Reduction in alveolar oxygen tension causes reversible hypoxic pulmonary vasoconstriction (HPV), which is thought to serve as an adaptive mechanism for diverting blood flow from poorly ventilated to better-ventilated regions of the lung to improve ventilation-perfusion matching. Since first described by Von Euler and Liljestrand 50 years ago, significant advances have been made in determining the O2 dependence, temporal characteristics, loci of response, physiological conditions, and neurohumoral factors that influence HPV in intact animal, isolated lung, and pulmonary arterial rings. Recent studies using isolated pulmonary arterial smooth muscle cells (PASMCs) indicate that hypoxia directly causes membrane depolarization, increase in intracellular \([\text{Ca}^{2+}]\), and cell-shortening; thus, the essential elements of HPV are contained in PASMCs, even though one or more endothelially derived mediators, such as ET-1 and/or an unknown factor,2,3 are required for the full expression of hypoxic response. However, despite this progress, the precise cellular mechanism of O2 sensing for HPV remains controversial.

There are several proposed mechanisms for O2 sensing in PASMCs, most of which are related to reactive oxygen species (ROS).4 The first hypothesis proposed that a smooth muscle microsomal NADH oxidoreductase, which generates H2O2 via a Po2-dependent production of superoxide, acts as the O2 sensor; H2O2 interacts with catalase to activate soluble guanylate cyclase and increase cGMP to provide tonic vasorelaxation during normoxia.5,6 Under hypoxic conditions, production of H2O2 from NADH oxidase would be reduced, leading to the removal of the vasorelaxant influence, and pulmonary vasoconstriction.

The second hypothesis proposed that hypoxia activates a sarcolemmal NADPH oxidase, similar to that found in neutrophils, to paradoxically increase superoxide production to signal HPV. This notion is supported by the evidence that NADPH oxidase was detected in pulmonary arteries by Western blot analysis and immunostaining of gp91phox subunit, and by spectrometric analysis of cytochrome \(b_{-245}\). Hypoxia was found to increase superoxide anion production, which was blocked by the inhibitor of NADPH oxidase, diphenyleneiodium (DPI), in cultured PASMCs. Moreover, DPI blocked hypoxic responses in isolated lung, isolated arteries, and PASMCs.8,9 However, the supporting evidence is weakened by the fact that DPI is a nonspecific blocker for many flavoprotein-dependent enzymes including complex I of the mitochondrial electron transport chain (ETC) and NO synthase (NOS), and other proteins such as K+ and Ca2+ channels, and by the unaltered HPV in the phagocyte NADPH oxidase gp91phox subunit knockout mice.10,11

The third hypothesis has received the most attention. It proposed a redox-based regulation of \(K_v\) channel as the O2 sensing mechanism for HPV.12 According to this hypothesis, hypoxia reduces mitochondrial electron transport, decreases ROS generation, and shifts the ratio of redox couples (ie, GSSG:GSH and NAD+:NADH) toward a more reduced state, leading to inhibition of \(K_v\) channels, membrane depolarization, Ca2+ influx via L-type Ca2+ channels, increase in cytosolic \([\text{Ca}^{2+}]\), and vasoconstriction. Consistent with this hypothesis, hypoxia or mitochondrial ETC inhibitors, rotenone and antimycin A, caused inhibition of \(K_v\) current in PASMCs, vasoconstriction, and decreased luminol-enhanced chemiluminescence in isolated lungs, suggesting a decrease in ROS production.12,13 Agents that promoted cytosolic oxidation were reported to activate, whereas agents that promote cytosolic reduction caused inhibition of \(K_v\) currents.14 However, the role of \(K_v\) channel in initiating HPV has been questioned because an antagonist of \(K_v\) channels failed to inhibit HPV.2,15 and a functional sarcoplasmic reticulum (SR) was found to be required for HPV.16 Hence, it is proposed alternatively that \(K_v\) channel inhibition during hypoxia is secondary to Ca2+ release from SR. Beside \(K_v\) channels, ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase may also act as redox sensors for HPV.17 A reduction of \(\beta\)-NAD+:\(\beta\)-NADH ratio during hypoxia was reported to stimulate ADP-ribosyl cyclase and inhibit cyclic ADP-ribose hydrolase, leading to accumulation of cyclic ADP-ribose, an endogenous activator of ryanodine receptors, to activate Ca2+ release from SR.

Recently, Waypa et al18 proposed the fourth hypothesis of O2 sensing. Using parallel experiments in isolated perfused lungs and PASMCs, they found that HPV was blocked by inhibitors acting at ETC sites proximal, but not distal, to superoxide production; mutant PASMCs lacking mitochondrial ETC failed to contract to hypoxia, whereas contraction to U46619 was unaffected. Moreover, \(2',7'-\text{dichlorofluorescin diacetate (DCF)}\) in PASMCs detected an increase in cytosolic ROS, which was blocked by myxothiazol, during hypoxia. Antioxidants and a Cu,Zn superoxide dismutase (SOD) inhibitor also blocked HPV. Based on these results, they proposed that mitochondria indeed function as O2...
sensors, but it is the increase instead of decrease in mitochondrial ROS generation that triggers HPV, and H_2O_2 is probably the signal molecule. Even though many questions have yet to be answered (see previous editorial), this hypothesis gained support from Leach et al who found similarly in isolated intrapulmonary arteries that inhibition of complex I and III did not cause vasoconstriction but abolished HPV, whereas inhibition of cytochrome oxidase potentiated hypoxic constriction. More interestingly, succinate, a substrate for complex II, reversed the effects of complex I but not complex III inhibition on HPV. In this issue of Circulation Research, Waypa et al push their case further by demonstrating that the proximal ROS-generating sites of ETC are required for the hypoxia-induced increase in cytoplasmic [Ca^{2+}], and verified the link between H_2O_2 and the increase in cytoplasmic [Ca^{2+}] by showing exogenous application of a low concentration of H_2O_2 (50 μmol/L) elicited a Ca^{2+} response and overexpression of catalase in PASMCs attenuated the hypoxia and H_2O_2, but not the angiotensin II, induced Ca^{2+} responses.

The major controversy in the above mentioned hypotheses of O_2 sensing is whether ROS go up or down in PASMCs during hypoxia. Such discrepency is likely related to differences in preparations and detection methods, each of which has its own severe specific limitations. To date, three reported measurements in cultured PASMCs with either lucigenin chemiluminescence or DCF fluorescence all showed increases in ROS release during hypoxia. Similar observations were also made in other cell systems. These results provide a strong case for an augmented ROS release in PASMCs during hypoxia. In contrast, observations of a reduction in ROS during hypoxia were made mainly by detection of lucigenin or luminol enhanced chemiluminescence in isolated perfused lungs. Despite the known problem of redox cycling of lucigenin, the chemiluminescence signal of such preparations reflected the global superoxide anion generated by all pulmonary cells (including airway epithelial and smooth muscle, endothelial, and vascular smooth muscle cells) in the lung surface. Hence, it did not provide a direct measurement of ROS in PASMCs. However, Michelakis et al recently measured ROS in endothelium-denuded resistance PA rings using three different detection methods (lucigenin-enhanced chemiluminescence, AmplexRed H_2O_2 assay, and DCF fluorescence), and all showed a decrease in ROS release during hypoxia. This motivates consideration of other factors that could lead to different results in ROS detection in pulmonary arteries/PASMCs during hypoxia. ROS are produced from a variety of biosynthetic sources, including mitochondrial ETC, NADPH and NADH oxidases, cyclooxygenase, lipoxygenase, xanthine oxidase, and nitric oxide synthase, and each of these sources may respond differently to hypoxia. For example, hypoxia may cause a decrease in ROS production from NADH oxidase but an increase in ROS release from mitochondrial ETC. In this case, it may be possible that depending on subcellular locations of these ROS sources, and different compartmentation of the detection probes (cytosolic versus intracellular; intra- versus extracellular compartments), the net signals are different in arterial tissues and in cultured cells. Moreover, if one considers the possibility of local ROS signaling due to spatial confinement, similar to the differential signaling of sarcolemmal NOS-3 to L-type Ca^{2+} channels and sarcoplasmic ROS-1 to ryanodine receptors reported in cardiac myocytes, one can even speculate about oxidation of effector molecules by ROS generated from a spatially-confined source in a globally-reduced environment. However, detection of subcellular ROS or redox transient in PASMCs has not yet been made.

Extending their previous experiments on antioxidants and inhibitors of SOD, Waypa et al using catalase overexpressed PASMCs, have provided further evidence suggesting that H_2O_2 (or hydroxyl radical) is the signaling molecule for HPV. It is tempting to speculate that ROS release from mitochondrial ETC activates Ca^{2+} release via ryanodine receptors to initiate HPV. This idea is especially compelling because H_2O_2 can activate ryanodine receptors directly by oxidation of sulfhydryls of the channel and mitochondria and sarcoplasmic reticulum are functionally coupled in vascular smooth muscle cell, local Ca^{2+} release from ryanodine receptors (Ca^{2+} sparks) causes membrane depolarization, instead of hyperpolarization, in PASMCs, and a functional SR is required for HPV. ROS may also stimulate cyclic ADP-ribose synthesis to activate ryanodine receptors in a calmodulin-dependent manner. The notion of mitochondrial/SR interaction fits nicely with previous findings to form a coherent hypothesis that hypoxia enhances ROS (probably H_2O_2) generation from mitochondrial ETC, activates ryanodine receptors to release SR Ca^{2+}, which then leads to store-operated calcium influx, altered activity of sarcoplasmal (K_v or other) ion channels, membrane depolarization, and calcium influx through L-type Ca^{2+} channels.

However, the picture is complicated by the fact that H_2O_2 can cause Ca^{2+} release from mitochondria. Exogenous application of H_2O_2 to unstimulated pulmonary arteries was found to cause an endothelium-independent contraction, but it was unaltered by Ca^{2+}-free external solution or inhibiting Ca^{2+} release by ryanodine and thapsigargin, an obvious deviation from HPV. Moreover, H_2O_2 can cause a variety of effects on different Ca^{2+} pathways, including activation of L-type Ca^{2+} channel and nonselective cation channel, uncoupling Na^+-K^+-ATPase, and altered activities of SR Ca^{2+}-ATPase and Na^+-Ca^{2+} exchanger. Hence, the Ca^{2+} response elicited by H_2O_2 could be rather complex. Because the H_2O_2-induced Ca^{2+} response in this study had not been characterized, it is unclear whether it resembles the Ca^{2+} response associated with HPV, i.e., blocked by free Ca^{2+} external solution, L-type Ca^{2+} channel antagonist, ryanodine, and thapsigargin. It is also unfortunate, probably due to technical limitations, that kinetic data of ROS production and Ca^{2+} response elicited during hypoxia are not available for comparison.

In conclusion, Waypa et al have made a further step toward validating their hypothesis. But until precise characterizations of the temporal and spatial aspects of ROS generation and thorough comparison of ETC ROS- and hypoxia-induced Ca^{2+} responses are available, ROS will continue their ups and downs on the stage of HPV.
References


Hypoxic Pulmonary Vasoconstriction: Ups and Downs of Reactive Oxygen Species
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