

Oxidant and redox signaling in vascular oxygen sensing mechanisms: basic concepts, current controversies, and potential importance of cytosolic NADPH

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Wolin, Michael S., Mansoor Ahmad, and Sachin A. Gupte. Oxidant and redox signaling in vascular oxygen sensing mechanisms: basic concepts, current controversies, and potential importance of cytosolic NADPH. *Am J Physiol Lung Cell Mol Physiol* 289: L159–L173, 2005; doi:10.1152/ajplung.00060.2005.—Vascular smooth muscle (VSM) derived from pulmonary arteries generally contract to hypoxia, whereas VSM from systemic arteries usually relax, indicating the presence of basic oxygen-sensing mechanisms in VSM that are adapted to the environment from which they are derived. This review considers how fundamental processes associated with the generation of reactive oxygen species (ROS) by oxidase enzymes, the metabolic control of cytosolic NADH, NADPH and glutathione redox systems, and mitochondrial function interact with signaling systems regulating vascular force in a manner that is potentially adapted to be involved in Po_2 sensing. Evidence for opposing hypotheses of hypoxia, either decreasing or increasing mitochondrial ROS, is considered together with the Po_2 dependence of ROS production by Nox oxidases as sensors potentially contributing to hypoxic pulmonary vasoconstriction. Processes through which ROS and NAD(P)H redox changes potentially control interactive signaling systems, including soluble guanylate cyclase, potassium channels, and intracellular calcium are discussed together with the data supporting their regulation by redox in responses to hypoxia. Evidence for hypothesized potential differences between systemic and pulmonary arteries originating from properties of mitochondrial ROS generation and the redox sensitivity of potassium channels is compared with a new hypothesis in which differences in the control of cytosolic NADPH redox by the pentose phosphate pathway results in increased NADPH and Nox oxidase-derived ROS in pulmonary arteries, whereas lower levels of glucose-6-phosphate dehydrogenase in coronary arteries may permit hypoxia to activate a vasodilator mechanism controlled by oxidation of cytosolic NADPH.

hydrogen peroxide; hypoxia; Nox oxidase; mitochondria; pentose phosphate pathway

PROCESSES THAT POTENTIALLY FUNCTION AS OXYGEN SENSORS

The direct binding of O_2 to a protein such as the heme-containing soluble guanylate cyclase (sGC) (31, 89) that is directly linked to the control of cellular function would be an ideal way of sensing changes in Po_2 . However, a protein of this type has not yet been identified in vascular tissue. The substrate requirement for enzymes that metabolize oxygen is usually one of the most fundamental mechanisms through which Po_2 is sensed at the cellular level. Although the oxygen requirement for mitochondrial energy metabolism needed for the generation of force is the most basic mechanism of tissue oxygen sensing, there are only a few instances where this process appears to be the primary mechanism present in blood vessels controlling their response to acute changes in force elicited by hypoxia (95, 101). Thus other aspects of the activities of oxygen metabolizing enzyme having actions that influence cellular function need to be considered for their potential role in cellular sensing of Po_2 . Some types of enzymes that appear to

function as cellular Po_2 sensors include oxygenase enzymes generating mediators such as eicosanoids and nitric oxide that regulate signaling processes, hydroxylases generating regulatory mediators or modifying protein groups (e.g., proline) often associated with removal of these protein subunits, oxidases producing reactive oxygen species (ROS), and other aspects of mitochondrial metabolism and electron transport beyond their role in aerobic energy metabolism (1, 104, 107, 115). Because processes that are then influenced by the actions of oxygen metabolizing enzymes are potentially components of intracellular or tissue-derived mediator signaling mechanisms involved in Po_2 sensing, many cellular systems may appear to be participants in regulation by oxygen tension. Some of the intracellular processes in vascular smooth muscle that seem to be regulated by acute changes in Po_2 include K^+ channels, systems controlling intracellular Ca^{2+} , and the activity of sGC. Whereas many systems mentioned above (and others summarized in Table 1) seem to influence the control of organ blood flow by acute and chronic changes in Po_2 , pulmonary and systemic vascular smooth muscle cells appear to directly respond to changes in Po_2 through mechanisms that have a diversity of hypothesized origins often linked to changes in ROS and redox systems associated with energy metabolism. Review articles have considered the early literature in the

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Table 1. *Effects of hypoxia on force regulation by ROS and redox-controlled signaling mechanisms in pulmonary and systemic arterial smooth muscle*

Artery	Species	ROS or Redox-Controlled Signaling Response
Pulmonary	Rat	Decreased mitochondrial ROS closes K_v channels (64, 109) Increased mitochondrial ROS increases $[Ca^{2+}]_i$ (105, 106)
	Bovine	Decreased Nox-derived H_2O_2 decreases cGMP (16, 73)
Coronary	Bovine	Decreased cytosolic NADPH lowers $[Ca^{2+}]_i$ (37)
Renal	Rat	Increased mitochondrial ROS opens K^+ channels (64)

broad field of vascular oxygen sensing controlling systemic organ blood flow by hypoxia (103) and the role of ROS and redox signaling (115) functioning through the redox regulation of K^+ channels (65, 77) and cellular calcium homeostasis (101, 104, 107) in hypoxic pulmonary vasoconstriction (HPV). In this review, we focus on current knowledge and controversies in the area of how oxidases generating ROS and the function of key cellular redox systems interacting with ROS metabolism potentially function as O_2 sensors participating in vascular force responses elicited by acute changes in PO_2 , with a focus on systems that are present in vascular smooth muscle cells.

Initial suggestions that ROS formation and metabolism could function in PO_2 sensing mechanisms (96), potential roles of oxidant signaling in pancreatic cell function (93), and observations on how ROS (108) and thiol oxidation (110) cause relaxation in the pulmonary vasculature resulted in the proposal of a redox theory for the actions of hypoxia regulating K^+ channels (5, 9). In this theory, HPV was proposed by Archer et al. (5) to result from removing the oxidizing influence of mitochondria-derived ROS on cytosolic NAD(P)H and glutathione (GSH) redox systems. As shown in Fig. 1, the oxidation of these redox systems was proposed to maintain low levels of pulmonary artery force under normoxia by promoting hyperpolarization through opening K^+ channels by oxidizing thiols on these channels. Several observations have contributed to an emphasis of alternatives to the initial redox hypothesis. We initially found that hydrogen peroxide relaxed pulmonary arteries through increasing cGMP (15) and that hypoxia appeared to cause contraction associated with a lowering peroxide and cGMP in bovine pulmonary arteries (16, 17). Subsequently, we detected evidence for NAD(P)H oxidase (Nox) as the major source of basal ROS generation in this vascular segment (34, 70, 73, 85). Evidence for additional alternative mechanisms including hypoxia causing a ROS-mediated contraction (111), hypoxia promoting contraction through increasing ROS with either Nox oxidases (58) or mitochondria (105–107) being a major source of these ROS under hypoxia, have also contributed to the existence of extreme controversy in this field. There are also several different redox-associated theories for why systemic arterial smooth muscle relaxes to hypoxia, including hypoxia increasing mitochondria-derived ROS (64) and hypoxia causing relaxation through the oxidation of cytosolic NADPH (35, 37). Whereas limitations of the actions of pharmacological-type probes and ROS detection methods employed are often used to explain the diversity of theories that exist, the majority of mechanisms that have been investigated

in isolated endothelium-removed blood vessels and cultured vascular smooth muscle cells provides rather solid evidence for the existence of many key aspects of these diverse theories. Thus this review emphasizes the description of the known properties of the ROS and NAD(P)H-linked redox controlled systems potentially contributing to mechanisms of PO_2 sensing in vascular smooth muscle, and it attempts to then define the origins of the controversies that exist in this field by considering potential physiological aspects of how the systems were examined.

PROPERTIES AND POTENTIAL ROLE OF ROS GENERATING OXIDASES AS VASCULAR PO_2 SENSORS

Most cellular sources of ROS production appear to show a rate of generation of these species that changes over the range of oxygen tensions that are thought to regulate physiological processes. When the properties of purified oxidase systems are examined, they usually show increasing rates of generation of superoxide anion and/or hydrogen peroxide, the one- and two-electron-reduced products of molecular O_2 , respectively, with increasing PO_2 in a manner consistent with O_2 functioning as a substrate for these enzymatic systems. However, there is much evidence suggesting that the regulation of other aspects of ROS metabolism create conditions where the levels or actions of these species can increase under hypoxia. Although each method of ROS detection has limitations, there is a convincing level of combined detection and signaling evidence provided by the multiple approaches used that isolated pulmo-

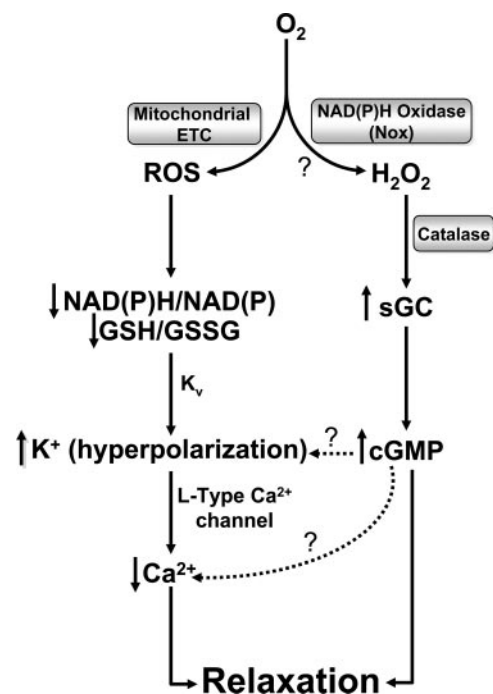


Fig. 1. Model showing initial concepts of the redox hypothesis for hypoxic pulmonary vasoconstriction, emphasizing hypoxia decreasing mitochondrial reactive oxygen species (ROS) production, which normally dilates pulmonary arteries under normoxic conditions through oxidizing cytosolic NAD(P)H and GSH redox systems. The oxidation of these systems was thought to maintain the opening of K^+ channels, causing hyperpolarization and relaxation through closing voltage-regulated Ca^{2+} channels. The model is expanded to include alternative roles for NAD(P)H (Nox oxidases) and cGMP. K_v , voltage-gated K^+ ; sGC, soluble guanylate cyclase; ETC, electron transport chain.

nary arteries or vascular smooth muscle cells can show either increased or decreased levels of ROS during hypoxia (56, 58, 64, 70, 73, 85, 106, 107). Thus one needs to consider how oxidases are functioning in vascular tissue under physiological conditions. Because the activities of oxidase systems appear to be highly regulated by physiological processes and the availability of electron-donating substrates used to reduce O₂, the role of each oxidase system as a potential Po₂ sensor will be highly controlled by factors beyond the Po₂ dependence of O₂ as a substrate for these oxidase systems unless these systems are active and have adequate electron donor [e.g., NAD(P)H] availability under baseline physiological conditions.

There are several different sources of ROS generation in cells present in vascular tissue that could function as a sensor of Po₂ through their substrate requirements for oxygen. Many of the known ROS-generating oxidases utilize electrons derived from NADPH or NADH for the production of ROS from molecular oxygen. There is now strong evidence that certain NAD(P)H oxidase systems (Nox) containing subunits initially identified in the phagocytic cell oxidase system show a baseline ROS generating activity in vascular cells that could function in Po₂ sensing (34, 58). Nox-1, Nox-2, and Nox-4 have been reported to be present in most of the cell types in the vascular wall, including smooth muscle (53). In general, the mitochondrial electron transport chain is potentially a significant cellular source of ROS. There is also substantial evidence based on probes that regulate mitochondrial electron transport that ROS generated in the proximal part of the electron transport chain have the ability to function in controlling signaling responses linked to vascular Po₂ sensing (65, 77, 107). Xanthine dehydrogenase/oxidase, cytochrome *P*-450, nitric oxide synthase (NOS), and cyclooxygenase are endothelial cell enzyme systems, also potentially present in vascular smooth muscle, that can generate ROS under certain circumstances. Although most of these other oxidases can be significant sources of ROS under specific physiological and pathophysiological conditions, there is generally an absence of evidence that they generate vasoactive levels of ROS under baseline conditions. Thus these other oxidase systems may not participate in the sensing of acute changes in Po₂ through changes in ROS generation under basal physiological conditions. It is important to consider what is known regarding processes that control ROS generating activity of these enzyme systems because they could become Po₂ sensors under conditions where physiological systems are stressed by pathophysiological conditions.

NOX OXIDASES

Properties of Nox oxidases. Vascular smooth muscle and other cell types in the blood vessel wall seem to contain NAD(P)H oxidase activity originating from Nox-1, Nox-2, and/or Nox-4 that appear to be actively generating superoxide and its metabolic products such as hydrogen peroxide (34, 38, 98). Although little is known regarding the actual role of the individual Nox oxidases in vascular Po₂ sensing, each oxidase has unique properties that may provide insight into how these enzymes contribute to the regulation of cellular function. Nox-2 is the most-studied oxidase as a result of its importance in generating ROS in phagocytic cells (10, 52). In unstimulated phagocytic cells, an inactive form of Nox-2 is present in

membranes containing gp91phox (now termed Nox-2) and p22phox subunits. All of the ROS generating Nox oxidase systems are thought have Nox subunits that contain a flavin electron transfer system to a b₅₅₈-type cytochrome, and most of these oxidases have been shown to also contain the p22phox subunit. Phagocytic cell activation promotes phosphorylation of a cytosolic p47phox subunit, associated with binding of this subunit to other cytosolic p67phox and p40phox subunits. Subsequent binding of this subunit complex to the membrane-bound subunits then permits electron transfer from NADPH into a flavin-linked cytochrome b₅₅₈ that subsequently reduces molecular oxygen into superoxide. The reduced (Fe²⁺) form of cytochrome b₅₅₈ appears to directly react with oxygen at a very rapid rate [$\sim 10^{-7} \text{ M}^{-1}\text{s}^{-1}$ at 10°C (46)]. This rapid reaction rate is consistent with an absence of evidence for the detection of Fe²⁺ cytochrome b₅₅₈ in cells or membrane preparations under aerobic conditions, and it suggests that the Po₂ dependence of this oxidase will be both a function of the rate of electron transfer to cytochrome b₅₅₈ and concentration of oxygen that reacts with the Fe²⁺ form of this cytochrome. This superoxide generating process is further enhanced through the cell activation as a result of promoting binding of the small GTPase Rac to a different site on the gp91phox subunit. In vascular smooth muscle, p47phox and Rac-1 appear to be involved in Nox-oxidase activation, and there is currently a lack of evidence for p67phox and p40phox subunits having a role in controlling Nox activity (53). Recent studies have provided evidence that a Nox-O1 subunit functions in a manner similar to the p47phox subunit in promoting Nox-1 activity, but it seems to be already bound to Nox-1 in the absence of cellular stimulation (52). It is thought that cell activation-stimulated binding of a Nox-A1 subunit to Nox-1+Nox-O1 further increases its superoxide generating activity in a manner similar to p67phox. However, the role of these subunits in the control of Nox-1 activity in vascular smooth muscle is not yet known. Nox-4 does not seem to have a site that has been linked to the binding of p47phox or Nox-O1 subunits (52), suggesting it may have another mechanism of regulation or that it is actively generating a basal level of ROS in the absence of cellular stimulation. Nox-4 was initially described as a renal Nox (renox), which was suggested to be a Po₂ sensor involved in controlling the production of erythropoietin (30). The emerging picture of how these oxidases function is that each oxidase may have specific subcellular sites that are colocalized with signaling systems that control the activity of the oxidase and signaling mechanisms regulated by the ROS generated by the oxidase (40, 114). Whereas the Nox oxidases seem to utilize both NADH and NADPH as substrates, the greater availability of cytosolic NADPH over NADH may cause it to be the primary substrate for the baseline activity of these oxidases seen under normoxic conditions (34). Changes in many physiological stimuli such as growth factors including angiotensin II, pressure and shear forces, nitric oxide, and Po₂ can potentially influence the expression, activation, and/or availability of NAD(P)H as substrates for these oxidases. On the basis of the properties of Nox oxidases, it appears that hypoxia could cause an acute increase in ROS production by augmenting rates of electron transport to cytochrome b₅₅₈, and this could occur through hypoxia promoting oxidase activation and/or increasing the availability of its NADH or NADPH substrates. Thus the Nox oxidases are potentially highly adapt-

able systems for different roles in vascular Po_2 sensing and the regulation of many redox-controlled regulatory processes.

Potential roles of Nox oxidases as vascular Po_2 sensors. There are hypotheses for Nox oxidases having key roles in either increasing (58, 111) or decreasing (34, 70, 73, 85) their production of vasoactive ROS in the pulmonary hypoxic vasoconstriction response. A general limitation in this area has been methods of probing the role of Nox oxidases. Hypotheses supporting the role of Nox oxidases as a source of either increased vasoconstrictor ROS or decreases in vasodilator ROS during hypoxia are based on potentially nonspecific inhibitors of these oxidases such as diphenyleneiodonium-type agents (58, 64, 70, 71, 100) selectively attenuating the detection of superoxide and/or the pulmonary vascular contraction to hypoxia, under conditions where they do not markedly alter the response to other vasoconstrictors. For example, in endothelium-removed bovine pulmonary arteries, we have observed (70, 72) that hypoxia decreases the detection of superoxide derived from what appears to be a Nox-type oxidase, which is inhibited by diphenyliodonium under conditions where this probe attenuates contraction to hypoxia. The primary sources of superoxide generation in these bovine pulmonary arteries appear to be cytosolic NAD(P)H redox-dependent Nox-type oxidases (34, 73, 74). Hypoxia appears to decrease hydrogen peroxide metabolism by catalase and a relaxation that seems to be mediated through this process stimulating cGMP production by sGC in these arteries (16, 17). In addition, lowering intracellular peroxide with ebselen and inhibiting catalase-mediated stimulation of guanylate cyclase with 3-amino-1,2,4-triazole mimics the effects of hypoxia on force in these bovine pulmonary arteries (16, 72). The support for Nox oxidases increasing ROS generation under hypoxia includes evidence from the laboratory of Marshall et al. (58) for showing in scintillation vial experiments that hypoxia increased the detection of superoxide from large and resistance bovine arterial smooth muscle in a manner inhibited by diphenylene iodonium and not by the mitochondrial inhibitor myxothiazol. This study also reported evidence that diphenylene iodonium also attenuated contraction to hypoxia in cat pulmonary arteries without increasing force. Observations by Weissmann and colleagues (111) in saline-perfused rabbit lungs of an attenuation of the contraction to hypoxia by an inhibitor of Nox oxidases [4-(2-aminoethyl) benzenesulfonyl fluoride] without increases in baseline perfusion pressure or agonist-induced contraction under normoxia have also been interpreted as originating from increased Nox-derived ROS under hypoxia. Since the pulmonary vessels from mice lacking gp91phox (Nox-2) have been demonstrated to show normal contractile responses to hypoxia associated with decreased detection of superoxide (8), there is rather strong evidence against a key role for changes in the generation of ROS by Nox-2 in the hypoxia-elicited contractile response. However, recent studies have detected Nox-4 (in addition to Nox-2) in pulmonary arteries that contract to hypoxia (34). Whereas apocynin appears to inhibit basal Nox-2 activity in bovine pulmonary arteries (34), this probe does not appear to attenuate hypoxia-elicited increases in cultured rat pulmonary arterial smooth muscle cells (105) or force in bovine pulmonary arteries (Ahmad and Wolin, unpublished observations) or mimic the effects of hypoxia in rat lungs (105). Thus the role of Nox-4 needs to be a focus of future studies examining the

role of Nox oxidases in pulmonary arterial responses to hypoxia.

MITOCHONDRIAL ROS GENERATION

Properties of ROS generation by mitochondria. Mitochondria are a potential source of ROS generation in all cells, and their ability to generate these species has many facets that need to be considered. Early work by Chance and colleagues (19) investigating peroxide release from mitochondria provided evidence that isolated mitochondria studied in the presence of substrate, ATP, and O_2 ("state 4") showed higher levels of hydrogen peroxide formation than mitochondria studied under similar conditions replacing ATP with ADP ("state 3"). This work led to evidence for two sites in the electron transport chain that appear to be the major sources of mitochondrial superoxide generation (see Fig. 2). One site is in the NADH dehydrogenase in the region of complex I proximal to the location where rotenone inhibits electron transport. The other major site identified was in the region of the Q-cycle distal to the myxothiazol and proximal to the antimycin A sites of electron transport inhibition. Superoxide formation from this site was thought to be closely associated with the levels of the ubisemiquinone or one-electron-reduced form of coenzyme Q (39, 78). Manganese superoxide dismutase (Mn-SOD) in the matrix of mitochondria appears to convert superoxide generated from both the complex I site and ubisemiquinone in the region of the Q_i site of complex III into hydrogen peroxide, whereas copper-zinc superoxide dismutase (Cu,Zn-SOD) on the cytosolic side of the mitochondrial inner membrane is thought to convert superoxide generated from ubisemiquinone in the region of the Q_o site into hydrogen peroxide. Mitochondria are thought to release peroxide as a result of permeability through membranes and superoxide through voltage-gated anion channels located on the outer membrane of mitochondria. Antimycin is thought to bind at the Q_i site in a manner that enhances the levels of ubisemiquinone and superoxide generation, whereas myxothiazol is thought to bind at the Q_o site and prevent ubisemiquinone formation. Some of the most recent work on the site of superoxide production in the NADH dehydrogenase suggests that superoxide is either generated at the flavin site linked to electron transfer system with the iron-sulfur center of complex I (50) or the site where complex I binds ubisemiquinone in a manner that is stabilized by rotenone (51). There is also recent evidence that complex I reduces coenzyme Q to its hydroquinone form (QH_2) through a ubisemiquinone intermediate that can be stabilized (83). Several factors appear to control superoxide production at these sites located in the proximal portion of the electron transport chain. It is currently thought that elevated superoxide production is related to increased reduction of the proximal portion of the electron transport chain, which is generally associated with high availability of mitochondrial NADH and protomotive force. Freeman and Crapo (29) showed how isolated mitochondria increase their production of ROS as a function of Po_2 , and work from Waypa et al. (105, 106) has provided evidence that hypoxia can promote increased mitochondrial ROS production primarily from the ubisemiquinone site associated with complex III. However, it is perhaps more important to highlight that baseline release of ROS from mitochondria seems to be essentially not detectable until these

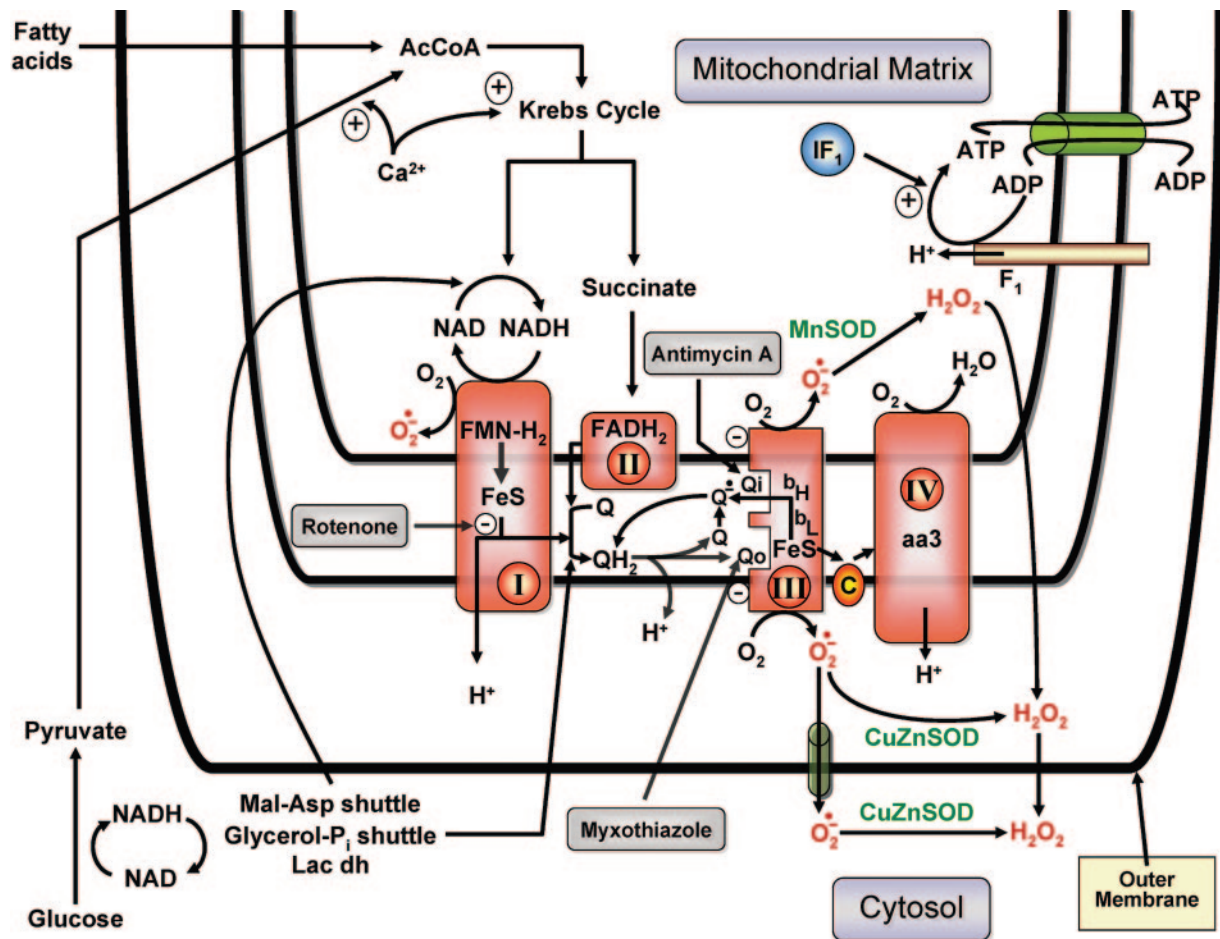


Fig. 2. Model emphasizing sites where mitochondrial electron transport inhibitors potentially function to modulate superoxide generation. Inhibitors are shown in gray boxes; mitochondrial complexes are shown in red and yellow boxes. Arrows in the region of complexes I-IV show pathways of electron transfer between flavins (FMN-H₂, FADH₂), iron-sulfur centers (Fe-S), coenzyme Q (Q-QH₂), cytochromes (b_H, b_L, c, aa₃) and molecular oxygen (O₂) associated with the generation of superoxide (O₂⁻). The data supporting hypoxia increasing mitochondria-derived ROS are most consistent with superoxide generation originating from complex III. The model also shows some key aspects of mitochondrial-cytosolic metabolic redox interactions. Mal-Asp shuttle, malate-aspartate; glycerol-P_i shuttle, glycerol phosphate; lac dh, lactate dehydrogenase; AcCoA, acetyl coenzyme A; F₁, ATP synthase site; IF₁, inhibitor subunit of the F₁ ATPase reaction; Mn-SOD, manganese superoxide dismutase; Cu,Zn-SOD, copper-zinc superoxide dismutase. See the text for further description.

organelles are exposed to inhibitors of electron transport or oxidative stress (50, 99). Mitochondrial levels of Ca²⁺ appear to control the activity of the Krebs cycle under physiological conditions, and multiple pathophysiological processes promote Ca²⁺-dependent increases in the generation of ROS by mitochondria (14). In a recent study in cultured neurons, it was demonstrated that a brief transient exposure to high physiological levels of intracellular Ca²⁺ promotes a prolonged increase in mitochondrial ROS generation from a site that is inhibited by rotenone (43). Thus other factors occurring under physiological conditions, such as brief exposure to high levels of intracellular Ca²⁺ or oxidants, may enhance mitochondrial ROS generation.

Evidence for hypoxia decreasing mitochondrial ROS supporting the redox hypothesis of pulmonary hypoxic vasoconstriction. Perspectives on the strengths and weaknesses of evidence supporting a role for mitochondria decreasing (77) and increasing (107) ROS generation in mediating the process of pulmonary hypoxic vasoconstriction are the subjects of two very recently published review articles. Thus our discussion will focus on concisely describing the strongest fundamental

evidence related to the role of mitochondria-derived ROS in responses of vascular smooth muscle to hypoxia in a manner that allows the discussion of alternative hypotheses for potential metabolic roles of mitochondria in influencing cytosolic NADP(H) redox-linked sensing mechanisms that are considered in this review article. Studies on the role of mitochondrial ROS in vascular Po₂ sensing evolved from the redox hypothesis of Archer et al. (9). Initially, it was observed that hypoxia, rotenone, and antimycin decreased the detection of ROS from perfused lungs detected by lucigenin and/or luminol chemiluminescence (5, 86), and subsequently these agents were shown to decrease ROS in isolated rat pulmonary arteries and mitochondria isolated from these arteries by several additional detection methods (64). Early studies by Rounds and McMurtry (91) had demonstrated that these mitochondrial inhibitors caused transient vasoconstriction responses in perfused rat lungs. On the basis of these observations and finding that rotenone and antimycin inhibited both the subsequent hypoxic vasoconstriction responses in perfused lungs and K⁺ currents in isolated pulmonary artery smooth muscle cells in a manner similar to hypoxia, it was hypothesized that hypoxia closed the

K⁺ channels by causing a shift to a more reduced cytosolic redox state through decreasing the release of mitochondria-derived ROS (5). Michelakis and colleagues (64) also reported evidence that pulmonary arteries had mitochondria that differed from renal artery mitochondria in that isolated pulmonary artery mitochondria produced more ROS from a source inhibited by antimycin, had lower levels of complex I and III protein subunits, and showed a more depolarized mitochondrial membrane potential in primary cell cultures than renal arterial smooth muscle cells. In addition, this group provided evidence that renal arteries show an increase in superoxide detected by lucigenin under hypoxia. Thus in rat pulmonary arteries hypoxia has been observed to decrease vasodilator ROS production from the proximal part of the electron transport chain in a manner that is very well supported by the properties of mitochondria present in the preparations employed for the studies supporting the redox theory for hypoxic vasoconstriction.

Evidence for hypoxia increasing mitochondrial ROS as a mediator of pulmonary arterial response to hypoxia. Waypa et al. (105, 106) investigated the role of mitochondrial ROS in rat pulmonary responses to hypoxia and found some similarities and differences that pointed toward mitochondria increasing ROS production in hypoxia. This group developed evidence primarily from the properties of pulmonary arterial smooth muscle cell contractile and intracellular Ca²⁺ responses that were very consistent with previous studies on ROS generation by mitochondria in multiple different nonvascular tissue preparations that defined the ubiquinone-complex III region of the mitochondrial electron transport chain as a key site of superoxide generation. In their studies, hypoxia was observed to promote an increase in ROS generation in cultured rat pulmonary arterial smooth muscle cells over a 30-min time period from a site inhibited by myxothiazol. This agent was shown to attenuate hypoxia-elicited vasoconstriction in perfused rat lungs and increases in intracellular Ca²⁺ detected with fura-2 and contraction in cultured rat pulmonary artery smooth muscle cells without inhibiting similar responses to the contractile agent U-46619. A dose of rotenone was also found to inhibit the effects of hypoxia on contraction and intracellular Ca²⁺ in a manner similar to myxothiazol, and similar results were observed by others in isolated rat pulmonary arteries (54). The actions of antimycin in these studies by Waypa et al. were more difficult to interpret because whereas low doses of antimycin did not alter contractile responses to hypoxia in perfused lungs or increases in Ca²⁺ in cultured pulmonary artery smooth muscle cells, higher doses caused contractile responses in perfused lungs and cells, associated with an attenuation of force generation by contractile agents. Pulmonary artery myocytes cultured with ethidium bromide for 2 wk to deplete essential electron transport chain subunits needed for mitochondrial function were shown to selectively lose their contractile response to hypoxia. In addition, the actions of agents used to inhibit anion channels and Cu,Zn-SOD and enhance the removal of hydrogen peroxide functioned in the direction of supporting a role for an increased hydrogen peroxide-elicited Ca²⁺ release mediating contraction during hypoxia originating from superoxide that had to be released from anion channels and converted to hydrogen peroxide by SOD. Overall, the studies by Waypa et al. are highly consistent with hypoxia increasing superoxide generation from the ubiquinone-complex III region of the mitochondrial electron transport

chain in the mechanism of hypoxia-elicited contractile responses in the rat pulmonary artery smooth muscle cell preparations utilized by this group.

Potential alternative explanations for the role of mitochondria in Po₂-elicited vascular responses. There is evidence that mitochondria may have additional mechanisms of contributing to vascular responses to hypoxia. It has been reported in a rabbit pulmonary artery primary cell culture preparation that hypoxia increased intracellular Ca²⁺ detected by fura-2 from what appears to be a sarcoplasmic reticulum source in a manner that was enhanced by the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP), suggesting that mitochondria were functioning to buffer the Ca²⁺ that was released (47). In this study, it was also reported that hypoxia did not release Ca²⁺ in primary cultures of rabbit coronary smooth muscle cells. It has been observed that mitochondrial electron transport inhibitors attenuate force generation in essentially all of the rat pulmonary vascular preparations where changes in mitochondria-derived ROS are reported to have a primary role in mediating contractile responses to hypoxia. In addition, most of these preparations also show a relaxation following the initial contraction to hypoxia, especially under extreme hypoxic conditions. This relaxation to extreme hypoxia has been shown to be associated with a glucose-dependent decrease in ATP levels (94). In contrast, studies supporting the Nox oxidases in hypoxic responses have generally been conducted in bovine pulmonary arteries where mitochondrial inhibitors seem to have minimal effects on force generation or the response to hypoxia (58, 60). One possible explanation for these differences is the potential absence in rats of the mitochondrial ATPase inhibitor subunit IF₁ that functions to prevent the consumption of cytosolic ATP for maintenance of mitochondrial membrane potential under conditions such as severe hypoxia (92). Our studies also indicate that force generation in endothelium-removed bovine coronary arteries is essentially not altered by inhibition of mitochondrial electron transport by antimycin A (33), suggesting that the inhibition of mitochondrial function does not mimic hypoxia in these arteries. Although most systemic and pulmonary vascular preparations studied seem to maintain their ATP levels under hypoxia, hypoxia may modulate the function of cytosolic energy metabolizing systems in a manner that influences the function of cytosolic redox control mechanisms (112). Leach et al. (54) observed that severe hypoxia appears to cause an increase in NAD(P)H fluorescence in isolated rat pulmonary arteries that seems to originate from inhibition of the mitochondrial electron transport chain. Mitochondria may also be functioning to buffer Ca²⁺ that is released from the sarcoplasmic reticulum (SR), and they are likely to have important metabolic interactions with the systems that control cytosolic NAD(H) and possibly NADP(H) redox, which could contribute to vascular responses to hypoxia.

PROPERTIES OF OTHER VASCULAR OXIDASES

The ROS generating activities in vascular tissue of xanthine dehydrogenase/oxidase, cytochrome P-450, NOS, and cyclooxygenase are normally thought to be quite low. However, these systems can be activated to produce ROS under conditions that stress the vasculature. The oxidase activity of xanthine dehydrogenase appears to be stimulated by p38 MAP

kinase phosphorylation and by modification of this protein by thiol oxidation and proteolysis (13). The expression and/or activity of xanthine oxidase in the vascular endothelium has been reported to be increased by conditions including hypoxia, inflammation, and hypertension. This enzyme normally uses hypoxanthine and xanthine, and perhaps cytosolic NADH, as substrates to generate superoxide and hydrogen peroxide when they are available. Conditions such as hypoxia or ischemia are thought to cause the accumulation of these substrates for xanthine oxidase and to increase the oxidase activity of this enzyme. The metabolism of substrates by cytochrome *P*-450 (11) and cyclooxygenase (48) can cause the generation of superoxide by these enzymes. Whereas the significance of these enzymes in regulating vascular function through the generation of ROS is not well understood, bradykinin has been reported to stimulate endothelial generation of ROS by these enzymes in certain preparations (25, 42, 48). The NOS reaction can become a major source of endothelial cell hydrogen peroxide generation when its production of NO becomes uncoupled and the superoxide generating NADPH oxidase activity of this enzyme increases (4). Oxidation or depletion of the tetrahydrobiopterin cofactor for the NOS reaction is one of the most fundamental ways of increasing the NADPH oxidase activity of this enzyme. Whereas endothelial cell oxidant stress and peroxynitrite formation promote loss of tetrahydrobiopterin, the oxidase activity of NOS also appears to be enhanced or inhibited by many of the known ways of regulating the NOS reaction. Currently, there is an absence of information documenting the role of the enzymes considered here as sources of ROS generation within vascular smooth muscle cells in amounts linked to the control of signaling systems. However, it has been demonstrated that the endothelium has the ability to generate vasoactive levels of ROS from each of these systems under specific pathophysiological conditions. Thus these systems could potentially become endothelial cell Po_2 sensors regulating vascular function through ROS when they are activated to produce these species.

MECHANISMS OF INTERACTIONS OF ROS WITH CELLULAR SIGNALING SYSTEMS THAT COULD MEDIATE VASCULAR Po_2 -ELICITED RESPONSES

The intracellular levels of ROS in cells under basal physiological conditions associated with Po_2 sensing for superoxide and peroxide are likely to be in the picomolar and low nanomolar ranges, respectively (19, 113, 116). Thus several factors are likely to be of major importance in controlling the interaction of these species with signaling systems under physiological conditions (113, 116). First, the rate constant for reaction of the ROS with a component of the signaling system needs to be extremely high, such as in the range of 10^{-6} to $10^{-9} \text{ M}^{-1}\text{sec}^{-1}$. Although metabolizing enzymes for ROS have rate constants in this region, there are few other interactions with protein functional groups that have known rates of reaction with ROS consistent with roles in physiological regulation. It is also possible that a subcellular colocalization of redox-regulated components of signaling systems with oxidases producing ROS could either allow the selective regulation of certain signaling systems or permit localized increases in ROS to levels that promote reactions with lower rate constants to occur. Interactions of individual ROS with cata-

lase, GSH peroxidase, peroxiredoxins, and other metal centers such as heme peroxidases or thiols activated by binding metals such as iron or zinc are enzymes or sites on proteins where ROS can potentially initiate the modulation of signaling processes. For example, the metabolism of peroxide by GSH peroxidase or peroxiredoxins results in the formation of GSSG and oxidized thioredoxin (Trx), respectively, and perhaps the oxidation of NADPH to NADP through the GSH reductase and Trx reductase reactions. The formation of GSSG is thought to promote regulation through the glutathionation of protein thiols, whereas the Trx/Trx reductase system is thought to control multiple aspects of regulation through redox processes, such as the redox status of specific protein thiols. Peroxide metabolism through catalase can stimulate sGC (15) and perhaps regulate the activity of protein phosphatases (117), and cyclooxygenase has a heme peroxidase activity that turns on and sustains the production of prostaglandins by this enzyme (59). Oxidation of the zinc finger or zinc-thiol sites on PKC enzymes appears to be a mechanism through which ROS can directly activate this important regulatory system (49). There is evidence that thiol redox can regulate many of the signaling systems thought to participate in Po_2 sensing, such as K^+ and Ca^{2+} channels, and Ca^{2+} ATPases that promote Ca^{2+} reuptake such as sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) (113, 116). SERCA is the major reuptake system for Ca^{2+} by the SR of vascular smooth muscle, and it could potentially to be a key site for regulation of vascular function by both redox and Po_2 . One of the most sensitive systems for regulation by ROS is the activity of sGC. Peroxide has several ways of stimulating sGC, including a process mediated by peroxide metabolism via catalase, and through peroxide activating the production of nitric oxide by endothelium (15, 18). In addition, the most sensitive known sites for the actions of increased superoxide include inhibition of these mechanisms of sGC stimulation (21). Thus multiple ROS and redox-linked cellular control systems could be regulated by changes in Po_2 .

MITOCHONDRIAL AND CYTOSOLIC REDOX SYSTEMS POTENTIALLY REGULATED BY HYPOXIA THAT COULD POTENTIALLY FUNCTION AS VASCULAR Po_2 SENSORS

Organization of mitochondrial and cytosolic NAD(P)H redox systems. Many cellular oxidation-reduction (redox) systems seem to interact in ways that suggest these processes are organized for participation in physiological regulatory processes, including the sensing of Po_2 . Whereas much is known about the properties of many of the enzymes that utilize the NAD(H) and NADP(H) pyridine nucleotides, there is only limited information on how these systems function in cellular regulation. Cellular metabolism is designed (80) to generate and use separate cytosolic and mitochondrial NADP/NADPH and NAD/NADH redox systems for the control of other redox components that are often more directly linked to signaling systems or to the control of physiological processes. The cytosolic pool of NADP(H) seems to be significantly reduced in most cellular systems that have been studied, whereas the cytosolic NAD(H) pool seems to be highly oxidized in most cells under baseline-type conditions (19, 80). Recent studies are consistent with vascular smooth muscle having cytosolic redox control mechanisms that are regulated in a manner similar to other cellular systems that have been previously

characterized (12). The mitochondrial pool of NAD(H) is thought to be significantly reduced by the flow of substrates (pyruvate and fatty acids) into the Krebs cycle, but the redox status of this system appears to be dynamically regulated. Factors such as the balance between cellular work and the ability to maintain oxygen delivery to mitochondria and the availability of substrates for Krebs cycle metabolism derived from glycolysis and fatty acids to support cellular work may all contribute to the dynamic regulation of NAD(H) redox in the matrix of mitochondria. Mitochondrial NADPH levels seem to be dependent of aspects of energy metabolism that are similar to mitochondrial NADH, including some equilibration through isocitrate dehydrogenase and transhydrogenase enzyme activities, but NADPH levels also seem to be controlled by other processes, such as its consumption, as a result of the metabolism of oxidants such as peroxide. The mitochondrial shuttles (maleate-aspartate and glycerol phosphate) are thought to work together with the lactate dehydrogenase reaction to maintain low levels of cytosolic NADH and to also function as systems that permit mitochondrial-cytosolic redox interactions (see Fig. 2). The cytosolic NADPH pool is thought to be primarily derived from the initial enzymatic reactions of the pentose phosphate pathway (PPP) beginning with the glucose-6-phosphate dehydrogenase reaction. Although many enzymes are known to utilize NADPH including GSH reductase, Trx reductase, cytochrome *P*-450, NADPH oxidase, etc., their contribution to the consumption of NADPH seems to depend on factors that control the activity of each system. Because cytosolic NADH concentrations are kept low, it is likely that conditions such as tissue hypoxia, increased levels of mitochondrial NADH, and/or the availability of increased lactate cause metabolic conditions that allow cytosolic NADH to accumulate and become a potential factor in regulating cellular processes. Many systems that influence pyridine nucleotide redox are closely associated with enzymes that metabolize oxygen, and this creates interactions that are likely to be of great importance in the conversion of cellular P_{O_2} sensing information into the expression of a physiological response.

The original redox theory for hypoxic vasoconstriction developed by Archer et al. (9) emphasizes removal by hypoxia of an oxidation of cytosolic NAD(P)H and GSH redox in pulmonary arteries by ROS derived from mitochondria and other sources now thought to be systems such as Nox oxidases (77). It has recently been reported that hypoxia increases NAD(P)H fluorescence in isolated rat pulmonary arteries in a manner that appears to originate from inhibition of the mitochondrial electron transport chain by a process similar to the actions of rotenone (54). Although this could be viewed as being consistent with the redox theory of HPV, the increased NAD(P)H fluorescence in these pulmonary arteries remained elevated under hypoxia after the observed transient contraction response decayed. In addition, rotenone did not mimic the contraction to hypoxia, and succinate reversed the inhibitory actions of rotenone on the contraction to hypoxia without altering the detection of NAD(P)H fluorescence. In addition, earlier studies have shown that hypoxia causes a rapid increase in rat pulmonary artery NADH levels, without alterations in NADPH (94). These observations could either originate from an absence of the ability to detect changes in cytosolic NAD(P)H redox localized to regions near mitochondria by the fluorescence methods used (77) or they may indicate that mitochondrial

control of cytosolic redox is not a primary factor in the mechanism of pulmonary artery contraction to hypoxia.

The influence of NAD(P)H redox changes on the response of systemic arteries to hypoxia does not appear to have been investigated in previous studies. It is well established that hypoxic or ischemic organs usually release lactate. In addition, lactate is a vasodilator of most systemic arteries that have been studied (20, 115). Our recent studies are detecting evidence that hypoxia relaxes bovine coronary arteries through a mechanism that seems to be associated with promoting the oxidation of cytosolic NADPH and GSH (35, 37). Hypoxia appears to lower the levels of superoxide and hydrogen peroxide in these arteries, and modulating the levels of these ROS species does not influence expression of the relaxation to hypoxia (67, 69). Thus changes in ROS generation by hypoxia do not appear to mediate the oxidation of NADPH and GSH that is observed. Our studies suggest that the oxidation of NADPH by hypoxia in coronary arteries is associated with decreases in glucose-6-phosphate, which is consistent with NADPH generation by the glucose-6-phosphate dehydrogenase reaction of the PPP being impaired by hypoxia. Inhibition of glycolysis with 2-deoxyglucose or mitochondrial electron transport with antimycin A does not promote coronary artery relaxation (33). Thus the response to hypoxia does not seem to occur through directly inhibiting glycolytic or mitochondrial energy metabolism, and it does not mimic the actions of these inhibitors on redox systems such as cytosolic NADPH. Interestingly, our studies detect that pyruvate inhibits relaxation to hypoxia in bovine coronary arteries in a manner associated with preventing the oxidation of NADPH by hypoxia (37). The actions of pyruvate further support the potential importance of a recently described PPP-regulated relaxing mechanism thought to be controlled by changes in cytosolic NAD(P)H redox (33) in the response to hypoxia. Although it has been known since its initial discovery by Warburg (103a) that oxygen regulates metabolism through the PPP and that glycolytic metabolism competes with the PPP, the actual P_{O_2} -sensing mechanism and metabolic processes that link this hypothesized sensor to the control of cytosolic NAD(P)H redox remain to be elucidated.

MECHANISMS OF INTERACTIONS OF NAD(P)H REDOX SYSTEMS WITH CELLULAR SIGNALING SYSTEMS

The localized concentrations of individual pyridine nucleotides can potentially have a major influence of regulatory systems that control the physiological behavior of cells such as processes that regulate vascular smooth muscle contractile function. Because contractile function is most directly controlled by cytosolic regulatory systems, this section will emphasize linkages of redox to the control of signaling in the cytosolic compartment. The availability of NADPH directly controls the maintenance of GSH and Trx redox through the reductases for these systems. The removal of peroxide by peroxiredoxins and GSH peroxidases is therefore linked to the ability of a cell to maintain its levels of NADPH. The levels of NADPH and NADH also appear to control the rates of generation of superoxide and peroxide generation by NADPH oxidases such as the Nox oxidases present in vascular smooth muscle (34, 97). Thus the initiation of redox signaling resulting from the generation of oxidants, the levels of individual ROS potentially interacting with signaling systems, and the reversal

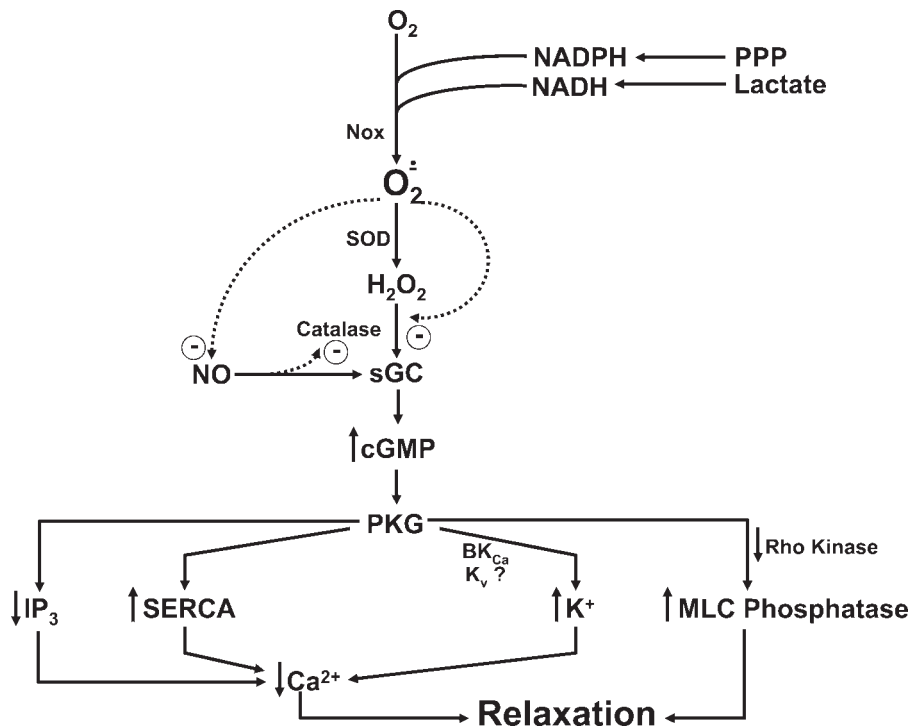


Fig. 3. Model showing how the oxygen requirements of Nox oxidases could control sGC activity through ROS and how cGMP coordinates multiple mechanisms of vascular smooth muscle relaxation. PPP, pentose phosphate pathway; NO, nitric oxide; PKG, cGMP-dependent protein kinase; IP₃, inositol 1,4,5-trisphosphate; BK_{Ca} and K_v, Ca²⁺- and voltage-activated K⁺ channels; SERCA, sarco(endo)plasmic reticulum Ca²⁺-ATPase; MLC, myosin light chain.

of redox changes caused by oxidants are all controlled by the local concentrations of NADPH and NADH. The activation of Nox oxidases seems to be able to cause cytosolic NAD(P)H redox changes (12), but it is not known whether these changes are due to consumption by oxidases or the signaling actions of ROS that are generated. In addition, NADPH is a cofactor for the generation of vasoactive mediators produced in the vessel wall through enzymes such as NOS, cytochrome *P*-450, heme oxygenase, etc., and there are observations suggesting that specific pyridine nucleotides can control the activities of some key systems regulating intracellular K⁺ and Ca²⁺.

CELLULAR SYSTEMS REGULATED BY ROS AND/OR REDOX THAT APPEAR TO CONTRIBUTE TO VASCULAR Po_2 SENSING

sGC-derived cGMP. A cellular signaling system regulated by Po_2 and by multiple cellular redox systems and ROS is sGC. It has been known for ~30 yr that cellular cGMP levels appear to be controlled by Po_2 (22). This enzyme can be directly regulated (57) through a heme group that binds nitric oxide and CO in its ferrous (Fe^{2+}) oxidation state (23), mechanisms described above for stimulation by peroxide, and there are superoxide and thiol redox-dependent mechanisms inhibiting rates of cGMP production. All these mechanisms that directly control rates of production of cGMP by sGC are also linked in additional ways to redox systems and Po_2 -regulated processes. Oxygen is required for the biosynthesis of nitric oxide, CO, and the ROS that regulate sGC, and it may control other cytosolic NADPH and NADH redox-linked processes that influence sGC activity. However, O_2 does not appear to directly bind to the heme of the mammalian form of sGC present in vascular tissue (31, 89). The heme of sGC appears to be maintained in its ferrous oxidation state by a cytosolic NADPH-dependent methemoglobin reductase activity, and evidence has been detected in bovine coronary arteries for an oxygen-dependent oxidation

of the heme of sGC to its ferric form known to be resistant to stimulation by nitric oxide (36, 44). In addition to cytosolic NADPH and NADH being substrates for Nox oxidases generating ROS that potentially activate sGC, NADPH also has a key role in controlling the redox status of GSH for the metabolism of peroxide that regulates sGC and perhaps for modulating a thiol oxidation mechanism potentially promoted by GSSG that seems to directly inhibit the activity of sGC (66). The literature contains evidence for cGMP mediating vascular relaxation through multiple systems shown in Fig. 3 that are associated with causing hyperpolarization by opening K⁺ channels and lowering vascular smooth muscle calcium through multiple mechanisms that have also been linked to regulatory systems thought to mediate Po_2 -elicited responses. We have provided evidence that hypoxia decreased cGMP in endothelium-removed bovine pulmonary arteries through processes that seem to originate from decreases in peroxide metabolism by catalase (16, 17, 75). However, the role of this catalase-mediated system in controlling contractile responses was only supported with mechanistic probes available 15 yr ago that could potentially have other actions, and further study is needed to establish whether the regulation of sGC is mediating pulmonary artery responses to changes in Po_2 . An interesting aspect of this mechanism of regulating sGC is that it appears to be inhibited by nitric oxide in isolated pulmonary arteries and perfused rat lungs (68, 75). The cGMP system appears to coordinate smooth muscle relaxation through its ability to regulate many processes that control force generation, generally as a result of activating cGMP-dependent protein kinases (PKG). Some systems that seem to be regulated by cGMP include mechanisms that control the release and reuptake of Ca²⁺ and the sensitivity of the contractile apparatus to Ca²⁺ (41, 55, 79). In addition, there is evidence for the direct phosphorylation of subunits of the SERCA pump, the inositol

1,4,5-trisphosphate (IP₃) receptor, Ca²⁺-regulated K⁺ channels, and myosin phosphatase by PKG under conditions where cGMP promotes vascular relaxation. Phosphorylation by PKG can potentially increase SR Ca²⁺ reuptake by SERCA, inhibit SR Ca²⁺ release by the IP₃ receptor, cause hyperpolarization by opening K⁺ channels, and desensitize the contractile apparatus by stimulating myosin phosphatase. In addition, cGMP may function to inhibit the receptor-mediated generation of IP₃ by contractile agents. Thus when P_O₂ controls ROS and/or redox systems that regulate the activity of sGC, changes in cGMP are likely to result in a coordination of mechanisms that regulate vascular smooth muscle force.

K⁺ channels. The original redox hypothesis for pulmonary hypoxic vasoconstriction reported by Archer et al. (5, 9) included a thiol redox regulation of membrane K⁺ channels by mitochondrial oxidants modulating cytosolic redox. Since that time, much effort has been devoted to providing evidence that K⁺ channels are regulated through redox processes controlled by P_O₂. As the controversy over the P_O₂ sensor continues, the end effectors responsible for modulation of vascular function to changes in P_O₂ are also elusive. Initially, K⁺ channels were considered as potential targets for sensing P_O₂ in carotid bodies. Post et al. (90) identified that voltage-gated K⁺ channel (K_v) currents were reduced by hypoxia in the adult dog pulmonary artery smooth muscle cells (PASMC) but not in renal artery smooth muscle cells. Others demonstrated that K_v, Ca²⁺-activated K⁺ (K_{Ca}), and noninactivating K⁺ channel currents are modulated in rat and rabbit PASMC by hypoxia (62). Inhibition of different K⁺ channels in rat and rabbit could be due to variation in species, gender and age of the animal, and location of pulmonary artery segments used in the studies. It has been reported that there are differences in the expression of K⁺ channels in the conduit and resistance PASMC and that there is a maturational shift in the expression from K_v to K_{Ca} channels (65, 109). The debate over K⁺ channels as potential candidates for responding to P_O₂ changes is another aspect of the controversy in this field, which is the focus of a recent review (61). Our review is trying to define the actual targets of ROS and redox. The generation of oxidase-derived ROS in vascular smooth muscle and changes in the ratio of cellular reducing cofactors, including NAD⁺/NADH, NADP⁺/NADPH, and GSH/GSSG, caused by changes in P_O₂ are currently thought to regulate the activity of K_{Ca} and K_v channels and thereby modulate membrane potential (62, 109). Evidence indicates that oxidizing agents such as diamide, NAD⁺, and GSSG open K_v and K_{Ca} channels of isolated rabbit PASMC by binding to a channel protein and/or by regulating the redox state of a cysteine residue on β-subunit (5, 87, 88). It is thus proposed that inhibition of K_v channels depolarizes membrane potential and increases Ca²⁺ influx through L-type Ca²⁺ channels, thereby inducing HPV. Further studies have identified that K_v1.5 play an important role in mediating HPV because it is a potential target for redox changes and HPV is abolished in K_v1.5 knockout mice (7). Nonetheless, although studies link hypoxia and redox agents to K_v channels, there is no direct evidence demonstrating whether redox changes occur on the channel protein. Thus the actual mechanisms through which redox signaling regulates opening and closing of K⁺ channels are currently not understood. Furthermore, some studies have shown that blocking K_v channels with 4-aminopyridine does not prevent the expression of HPV (62, 104).

PROCESSES CONTROLLING INTRACELLULAR CA²⁺

The major role calcium has in controlling vascular force makes it a key factor in any oxygen-sensing mechanism controlling vascular contractile function. Another hypothesis is hypoxia may directly modulate Ca²⁺ channels and mobilization. McMurtry and colleagues (63, 102) were the first to report that Ca²⁺ entry through L-type Ca²⁺ channels plays a role in HPV. Using whole cell patch-clamp technique, Franco-Obregon and coworkers (27, 28) showed that rabbit PASMC voltage-gated Ca²⁺ channels opened and closed by alternating a hypoxia-reoxygenation stimulus, respectively. They also demonstrated that resistance and conduit PASMC voltage-gated Ca²⁺ channels were opened and closed by exposure to reductions in P_O₂ (26), thus indicating that either the expression or regulation of L-type Ca²⁺ channels was different in conduit arteries that show minimal contraction to hypoxia compared with resistance pulmonary artery smooth muscle that generally contract to hypoxia. Additionally, some of the latest data also suggest that hypoxia stimulates opening of receptor-operated Ca²⁺ channels and modulates Ca²⁺ release from the ryanodine-sensitive Ca²⁺ stores that mediate HPV (76). An interesting redox-linked mechanism for opening ryanodine channels through cyclic ADP ribose has been proposed for explaining the pulmonary artery contraction to hypoxia (24, 112). In this model, hypoxia increases NADH, which functions to inhibit the breakdown of cyclic ADP ribose. Nevertheless, the precise mechanisms involved in regulating intracellular Ca²⁺ by hypoxia are still unclear, and redox mechanisms have been shown to modulate many aspects of Ca²⁺ mobilization in the response of pulmonary arteries to hypoxia (104). For example, there is evidence that the direct inhibition of L-type Ca²⁺ channels by thiol oxidation could be one of the potential mechanisms of regulating intracellular Ca²⁺ (45). Another signaling pathway that could modulate Ca²⁺ influx or release is by the intracellular second messenger cGMP, since PKG is known to regulate Ca²⁺- and voltage-activated K⁺ activity (6) and to promote SR uptake of Ca²⁺ by stimulating the SERCA pump through phosphorylation of phospholamban (41, 55, 79). The SERCA pump was identified 15 yr ago as a target for regulation by ROS in vascular tissue (32), and recent proteomic studies are documenting the actual functional groups that undergo redox changes when this system is exposed to ROS and reactive nitric oxide-derived species (3). The question to consider is whether calcium is controlled by other systems discussed above that are regulated by redox such as cGMP, K channels regulating membrane potential, and capacitive Ca²⁺ storage, or other systems also known to be potentially regulated by ROS or redox such as PKC, tyrosine phosphatases, Akt, Rho, and MAP kinases, etc., or if the Ca²⁺ transport systems are directly controlled by redox. Because hypoxia typically causes contraction in pulmonary arteries and relaxation in systemic arteries under a wide variety of conditions through mechanisms generally associated with changes in intracellular Ca²⁺, it is likely that hypoxia modulates force through systems such as cGMP or cytosolic NADPH, which function to coordinate multiple aspects of the regulation of cellular Ca²⁺.

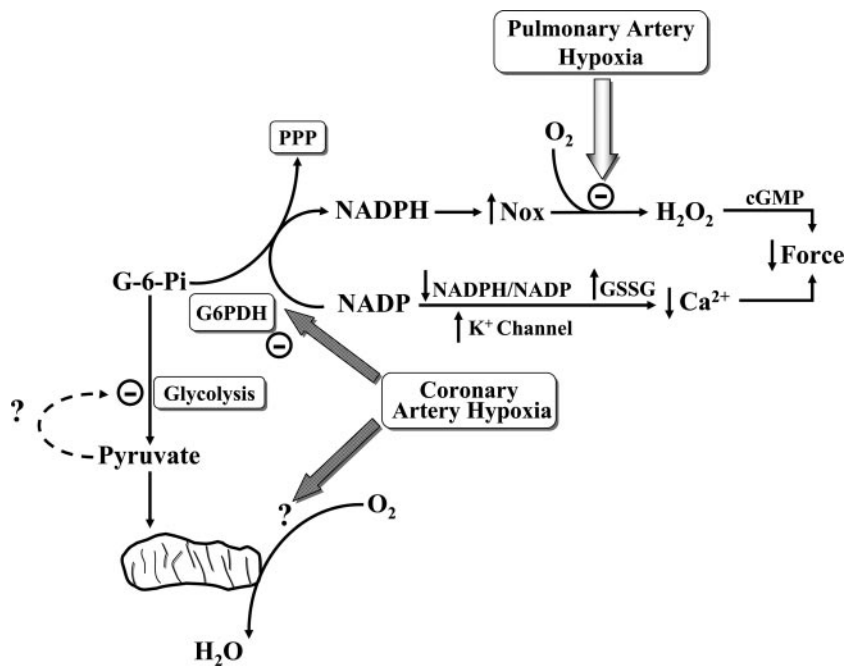


Fig. 4. Model showing the key role of cytosolic NADPH redox control by glucose-6-phosphate dehydrogenase (G6PDH) in the PPP maintaining elevated levels of NADPH and a Nox oxidase-mediated relaxation in bovine pulmonary arteries inhibited by hypoxia. In contrast, the lower levels of G6PDH in bovine coronary arteries permit hypoxia to promote a relaxation controlled by oxidation of NADPH.

CONTROL OF CYTOSOLIC NADPH REDOX BY PPP

Cytosolic NADP(H) redox regulation by glucose metabolism through the PPP appears to control a coordination of multiple systems that regulate intracellular Ca^{2+} levels and vascular smooth muscle force generation (33). Inhibition of the PPP permits oxidation of cytosolic NADPH and GSH to occur, and this appears to result in a lowering of intracellular Ca^{2+} through mechanisms potentially involving inhibition of voltage- and receptor-controlled Ca^{2+} influx and intracellular release, hyperpolarization through opening K^+ channels, and activation of Ca^{2+} reuptake by the SERCA pump (33, 35). There is evidence that many of these systems are controlled by either NADP(H) and/or GSH redox (2, 35). Comparison of the actions of PPP inhibitors on the vascular preparations studied suggests that the apparent importance of the mechanism promoting relaxation seems to be dependent on aspects of the regulatory processes promoting contraction under the experimental conditions examined. Our recent studies are providing evidence that hypoxia promotes NADP(H) and GSH oxidation in endothelium-removed bovine coronary arteries associated with the expression of relaxation by these vessels through Ca^{2+} -lowering mechanisms controlled by these systems (35). Previous studies have found evidence (35, 64, 101) for stimulation of the SERCA pump, K^+ channel opening, and decreases in the activity of Rho kinase in the relaxation of systemic arteries to hypoxia, and it is possible that all these processes could be controlled by redox changes in the cytosolic NADP(H) and GSH systems.

FUNDAMENTAL DIFFERENCES BETWEEN PULMONARY AND SYSTEMIC ARTERIES

Observations of hypoxia generally causing contraction in endothelium-removed pulmonary arteries and relaxation of systemic arteries suggest that differences are likely to exist in the sensors of Po_2 and/or in the signaling processes that link these sensors to the control of vascular force generation.

Whereas multiple systems may influence vascular responses to changes in Po_2 , several of the ROS and metabolic-linked redox systems considered in this review may be key factors in the origins of differences between pulmonary and systemic arteries. On the basis of studies conducted under conditions comparable to each other, evidence has been provided that rat renal arteries (64) and bovine coronary arteries (34) show lower levels of ROS than pulmonary arteries from these same species. Studies in the rat vessels suggest these differences originate from the mitochondria present in these arteries (64). Mitochondria in the rat pulmonary arteries show a more depolarized membrane potential. In this study, rotenone was observed to mimic the effect of hypoxia in constricting the pulmonary and dilating the renal circulations, and depolarizing and hyperpolarizing, respectively, smooth muscle cells freshly isolated from arteries in these organs. In addition, mitochondria isolated from rat lungs generate more superoxide and peroxide, and they have lower levels of key subunits of components of the proximal part of the electron transport chain and greater levels of Mn-SOD than mitochondria isolated from rat kidneys. Our studies in endothelium-removed bovine arteries have observed other fundamental differences in the response of pulmonary and coronary arteries to hypoxia. The pulmonary vessels contract to hypoxia through processes that appear to originate from a lowering of basal NADPH-dependent Nox oxidase-derived peroxide levels (34, 72, 73), and coronary arteries seem to be relaxing through a mechanism associated with a lowering of basal levels of NADPH (35, 37). We have detected a variation in the control of cytosolic NADPH by the PPP between coronary and pulmonary arteries that may be a key factor in generating the differences in responses to hypoxia that are observed. A model for how these mechanisms function is shown in Fig. 4. The pulmonary arteries appear to maintain a higher basal level of Nox-derived peroxide as a consequence of the higher levels of NADPH and glucose-6-phosphate dehydrogenase present in these arteries (34), whereas we hypoth-

esize that hypoxia may lower NADPH in coronary arteries as a result of the less effective ability of the PPP to compete with glycolysis for glucose-6-phosphate originating from the lower levels of glucose-6-phosphate dehydrogenase that appear to be present in these vessels. Our studies have detected evidence that reoxygenation following a hypoxic period of ~15 min or greater causes a transient increase in superoxide- and peroxide-mediated relaxation in both bovine pulmonary and coronary arteries from sources that have the properties of Nox oxidases (67, 69, 70, 72, 73). Thus there is evidence for several differences between pulmonary and systemic arteries related to the properties of mitochondria, sources of ROS, and the control of cytosolic NADPH redox.

There is also evidence for major differences in the processes through which ROS and redox changes regulate vasoactive responses in pulmonary and systemic arteries. The observations made in rat arteries by Michelakis and colleagues (64) have been hypothesized to originate from hypoxia causing contraction in pulmonary arteries by decreasing the ROS and their actions on opening voltage-regulated K^+ channels, whereas renal arteries show increased ROS under hypoxia, and this is suggested to cause relaxation through opening K^+ channels. There is also evidence for differences in K^+ channel responses in rat pulmonary arteries compared with ductus arteriosus originating from conditions that modulate thiol redox (84). In the ductus arteriosus, increasing PO_2 and thiol oxidation causes decreases in K^+ currents, increases in intracellular Ca^{2+} , depolarization, and contraction, whereas the opposite responses are seen in pulmonary artery smooth muscle. Our studies in bovine arteries have suggested that responses linked with hydrogen peroxide appear to be mediated through it stimulating sGC to increase cGMP, whereas the relaxation resulting from the oxidation of cytosolic NADPH appears to control a coordinated set of mechanisms that lower intracellular Ca^{2+} (33). Our previous work has also detected contractile responses to peroxide in both bovine pulmonary and coronary arteries; however, these responses seem to require rather large concentrations of peroxide or a selective activation of what appears to be Nox-2 by stretch (15, 81, 82). Thus there is also evidence for several differences between pulmonary and systemic arteries related to the way ROS and redox changes influence the control of vascular force.

POTENTIAL ORIGINS OF CURRENT CONTROVERSIES IN ROS AND REDOX MECHANISMS OF VASCULAR PO_2 SENSING

Responses of vascular smooth muscle to changes in PO_2 mediated through ROS and redox appear to involve one or more O_2 sensors that control signaling systems that potentially contain components that interact with each other. Some systems that are components of PO_2 -regulated processes are likely to adapt to conditions in the physiological environment seen by the vascular smooth muscle, such as low PO_2 in the pulmonary artery, fluctuations in PO_2 caused by respiration in the lung, and tissue metabolism in the microcirculation of organs or vascular beds such as muscle, pressure-induced stretch in the systemic arterial circulation, autacoids, and tissue-derived metabolites. There are many observations indicating that autacoids such as nitric oxide, eicosanoids and ROS derived from endothelium, and tissue-derived metabolites including lactate, adenosine, ROS, and decreased pH are potentially contributing factors in

vascular responses to acute exposure to hypoxia in preparations where these substances have the potential to regulate vascular force. When vascular smooth muscle is cultured, the culture conditions may cause adaptations in regulatory systems originating from growth-promoting conditions, an absence of physiological stretch, and the PO_2 and metabolic environment of the cell culture. These adaptations could affect the functions of key signaling systems including oxidases and components of energy metabolism such as glycolytic and mitochondrial enzyme expression linked to the control of redox. When vasoactive stimuli such as contractile agents or perturbations of ion transport systems are used in studies examining mechanisms involved in responses to changes in PO_2 , the contributions of signaling mechanisms controlling force or membrane potential may be altered in a manner that changes the apparent significance of the control mechanisms regulated by PO_2 . These factors should be considered in looking for explanations for some of the differences observed in vascular ROS and redox-associated PO_2 -sensing mechanisms documented with in-depth, carefully conducted studies considered in this review.

The current hypotheses on ROS and metabolism-linked redox PO_2 -sensing mechanisms contributing to pulmonary artery smooth muscle-derived force responses to hypoxia need to be investigated in a manner that gives further consideration to relationships between the fundamental processes that are potentially involved. There is what appears to be conflicting evidence for PO_2 sensing by multiple sources of ROS generation. Initial studies with crude pharmacological-type probes supporting a role for NAD(P)H oxidases (now termed Nox oxidases) in providing ROS changes that control the pulmonary artery contractions to hypoxia need to be supported by identifying a specific role for at least one of the Nox oxidases present in vascular tissue in these responses. Since hypoxic contraction was seen in mice lacking Nox-2 (gp91phox), this form of Nox oxidase does not appear to be a primary PO_2 sensor in hypoxic vasoconstriction. Our recent studies have also identified Nox-4 in endothelium-removed bovine pulmonary arteries (34). Because this form of Nox (initially called renox) has been proposed as a tissue PO_2 sensor in the renal erythropoietin system (30), further studies are needed to define the role of this oxidase. Although there is very convincing data that exposure to hypoxia causes cultured pulmonary artery cells to show that hypoxia increases mitochondrial ROS production linked to increases in intracellular Ca^{2+} , these studies need to be extended into functional pulmonary vascular preparations that show reproducible rapidly developing contractions on exposure to hypoxia. These studies also need to be extended into systemic vascular smooth muscle cells to provide evidence that pulmonary cells have a unique property associated with ROS generation that can contribute to hypoxia-elicited contraction. Studies supporting a role for hypoxia decreasing mitochondria-derived ROS are generally based on mitochondrial probes such as rotenone-altering responses and the detection of ROS. Although these data suggest an important role for mitochondrial function in the responses observed, actions of the probes used in these studies do not seem to be consistent with their observed effects on mitochondria-linked ROS generation because rotenone (and antimycin) could potentially increase Krebs cycle-derived NADH-dependent ROS generation. Mitochondrial probes are also likely to have major effects on both energy metabolism and the control of cytosolic

NAD(P)H redox systems that could influence PO_2 -linked vascular responses in ways that remain to be defined. The extensive studies supporting key roles for ROS- and/or redox-regulated K^+ channels provide very convincing data for their contributions to PO_2 -elicited changes in ion channel and contractile function. However, the actual mechanisms controlling the channels have not yet been defined. K^+ channels, systems controlling intracellular Ca^{2+} , and the actions of Ca^{2+} on the contractile apparatus have many components controlling their influence on force that could be regulated through ROS- and metabolism-linked redox systems. For example, K^+ channels are components of highly interactive systems associated with the function of capacitive Ca^{2+} stores, cell membrane potential, and calcium-linked control of ion channel function. There are many sites through which ROS and/or redox could control this interactive system, including mechanisms mediated through cGMP. Although our studies in bovine pulmonary arteries support a mechanism where hypoxia lowers ROS generation associated with decreases in a peroxide stimulation of sGC, the actual role of cGMP in controlling the force response to hypoxia needs to be better defined. Thus the complexities of the way ROS and redox systems potentially influence the interactive systems that control intracellular Ca^{2+} and force may be a major factor in the diversity of observations that exist in the area of how pulmonary arteries respond to hypoxia. There is also evidence for several different mechanisms through which pulmonary arteries contract to hypoxia and systemic arteries relax under similar conditions related to processes involving ROS and cellular redox systems. Our elucidation (33) of how modulating cytosolic NADPH redox through the function of the PPP regulates the coordination of multiple NADPH and thiol redox-controlled signaling mechanisms that lower calcium and Nox oxidase activity through limiting the availability of NADPH has provided new mechanisms that could potentially contribute to explaining some of the poorly understood issues in the diversity of vascular responses to hypoxia. Observations that hypoxia typically causes smooth muscle contraction in pulmonary and relaxation in systemic arteries across multiple species and experimental conditions suggest that hypoxia should function through systems such as cGMP or cytosolic NADPH, which seem to be designed to coordinate multiple aspects of the regulation of force generation.

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REFERENCES

1. Acker T and Plate KH. Hypoxia and hypoxia inducible factors (HIF) as important regulators of tumor physiology. *Cancer Treat Res* 117: 219–248, 2004.
2. Adachi T, Matsui R, Xu S, Kirber M, Lazar HL, Sharov VS, Schoneich C, and Cohen RA. Antioxidant improves smooth muscle sarco/endoplasmic reticulum Ca^{2+} -ATPase function and lowers tyrosine nitration in hypercholesterolemia and improves nitric oxide-induced relaxation. *Circ Res* 90: 1114–1121, 2002.
3. Adachi T, Weisbrod RM, Pimentel DR, Ying J, Sharov VS, Schoneich C, and Cohen RA. S-glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med* 10: 1200–1207, 2004.
4. Alp NJ and Channon KM. Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. *Arterioscler Thromb Vasc Biol* 24: 413–420, 2004.
5. Archer SL, Huang J, Henry T, Peterson D, and Weir EK. A redox-based O_2 sensor in rat pulmonary vasculature. *Circ Res* 73: 1100–1112, 1993.
6. Archer SL, Huang JM, Hampl V, Nelson DP, Shultz PJ, and Weir EK. Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc Natl Acad Sci USA* 91: 7583–7587, 1994.
7. Archer SL, London B, Hampl V, Wu X, Nsair A, Puttagunta L, Hashimoto K, Waite RE, and Michelakis ED. Impairment of hypoxic pulmonary vasoconstriction in mice lacking the voltage-gated potassium channel Kv1.5. *FASEB J* 15: 1801–1803, 2001.
8. Archer SL, Reeve HL, Michelakis E, Puttagunta L, Waite R, Nelson DP, Dinauer MC, and Weir EK. O_2 sensing is preserved in mice lacking the gp91 phox subunit of NADPH oxidase. *Proc Natl Acad Sci USA* 96: 7944–7949, 1999.
9. Archer SL, Will JA, and Weir EK. Redox status in the control of pulmonary vascular tone. *Herz* 11: 127–141, 1986.
10. Babior BM. NADPH oxidase: an update. *Blood* 93: 1464–1476, 1999.
11. Bai J and Cederbaum AI. Adenovirus-mediated overexpression of catalase in the cytosolic or mitochondrial compartment protects against cytochrome P450 2E1-dependent toxicity in HepG2 cells. *J Biol Chem* 276: 4315–4321, 2001.
12. Barron JT, Sasse MF, and Nair A. Effect of angiotensin II on energetics, glucose metabolism and cytosolic NADH/NAD and NADPH/NADP redox in vascular smooth muscle. *Mol Cell Biochem* 262: 91–99, 2004.
13. Berry CE and Hare JM. Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. *J Physiol* 555: 589–606, 2004.
14. Brookes PS, Yoon Y, Robotham JL, Anders MW, and Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol* 287: C817–C833, 2004.
15. Burke TM and Wolin MS. Hydrogen peroxide elicits pulmonary arterial relaxation and guanylate cyclase activation. *Am J Physiol Heart Circ Physiol* 252: H721–H732, 1987.
16. Burke-Wolin T and Wolin MS. H_2O_2 and cGMP may function as an O_2 sensor in the pulmonary artery. *J Appl Physiol* 66: 167–170, 1989.
17. Burke-Wolin TM and Wolin MS. Inhibition of cGMP-associated pulmonary arterial relaxation to H_2O_2 and O_2 by ethanol. *Am J Physiol Heart Circ Physiol* 258: H1267–H1273, 1990.
18. Cai H, Li Z, Dikalov S, Holland SM, Hwang J, Jo H, Dudley SC Jr, and Harrison DG. NAD(P)H oxidase-derived hydrogen peroxide mediates endothelial nitric oxide production in response to angiotensin II. *J Biol Chem* 277: 48311–48317, 2002.
19. Chance B, Sies H, and Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59: 527–605, 1979.
20. Chen YL, Wolin MS, and Messina EJ. Evidence for cGMP mediation of skeletal muscle arteriolar dilation to lactate. *J Appl Physiol* 81: 349–354, 1996.
21. Cherry PD, Omar HA, Farrell KA, Stuart JS, and Wolin MS. Superoxide anion inhibits cGMP-associated bovine pulmonary arterial relaxation. *Am J Physiol Heart Circ Physiol* 259: H1056–H1062, 1990.
22. Clyman RI, Blacksin AS, Manganiello VC, and Vaughan M. Oxygen and cyclic nucleotides in human umbilical artery. *Proc Natl Acad Sci USA* 72: 3883–3887, 1975.
23. Denninger JW and Marletta MA. Guanylate cyclase and the NO/cGMP signaling pathway. *Biochim Biophys Acta* 1411: 334–350, 1999.
24. Dipp M and Evans AM. Cyclic ADP-ribose is the primary trigger for hypoxic pulmonary vasoconstriction in the rat lung in situ. *Circ Res* 89: 77–83, 2001.
25. Fleming I, Michaelis UR, Bredenkotter D, Fisslthaler B, Dehghani F, Brandes RP, and Busse R. Endothelium-derived hyperpolarizing factor synthase (cytochrome P450 2C9) is a functionally significant source of reactive oxygen species in coronary arteries. *Circ Res* 88: 44–51, 2001.
26. Franco-Obregon A and Lopez-Barneo J. Differential oxygen sensitivity of calcium channels in rabbit smooth muscle cells of conduit and resistance pulmonary arteries. *J Physiol* 491: 511–518, 1996.
27. Franco-Obregon A and Lopez-Barneo J. Low PO_2 inhibits calcium channel activity in arterial smooth muscle cells. *Am J Physiol Heart Circ Physiol* 271: H2290–H2299, 1996.
28. Franco-Obregon A, Urena J, and Lopez-Barneo J. Oxygen-sensitive calcium channels in vascular smooth muscle and their possible role in hypoxic arterial relaxation. *Proc Natl Acad Sci USA* 92: 4715–4719, 1995.

29. Freeman BA and Crapo JD. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J Biol Chem* 256: 10986–10992, 1981.
30. Geiszt M, Kopp JB, Varnai P, and Leto TL. Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci USA* 97: 8010–8014, 2000.
31. Gray JM, Karow DS, Lu H, Chang AJ, Chang JS, Ellis RE, Marletta MA, and Bargmann CI. Oxygen sensation and social feeding mediated by a *C. elegans* guanylate cyclase homologue. *Nature* 430: 317–322, 2004.
32. Grover AK, Samson SE, Fomin VP, and Werstki ES. Effects of peroxide and superoxide on coronary artery: ANG II response and sarcoplasmic reticulum Ca^{2+} pump. *Am J Physiol Cell Physiol* 269: C546–C553, 1995.
33. Gupte SA, Arshad M, Viola S, Kaminski PM, Ungvari Z, Rabbani G, Koller A, and Wolin MS. Pentose phosphate pathway coordinates multiple redox-controlled relaxing mechanisms in bovine coronary arteries. *Am J Physiol Heart Circ Physiol* 285: H2316–H2326, 2003.
34. Gupte SA, Kaminski PM, Floyd B, Agarwal R, Ali N, Ahmad M, Edwards J, and Wolin MS. Cytosolic NADPH may regulate differences in basal Nox oxidase-derived superoxide generation in bovine coronary and pulmonary arteries. *Am J Physiol Heart Circ Physiol* 288: H13–H21, 2005.
35. Gupte SA, Rabbani G, Arshad M, and Wolin MS. Changes in NADPH and glutathione redox mediate bovine coronary artery relaxation to hypoxia (Abstract). *Circulation* 106: II-495, 2002.
36. Gupte SA, Rupawalla T, Phillibert D Jr, and Wolin MS. NADPH and heme redox modulate pulmonary artery relaxation and guanylate cyclase activation by NO. *Am J Physiol Lung Cell Mol Physiol* 277: L1124–L1132, 1999.
37. Gupte SA and Wolin MS. Role of pentose phosphate pathway (PPP) and NADPH redox in hypoxic vasodilation (Abstract). *FASEB J* 19: A1277, 2005.
38. Guzik TJ, Sadowski J, Kapelak B, Jopek A, Rudzinski P, Pillai R, Korb R, and Channon KM. Systemic regulation of vascular NAD(P)H oxidase activity and nox isoform expression in human arteries and veins. *Arterioscler Thromb Vasc Biol* 24: 1614–1620, 2004.
39. Han D, Antunes F, Canali R, Rettori D, and Cadenas E. Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. *J Biol Chem* 278: 5557–5563, 2003.
40. Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, and Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 24: 677–683, 2004.
41. Hofmann F. The biology of cyclic GMP-dependent protein kinases. *J Biol Chem* 280: 1–4, 2005.
42. Holland JA, Pritchard KA, Pappolla MA, Wolin MS, Rogers NJ, and Stemberman MB. Bradykinin induces superoxide anion release from human endothelial cells. *J Cell Physiol* 143: 21–25, 1990.
43. Hongpaisan J, Winters CA, and Andrews SB. Strong calcium entry activates mitochondrial superoxide generation, upregulating kinase signaling in hippocampal neurons. *J Neurosci* 24: 10878–10887, 2004.
44. Iesaki T, Gupte SA, and Wolin MS. A flavoprotein mechanism appears to prevent an oxygen-dependent inhibition of cGMP-associated nitric oxide-elicited relaxation of bovine coronary arteries. *Circ Res* 85: 1027–1031, 1999.
45. Iesaki T and Wolin MS. Thiol oxidation activates a novel redox-regulated coronary vasodilator mechanism involving inhibition of Ca^{2+} influx. *Arterioscler Thromb Vasc Biol* 20: 2359–2365, 2000.
46. Isogai Y, Iizuka T, Makino R, Iyanagi T, and Orii Y. Superoxide-producing cytochrome b. Enzymatic and electron paramagnetic resonance properties of cytochrome b558 purified from neutrophils. *J Biol Chem* 268: 4025–4031, 1993.
47. Kang TM, Park MK, and Uhm DY. Characterization of hypoxia-induced $[Ca^{2+}]_i$ rise in rabbit pulmonary arterial smooth muscle cells. *Life Sci* 70: 2321–2333, 2002.
48. Kontos HA, Wei EP, Kukreja RC, Ellis EF, and Hess ML. Differences in endothelium-dependent cerebral dilation by bradykinin and acetylcholine. *Am J Physiol Heart Circ Physiol* 258: H1261–H1266, 1990.
49. Korichneva I, Hoyos B, Chua R, Levi E, and Hammerling U. Zinc release from protein kinase C as the common event during activation by lipid second messenger or reactive oxygen. *J Biol Chem* 277: 44327–44331, 2002.
50. Kudin AP, Bimpong-Buta NY, Vielhaber S, Elger CE, and Kunz WS. Characterization of superoxide-producing sites in isolated brain mitochondria. *J Biol Chem* 279: 4127–4135, 2004.
51. Lambert AJ and Brand MD. Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I). *J Biol Chem* 279: 39414–39420, 2004.
52. Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 4: 181–189, 2004.
53. Lassegue B and Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol* 285: R277–R297, 2003.
54. Leach RM, Hill HM, Snetkov VA, Robertson TP, and Ward JP. Divergent roles of glycolysis and the mitochondrial electron transport chain in hypoxic pulmonary vasoconstriction of the rat: identity of the hypoxic sensor. *J Physiol* 536: 211–224, 2001.
55. Lincoln TM, Dey N, and Sellak H. Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. *J Appl Physiol* 91: 1421–1430, 2001.
56. Liu JQ, Sham JS, Shimoda LA, Kuppasamy P, and Sylvester JT. Hypoxic constriction and reactive oxygen species in porcine distal pulmonary arteries. *Am J Physiol Lung Cell Mol Physiol* 285: L322–L333, 2003.
57. Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepenik KP, and Waldman SA. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev* 52: 375–414, 2000.
58. Marshall C, Marmar AJ, Verhoeven AJ, and Marshall BE. Pulmonary artery NADPH-oxidase is activated in hypoxic pulmonary vasoconstriction. *Am J Respir Cell Mol Biol* 15: 633–644, 1996.
59. Marshall PJ, Kulmacz RJ, and Lands WE. Constraints on prostaglandin biosynthesis in tissues. *J Biol Chem* 262: 3510–3517, 1987.
60. Matejak BE, Kaminski PM, and Wolin MS. Effects of inhibitors of mitochondrial electron transport on the response of endothelium-removed bovine pulmonary arteries to changes in oxygen tension (Abstract). *Am J Respir Crit Care Med* 161: A627, 2000.
61. Mauban JR, Remillard CV, and Yuan JX. Hypoxic pulmonary vasoconstriction: role of ion channels. *J Appl Physiol* 98: 415–420, 2005.
62. McCulloch KM, Osipenko ON, and Gurney AM. Oxygen-sensing potassium currents in pulmonary artery. *Gen Pharmacol* 32: 403–411, 1999.
63. McMurtry IF, Davidson AB, Reeves JT, and Grover RF. Inhibition of hypoxic pulmonary vasoconstriction by calcium antagonists in isolated rat lungs. *Circ Res* 38: 99–104, 1976.
64. Michelakis ED, Hampl V, Nsair A, Wu X, Harry G, Haromy A, Gurtu R, and Archer SL. Diversity in mitochondrial function explains differences in vascular oxygen sensing. *Circ Res* 90: 1307–1315, 2002.
65. Michelakis ED, Thebaud B, Weir EK, and Archer SL. Hypoxic pulmonary vasoconstriction: redox regulation of O_2 -sensitive K^+ channels by a mitochondrial O_2 -sensor in resistance artery smooth muscle cells. *J Mol Cell Cardiol* 37: 1119–1136, 2004.
66. Mingone CJ, Gupte SA, Ali N, Oeckler RA, Olson S, and Wolin MS. Guanylate cyclase activation by nitric oxide is modulated through oxidation of vicinal thiols (Abstract). *Circulation* 110: III-249, 2004.
67. Mohazzab-H KM, Agarwal R, and Wolin MS. Influence of glutathione peroxidase on coronary artery responses to alterations in PO_2 and H_2O_2 . *Am J Physiol Heart Circ Physiol* 276: H235–H241, 1999.
68. Mohazzab-H KM, Fayngersh RP, and Wolin MS. Nitric oxide inhibits pulmonary artery catalase and H_2O_2 -associated relaxation. *Am J Physiol Heart Circ Physiol* 271: H1900–H1906, 1996.
69. Mohazzab-H KM, Kaminski PM, Fayngersh RP, and Wolin MS. Oxygen-elicited responses in calf coronary arteries: role of H_2O_2 production via NADH-derived superoxide. *Am J Physiol Heart Circ Physiol* 270: H1044–H1053, 1996.
70. Mohazzab-H KM, Fayngersh RP, Kaminski PM, and Wolin MS. Potential role of NADH oxidoreductase-derived reactive O_2 species in calf pulmonary arterial PO_2 -elicited responses. *Am J Physiol Lung Cell Mol Physiol* 269: L637–L644, 1995.
71. Mohazzab-H KM, Kaminski PM, and Wolin MS. NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. *Am J Physiol Heart Circ Physiol* 266: H2568–H2572, 1994.
72. Mohazzab-H KM and Wolin MS. Oxidant signalling and vascular oxygen sensing. Role of H_2O_2 in responses of the bovine pulmonary artery to changes in PO_2 . *Adv Exp Med Biol* 475: 249–258, 2000.

73. **Mohazzab-H KM and Wolin MS.** Properties of a superoxide anion-generating microsomal NADH oxidoreductase, a potential pulmonary artery Po_2 sensor. *Am J Physiol Lung Cell Mol Physiol* 267: L823–L831, 1994.
74. **Mohazzab-H KM and Wolin MS.** Sites of superoxide anion production detected by lucigenin in calf pulmonary artery smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 267: L815–L822, 1994.
75. **Monaco JA and Burke-Wolin T.** NO and H_2O_2 mechanisms of guanylate cyclase activation in oxygen-dependent responses of rat pulmonary circulation. *Am J Physiol Lung Cell Mol Physiol* 268: L546–L550, 1995.
76. **Morio Y and McMurtry IF.** Ca^{2+} release from ryanodine-sensitive store contributes to mechanism of hypoxic vasoconstriction in rat lungs. *J Appl Physiol* 92: 527–534, 2002.
77. **Moudgil R, Michelakis ED, and Archer SL.** Hypoxic pulmonary vasoconstriction. *J Appl Physiol* 98: 390–403, 2005.
78. **Muller FL, Liu Y, and Van Remmen H.** Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J Biol Chem* 279: 49064–49073, 2004.
79. **Munzel T, Feil R, Mulsch A, Lohmann SM, Hofmann F, and Walter U.** Physiology and pathophysiology of vascular signaling controlled by guanosine 3',5'-cyclic monophosphate-dependent protein kinase. *Circulation* 108: 2172–2183, 2003. [Corrigenda. *Circulation* 108: December 2003, p. 3165.]
80. **Nelson DL and Cox MM.** *Lehninger Principles of Biochemistry* (4th ed.). New York: Freeman, 2005, p. 521–750.
81. **Oeckler RA, Arcuino E, Ahmad M, Olson SC, and Wolin MS.** Cytosolic NADH redox and thiol oxidation regulate pulmonary arterial force through ERK MAP kinase. *Am J Physiol Lung Cell Mol Physiol* 288: L1017–L1025, 2005.
82. **Oeckler RA, Kaminski PM, and Wolin MS.** Stretch enhances contraction of bovine coronary arteries via an NAD(P)H oxidase-mediated activation of the extracellular signal-regulated kinase mitogen-activated protein kinase cascade. *Circ Res* 92: 23–31, 2003.
83. **Ohnishi T, Johnson JE Jr, Yano T, Lobrutto R, and Widger WR.** Thermodynamic and EPR studies of slowly relaxing ubisemiquinone species in the isolated bovine heart complex I. *FEBS Lett* 579: 500–506, 2005.
84. **Olschewski A, Hong Z, Peterson DA, Nelson DP, Porter VA, and Weir EK.** Opposite effects of redox status on membrane potential, cytosolic calcium, and tone in pulmonary arteries and ductus arteriosus. *Am J Physiol Lung Cell Mol Physiol* 286: L15–L22, 2004.
85. **Omar HA, Mohazzab-H KM, Mortelliti MP, and Wolin MS.** O_2 -dependent modulation of calf pulmonary artery tone by lactate: potential role of H_2O_2 and cGMP. *Am J Physiol Lung Cell Mol Physiol* 264: L141–L145, 1993.
86. **Paky A, Michael JR, Burke-Wolin TM, Wolin MS, and Gurtner GH.** Endogenous production of superoxide by rabbit lungs: effects of hypoxia or metabolic inhibitors. *J Appl Physiol* 74: 2868–2874, 1993.
87. **Park MK, Lee SH, Ho WK, and Earm YE.** Redox agents as a link between hypoxia and the responses of ionic channels in rabbit pulmonary vascular smooth muscle. *Exp Physiol* 80: 835–842, 1995.
88. **Park MK, Lee SH, Lee SJ, Ho WK, and Earm YE.** Different modulation of Ca-activated K channels by the intracellular redox potential in pulmonary and ear arterial smooth muscle cells of the rabbit. *Pflügers Arch* 430: 308–314, 1995.
89. **Pellicena P, Karow DS, Boon EM, Marletta MA, and Kuriyan J.** Crystal structure of an oxygen-binding heme domain related to soluble guanylate cyclases. *Proc Natl Acad Sci USA* 101: 12854–12859, 2004.
90. **Post JM, Hume JR, Archer SL, and Weir EK.** Direct role for potassium channel inhibition in hypoxic pulmonary vasoconstriction. *Am J Physiol Cell Physiol* 262: C882–C890, 1992.
91. **Rounds S and McMurtry IF.** Inhibitors of oxidative ATP production cause transient vasoconstriction and block subsequent pressor responses in rat lungs. *Circ Res* 48: 393–400, 1981.
92. **Rouslin W and Broge CW.** IF_1 function in situ in uncoupler-challenged ischemic rabbit, rat, and pigeon hearts. *J Biol Chem* 271: 23638–23641, 1996.
93. **Shibuya I and Kanno T.** Calcium-dependent secretory and redox response to CCK-8 in isolated perfused rat pancreas. *Am J Physiol Cell Physiol* 248: C228–C234, 1985.
94. **Shigemori K, Ishizaki T, Matsukawa S, Sakai A, Nakai T, and Miyabo S.** Adenine nucleotides via activation of ATP-sensitive K^+ channels modulate hypoxic response in rat pulmonary artery. *Am J Physiol Lung Cell Mol Physiol* 270: L803–L809, 1996.
95. **Shimizu S, Bowman PS, Thorne G III, and Paul RJ.** Effects of hypoxia on isometric force, intracellular Ca^{2+} , pH, and energetics in porcine coronary artery. *Circ Res* 86: 862–870, 2000.
96. **Sies H.** Peroxisomal enzymes and oxygen metabolism in liver. *Adv Exp Med Biol* 78: 51–60, 1977.
97. **Sorescu D, Somers MJ, Lassegue B, Grant S, Harrison DG, and Griendling KK.** Electron spin resonance characterization of the NAD(P)H oxidase in vascular smooth muscle cells. *Free Radic Biol Med* 30: 603–612, 2001.
98. **Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, and Griendling KK.** Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation* 105: 1429–1435, 2002.
99. **St-Pierre J, Buckingham JA, Roebuck SJ, and Brand MD.** Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem* 277: 44784–44790, 2002.
100. **Thomas HM III, Carson RC, Fried ED, and Novitch RS.** Inhibition of hypoxic pulmonary vasoconstriction by diphenyleneiodonium. *Biochem Pharmacol* 42: R9–R12, 1991.
101. **Thorne GD, Ishida Y, and Paul RJ.** Hypoxic vasorelaxation: Ca^{2+} -dependent and Ca^{2+} -independent mechanisms. *Cell Calcium* 36: 201–208, 2004.
102. **Tucker A, McMurtry IF, Grover RF, and Reeves JT.** Attenuation of hypoxic pulmonary vasoconstriction by verapamil in intact dogs. *Proc Soc Exp Biol Med* 151: 611–614, 1976.
103. **Tune JD, Gorman MW, and Feigl EO.** Matching coronary blood flow to myocardial oxygen consumption. *J Appl Physiol* 97: 404–415, 2004.
- 103a. **Warburg O and Christian W.** (Über aktivierung der robisonischen hexose-mono-phosphosäure in roten blutzellen und die gewinnung aktivierender fermentlösungen.) *Biochem Z* 242: 206–227, 1931.
104. **Ward JP, Snetkov VA, and Aaronson PI.** Calcium, mitochondria and oxygen sensing in the pulmonary circulation. *Cell Calcium* 36: 209–220, 2004.
105. **Waypa GB, Chandel NS, and Schumacker PT.** Model for hypoxic pulmonary vasoconstriction involving mitochondrial oxygen sensing. *Circ Res* 88: 1259–1266, 2001.
106. **Waypa GB, Marks JD, Mack MM, Boriboun C, Mungai PT, and Schumacker PT.** Mitochondrial reactive oxygen species trigger calcium increases during hypoxia in pulmonary arterial myocytes. *Circ Res* 91: 719–726, 2002.
107. **Waypa GB and Schumacker PT.** Hypoxic pulmonary vasoconstriction: redox events in oxygen sensing. *J Appl Physiol* 98: 404–414, 2005.
108. **Weir EK, Eaton JW, and Chesler E.** Redox status and pulmonary vascular reactivity. *Chest* 88: 249S–252S, 1985.
109. **Weir EK, Hong Z, Porter VA, and Reeve HL.** Redox signaling in oxygen sensing by vessels. *Respir Physiol Neurobiol* 132: 121–130, 2002.
110. **Weir EK, Will JA, Lundquist LJ, Eaton JW, and Chesler E.** Diamide inhibits pulmonary vasoconstriction induced by hypoxia or prostaglandin $\text{F}_{2\alpha}$. *Proc Soc Exp Biol Med* 173: 96–103, 1983.
111. **Weissmann N, Tadic A, Hanze J, Rose F, Winterhalder S, Nollen M, Schermuly RT, Ghofrani HA, Seeger W, and Grimminger F.** Hypoxic vasoconstriction in intact lungs: a role for NADPH oxidase-derived H_2O_2 ? *Am J Physiol Lung Cell Mol Physiol* 279: L683–L690, 2000.
112. **Wilson HL, Dipp M, Thomas JM, Lad C, Galione A, and Evans AM.** ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase act as a redox sensor. A primary role for cyclic ADP-ribose in hypoxic pulmonary vasoconstriction. *J Biol Chem* 276: 11180–11188, 2001.
113. **Wolin MS.** Interactions of oxidants with vascular signaling systems. *Arterioscler Thromb Vasc Biol* 20: 1430–1442, 2000.
114. **Wolin MS.** Subcellular localization of Nox-containing oxidases provides unique insight into their role in vascular oxidant signaling. *Arterioscler Thromb Vasc Biol* 24: 625–627, 2004.
115. **Wolin MS, Burke-Wolin TM, and Mohazzab-H KM.** Roles for NAD(P)H oxidases and reactive oxygen species in vascular oxygen sensing mechanisms. *Respir Physiol* 115: 229–238, 1999.
116. **Wolin MS, Gupta SA, and Oeckler RA.** Superoxide in the vascular system. *J Vasc Res* 39: 191–207, 2002.
117. **Yano S, Arroyo N, and Yano N.** SHP2 binds catalase and acquires a hydrogen peroxide-resistant phosphatase activity via integrin-signaling. *FEBS Lett* 577: 327–332, 2004.