experiments of the effector limb of HPV or of normoxic DA contraction, such as K_v or KCa channels, L-type calcium channels or store-operated calcium channels, are in the cell membrane. RhoA activation commonly occurs at the cell membrane and can be initiated by membrane depolarization (Urban et al., 2003) or directly by ROS (Aghajanian et al., 2009). Consequently these components of HPV could be influenced by the redox status of the cell membrane. The release of O_2^- and H_2O_2 from the cell membrane is well described (Zhang et al., 1999) (Zulueta et al., 1995). There are several plasma membrane electron transport systems that transfer intracellular reducing equivalents to extracellular electron acceptors (Merker et al., 2002). When the external acceptor is oxygen, O_2^- is formed (Herst et al., 2004). The transplasma membrane electron transport (TPMET) pathway is particularly active when there is a build-up of reducing equivalents (eg NADH) as in glycolytic metabolism. The NADPH quinone oxidoreductase 1 (NQO1) is more closely related to shuttling electrons from NADPH. In some cells, but not others, NQO1 transport leads to generation of H_2O_2 outside the cell, inhibitable by SOD, which suppresses quinone redox cycling (Tan & Berridge, 2010). Thus there are complex systems that relate the redox status of the cytoplasm to that of the cell membrane and are likely to alter oxygen sensing (Figure 5).

7.1 Pathophysiology of Oxygen Sensing

There are several conditions which seem to be related to abnormalities of oxygen sensing. In one example, an inherited overactive sensing of hypoxia leads to chronic hypoxic pulmonary hypertension (CHPHT), (brisket disease) (Weir et al., 1974). The important role of SOD in CHPHT, in which supplementation of SOD reduces pulmonary hypertension, has already been discussed. Perhaps related to this, is the finding of low levels of SOD in the blood of patients with acute high altitude pulmonary edema, that returned to normal after recovery (Fu et al., 2002). On the other hand, the occurrence of patent ductus arteriosus (PDA)

persisting in neonatal life, may represent a failure of oxygen sensing. PDA is more common in premature infants and may reflect a developmental lack of oxygen sensitivity in Kv channels and L-type calcium channels (Thebaud et al., 2008). PDA is also more common in infants born at high altitude where the normoxic stimulus to DA contraction is weaker. It seems likely that at altitude, the level of H_2O_2 in the DASMCS which initiates contraction, will be diminished (Reeve et al., 2001b) (Kajimoto et al., 2007). It would be interesting to examine the effect of exogenous SOD on PDA closure.

Another and perhaps more speculative example of diminished oxygen sensing is preeclampsia. Preeclampsia (PET) is more frequent in women living at high altitude (Keyes et al., 2003). In measurements made in pregnant women at altitude (3,100m), before the development of any hypertension, it was found that those who subsequently developed PET had less redistribution of common iliac blood flow to the uterine artery, that supplies the placenta (Zamudio et al., 1995). It is considered that the placenta becomes hypoxic in PET in part because of this failure of redistribution and in part because of inadequate trophoblast invasion. Evidence for lipid peroxidation in the placenta in PET, increased ROS and decreased SOD in the blood and placenta has accumulated over nearly 20 years (Wang et al., 1992) (Wang & Walsh, 1996) (Sikkema et al., 2001) (Nakamura et al., 2009) (Padmini et al., 2009). Similar findings are reported in a rat model of PET (Sedeek et al., 2008). In the latter study the exciting observation is that treatment with the SOD mimetic, tempol, significantly reduces the systemic mean arterial pressure, which is elevated in the PET rats. Thus it appears that derangement of normal oxygen sensing systems is likely to be involved in a variety of disorders.

8.1 Conclusions

The evidence that redox changes are a key link in the cascade of oxygen sensing is strong. In neonatal adrenomedullary cells and neuroepithelial bodies a decrease in H_2O_2 (a more reduced environment) signals the onset of hypoxia and results in a decrease in IK, calcium entry and secretion. In PASMCs the effector mechanism for hypoxia has more components, including release of intracellular calcium, activation of RhoA and activation of the L-type calcium channel unrelated to membrane potential, as well as activation secondary to the inhibition of IK and membrane depolarization. However, we conclude that the crucial signal of hypoxia in the relevant microdomains (including the cytosol) is still reduction. Differences in study conditions and techniques may explain some of the disparate findings regarding the changes in ROS that follow changes in oxygen tension. However, an intriguing possibility is that changes in oxygen may change SOD activity and/or expression. In the PA this would mean that hypoxia would be associated with a decrease in SOD activity, such that the vasoconstrictor/ proliferative species O_2^- and its downstream ROS are increased and the vasodilator/proapoptotic species H_2O_2 is decreased. Although both O_2^- and H_2O_2 can be oxidizing agents, their signaling interactions are quite different and O_2^- can also act as a reducing agent. If correct, this concept would place SOD at the center of oxygen sensing (Weir et al., 2010). The possibility is all the more exciting because of a recent description that the expression of MnSOD is epigenetically controlled (Archer et al., 2010).

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Fig 1.

The Redox Hypothesis applies to the specialized tissues of the Oxygen Homeostatic System. Changes in ROS or Redox Couples, often mediated by the mitochondria, regulate ion channels, transporters and transcription factors, usually by altering the reduction or oxidation of key sulfhydryl groups that regulate protein function. These changes, are transduced into tissue-specific biologic activities including vasoconstriction, vasodilatation and secretion. In each tissue the net effect of activation is to enhance O₂ uptake and delivery.

Fig 2.
Mitochondrial-targeted GFP shows the mitochondrial network in a normal pulmonary artery smooth muscle cell cultured from a rat resistance pulmonary artery.

Fig 3.

The ETC is comprised of four mega-complexes that mediate transfer of electrons down a redox potential gradient through series of carriers resulting in final acceptance of the electron by O_2 , producing ATP and water. This electron transport leads to extrusion of hydrogen ions which creates the mitochondrial membrane potential (Ψ_m) and accounts for the negative membrane potential of mitochondria. This potential energy is later used as the driving force to power the F1F0 ATPase. A small percentage (<3%) of total electron flux involves unpaired electrons, resulting in the generation of ROS within the mitochondrion (e.g. superoxide radical). Mitochondrial manganese superoxide dismutase (SOD2) rapidly transforms this superoxide to hydrogen peroxide which is a diffusible signaling mediator (unless over produced in which case it becomes toxic to the cell). P_{O_2} -dependent changes in ETC activity and ROS production, together with the actions of a variety of “anti-oxidant” enzymes, vary levels of hydrogen peroxide and redox couples which alter the function of ion channels, such as Kv1.5 and the large conductance, voltage-gated calcium channel. The resulting changes in intracellular calcium lead to changes in secretion and vascular tone.

Fig 4.

SOD plays a key role in determining the predominance of either O_2^- or H_2O_2 in the cascade of oxygen signaling. In this figure, the end effector is shown as the oxygen-sensitive potassium channel. The intermediate links that are susceptible to redox changes might include enzymes, such as tyrosine kinases and phosphatases.

Fig 5.

The most important concept in the redox hypothesis of oxygen signaling is that it is the control of electron flow which determines the activation or inhibition of the effector mechanisms (including K⁺ channels, Ca²⁺ channels, SR and small G proteins).