Hypoxia Triggers Subcellular Compartmental Redox Signaling in Vascular Smooth Muscle Cells

Gregory B. Waypa, Jeremy D. Marks, Robert Guzy, Paul T. Mungai, Jacqueline Schriewer, Danijela Dokic, Paul T. Schumacker

Rationale: Recent studies have implicated mitochondrial reactive oxygen species (ROS) in regulating hypoxic pulmonary vasoconstriction (HPV), but controversy exists regarding whether hypoxia increases or decreases ROS generation.

Objective: This study tested the hypothesis that hypoxia induces redox changes that differ among subcellular compartments in pulmonary (PASMCs) and systemic (SASMCs) smooth muscle cells.

Methods and Results: We used a novel, redox-sensitive, ratiometric fluorescent protein sensor (RoGFP) to assess the effects of hypoxia on redox signaling in cultured PASMCs and SASMCs. Using genetic targeting sequences, RoGFP was expressed in the cytosol (Cyto-RoGFP), the mitochondrial matrix (Mito-RoGFP), or the mitochondrial intermembrane space (IMS-RoGFP), allowing assessment of oxidant signaling in distinct intracellular compartments. Superfusion of PASMCs or SASMCs with hypoxic media increased oxidation of both Cyto-RoGFP and IMS-RoGFP. However, hypoxia decreased oxidation of Mito-RoGFP in both cell types. The hypoxia-induced oxidation of Cyto-RoGFP was attenuated through the overexpression of cytosolic catalase in PASMCs.

Conclusions: These results indicate that hypoxia causes a decrease in nonspecific ROS generation in the matrix compartment, whereas it increases regulated ROS production in the IMS, which diffuses to the cytosol of both PASMCs and SASMCs. (Circ Res. 2010;106:526-535.)

Key Words: mitochondria ■ reactive oxygen species ■ ratiometric fluorescent protein sensor ■ hypoxic pulmonary vasoconstriction

Small pulmonary arteries constrict in response to a decrease in alveolar oxygen tension through a physiological response termed hypoxic pulmonary vasoconstriction (HPV). Despite intensive study since the first description of HPV in 1946, the mechanism of O2 sensing remains unclear. Mitochondria have been considered a putative site of cellular oxygen sensing because they consume O2 and therefore represent the intracellular site with the lowest oxygen tension. Mitochondria generate superoxide and hydrogen peroxide during cellular respiration, so changes in the production of reactive oxygen species (ROS) during hypoxia could potentially signal changes in O2 tension and trigger cellular responses. However, whether hypoxia increases or decreases ROS generation during HPV remains unclear.

Using chemiluminescence in whole perfused lungs and endothelium-denuded rings of distal pulmonary arteries, Michelakis, Archer, and Weir and colleagues detected decreases in ROS generation during hypoxia.1–4 Similarly, in cultured pulmonary arterial pulmonary artery smooth muscle cells (PASMCs), Mehta et al detected a decrease in ROS generation as determined by dihydrodichlorofluorescein diacetate (DCF), dihydroethidium, and Amplex red.5 By contrast, using cultured PASMCs, we found that hypoxia triggers an increase in ROS generation.6–8 Our studies used the ROS-sensitive indicator dichlorofluorescein (DCF), mitochondrial inhibitors, enzymatic and chemical antioxidants and mitochondria-deficient (ρ0) cells. Other investigators have detected similar increases in ROS generation during hypoxia by using lucigenin-derived chemiluminescence and electron paramagnetic resonance spectrometry in small pulmonary arteries, as well as DCF in PASMCs.8,9

Attempts to resolve the question of whether ROS levels increase or decrease during hypoxia have been hindered by the technical limitations of the tools used to assess oxidant stress. Although lucigenin can detect ROS, lucigenin itself is capable of producing superoxide in cells.9 Furthermore,
questions regarding how these probes distribute between intracellular and extracellular compartments have led to concerns regarding the interpretation of data arising from their use. Other probes such as DCF and dihydroethidium lack specificity and can potentially accumulate within organelles. In addition, none of these probes exhibits ratiometric behavior, so changes in fluorescence arising from changes in intracellular dye concentration, excitation intensity, or fluorescence path length caused by changes in cell volume cannot be distinguished from changes in redox state. Finally, obtaining a global measure of tissue ROS production during hypoxia may mask important differences in ROS production occurring among cellular subcompartments. The goal of the present study was to test the hypothesis that hypoxia differentially alters ROS generation, depending on the subcellular compartment. To address this, we used a novel redox-sensitive protein sensor, which we separately targeted to the mitochondrial matrix, the mitochondrial intermembrane space, and the cytosol. This sensor, RoGFP, contains engineered cysteine residues, enabling dithiol formation in response to oxidant stress. Oxidation resulting in dithiol formation causes reciprocal changes in emission intensity when excited at two different wavelengths. Unlike DCF or lucigenin, the fluorescence excitation spectrum of RoGFP is strongly and reversibly dependent on the redox state of the introduced cysteines. Moreover, by exposing cells to strong reducing and oxidizing agents at the conclusion of an experiment, RoGFP oxidation state can be calibrated on a cell-by-cell basis in situ. We used these targeted sensors to assess redox changes in cellular subcompartments induced by hypoxia. We also compared the hypoxia-induced redox responses in PASMCs with those of systemic arterial smooth muscle cells (SASMCs).

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Pulmonary Microvessel Myocyte Isolation
The Northwestern University Institutional Animal Care and Use Committee approved all animal studies. PASMCs were isolated from rat lungs as described previously using a modification of the method of Marshall et al. Cells isolated by this method were confirmed to be PASMCs as previously described. Using a similar method, SASMCs were isolated from rat renal arteries and confirmed to be SASMCs as previously described. The isolated cells were cultured for 2 weeks (4 passages) and then used for the next 4 weeks (4 to 12 passages).

RoGFP Sensors
The RoGFP protein contains two engineered cysteine thiols, as first described by Remington et al (RoGFP2). The cDNA encoding the protein was created by introducing four mutations in the mammalian GFP expression vector (pEGFP-N1) (C48S, Q80R, S147C, and Q204C) using a QuikChange Multi Site-directed mutagenesis kit (Stratagene). The RoGFP construct was ligated into the VQ Ad5CMV K-NpA adenoviral shuttle vector between the KpnI and NotI sites; after sequencing and amplification, this plasmid was used to generate a recombinant adenovirus to permit widespread expression in our cells (ViraQuest Inc, North Liberty, Iowa). The resulting redox-sensitive protein has excitation maxima at 400 and 484 nm, with emission at 525 nm. In response to changes in redox conditions, RoGFP exhibits reciprocal changes in intensity at the two excitation maxima, and its ratiometric characteristics render it insensitive to expression levels. Although the fluorescence behavior of RoGFP is relatively independent of pH and it does not respond to authentic NO, reduced NADH, or the antioxidant N-acetyl-L-cysteine, its spectrum is slightly affected by reduced glutathione possibly attributable to thiol-disulfide exchange (Online Figures I and II).

RoGFP was expressed in the mitochondrial matrix (Mito-RoGFP) by appending a 48-bp region encoding the mitochondrial targeting sequence from cytochrome oxidase subunit IV, at the 5' end of the coding sequence. This construct was then ligated into the VQ Ad5CMV K-NpA plasmid between the KpnI and NotI sites, and used to generate an adenoviral vector. RoGFP was targeted to the mitochondrial intermembrane space (IMS-RoGFP) by appending it to glycerol phosphate dehydrogenase (GPD). A cDNA construct encoding GPD, an integral protein of the inner mitochondrial membrane whose C terminus protrudes into the intermembrane space, was ligated in-frame with cDNA encoding RoGFP. The corresponding polypeptide includes amino acids 1 to 626 of GPD, with RoGFP at the carboxy terminus. This method has been used previously to express YFP in the intermembrane space. (See the Online Data Supplement for characterization of the RoGFP sensors and experimental protocols.)

Statistics
Changes in RoGFP oxidation and YC2.3 fluorescence resonance energy transfer (FRET) ratio were analyzed by using a 2-way ANOVA with repeated measures. A Newman–Keuls multiple-range test was used to evaluate significant differences between groups and times. To control for differences in the hypoxic responses of cultured myocytes, experimental studies and control experiments were always carried out on the same day. Statistical significance was set at P<0.05.

Results
Hypoxia Induces Progressive Cytosolic Oxidation in PASMCs
To determine the effect of hypoxia on ROS production in the cytosol, isolated rat PASMCs expressing cytosolic RoGFP (Cyto-RoGFP) were subjected to hypoxia. Under baseline normoxic conditions, the Cyto-RoGFP sensor was 18.6±2.0% oxidized (Figure 1A). During hypoxia, Cyto-RoGFP demonstrated significantly progressive oxidation...
over a period of 30 minutes compared to normoxic PASMCs and reached 34.5±3.2% oxidized by the end of the experiment (Figure 1A). False-color ratiometric images of PASMCs demonstrate the dynamic redox range of the Cyto-RoGFP sensor. PASMCs expressing Cyto-RoGFP were superfused with normoxic (21% O₂) media (B), then with hypoxic (1.5% O₂) media for 30 minutes (C), followed by media containing DTT to fully reduce the sensor (D) and then with media containing tBH to fully oxidize the sensor (E). F, Representative time course, quantitative analysis of the fluorescence intensity ratio from representative individual PASMCs imaged in B through E. Img Fig indicates the time at which images B through E were captured during the experiment. G, Representative time course, quantitative analysis of the reciprocal changes in Cyto-RoGFP fluorescence intensity at the two excitation maxima (484 and 400 nm) for a single PASMC. Values are means±SE (n=6 cover slips, 4 to 10 cells/cover slip). *P<0.05 compared to normoxia.

Figure 1. Hypoxia shifts the cytosol to a more oxidized state in PASMCs. A, Combined results from multiple experiments in PASMCs expressing Cyto-RoGFP and superfused with either normoxic (21% O₂) or hypoxic (1.5% O₂) media. B through E, False-color ratiometric images of PASMCs demonstrate the dynamic redox range of the Cyto-RoGFP sensor. PASMCs expressing Cyto-RoGFP were superfused with normoxic (21% O₂) media (B), then with hypoxic (1.5% O₂) media for 30 minutes (C), followed by media containing DTT to fully reduce the sensor (D) and then with media containing tBH to fully oxidize the sensor (E). F, Representative time course, quantitative analysis of the fluorescence intensity ratio from representative individual PASMCs imaged in B through E. Img Fig indicates the time at which images B through E were captured during the experiment. G, Representative time course, quantitative analysis of the reciprocal changes in Cyto-RoGFP fluorescence intensity at the two excitation maxima (484 and 400 nm) for a single PASMC. Values are means±SE (n=6 cover slips, 4 to 10 cells/cover slip). *P<0.05 compared to normoxia.

The Mitochondrial IMS Becomes More Oxidized During Hypoxia in PASMCs

Having confirmed that hypoxia oxidizes thiol targets in the cytosol, we asked whether hypoxia induces ROS production in the IMS of the mitochondria. RoGFP expression was therefore targeted to the intermembrane space by appending it to GPD, an enzyme localized to the IMS. Confocal imaging demonstrated that IMS-RoGFP expression colocalized with mitochondrial cytochrome c, which is known to be localized to the intermembrane space (Figure 2A through 2C). To further verify correct targeting of IMS-RoGFP to the intermembrane space, we performed immuno-electron microscopy of cells using an antibody against GFP. At the dilutions used, no background labeling
outside mitochondria was observed in multiple images of separate mitochondria. Rather, the Immunogold labeling was restricted to the mitochondrial membrane at the mitochondrial periphery and cristae, demonstrating expression restricted to the mitochondrial intermembrane space (Figure 2D).

During normoxia, the RoGFP in the IMS was more oxidized (47.7±4.5%; Figure 2E) compared with the oxidation state demonstrated with Cyto-RoGFP (18.6±2.0%; Figure 1A). In response to hypoxia, IMS-RoGFP also significantly shifted to a more oxidized state compared to normoxic PASMCs and reached a level of 62.1±3.4% oxidized at the end of the experiment (Figure 2E). These results indicate that hypoxia shifts the mitochondrial IMS to a more oxidized state.

The Mitochondrial Matrix Becomes More Reduced During Hypoxia in PASMCs
Having found that hypoxia induces RoGFP oxidation in the intermembrane space, we next assessed the effect of hypoxia on redox status in the mitochondrial matrix. RoGFP was targeted to the mitochondrial matrix using the mitochondrial targeting sequence from cytochrome oxidase subunit IV. Confocal microscopy studies of expression of Mito-RoGFP and endogenous manganese superoxide dismutase, a protein expressed exclusively in the mitochondrial matrix, demonstrated colocalization of these two proteins at the resolution afforded by light microscopy (Figure 3A through 3C). Further assessment of its targeting was examined in Immunogold-labeled cryosections examined under electron microscopy (Figure 3D). Unlike the...
pattern of Immunogold labeling seen for IMS-RoGFP (Figure 2D), the Immunogold labeling in Mito-RoGFP-expressing cells was restricted to the matrix and was not associated with either cristae or peripheral mitochondrial membranes.

Under baseline normoxic conditions, the Mito-RoGFP sensor was 62.6 ± 1.5% oxidized (Figure 3E), significantly more oxidized than RoGFP localized to the mitochondrial intermembrane space (Figure 2E). However, unlike the Cyto- and IMS-RoGFP, which became more oxidized, during hypoxia Mito-RoGFP became progressively more reduced compared to normoxic PASMCs decreasing to 44.4 ± 1.9% oxidized by the end of the experiment (Figure 3E). These results reveal that the mitochondrial matrix undergoes a reductive stress during hypoxia.

Expression of Antioxidant Proteins Attenuates Hypoxia-Induced Oxidation of RoGFP

Our observation of hypoxia-induced oxidation of RoGFP in the cytosol and mitochondrial IMS, but not the matrix, suggested that hypoxia-induced ROS generation is localized to the cytosol and/or intermembrane space. To determine whether $H_2O_2$ signaling contributes to the oxidation response during hypoxia, catalase was first overexpressed in the cytosolic compartment. Cytosolic catalase overexpression completely blocked the hypoxia-induced increase in cytosolic oxidation (Figure 4A), indicating that hypoxia-induced oxidative stress in the cytosolic compartment is ultimately $H_2O_2$. Overexpression of catalase in the mitochondrial matrix, as previously achieved, had no effect on the hypoxia-induced decrease in oxidation of Mito-RoGFP during hypoxia (Figure 4B). These results indicate that the
progressive reduction in the mitochondrial matrix during hypoxia is not mediated by H$_2$O$_2$ signaling.

Hypoxia Shifts the Redox Status of Subcellular Compartments in SASMCs

Unlike pulmonary arteries, systemic arteries relax in response to hypoxia.$^4$ We assessed the redox response to hypoxia in smooth muscle cells isolated from renal arteries to determine whether they respond differently from PASMCs during identical hypoxic stimulation. Just as in the PASMCs, hypoxia induced a significant oxidation of Cyto-RoGFP from normoxic baseline (21.6±1.6% to 46.7±5.9% oxidized; Figure 5A). Similarly, hypoxia also induced oxidation of RoGFP localized to the mitochondrial IMS from normoxic baseline (27.5±3.5% to 50.4±4.6% oxidized; Figure 5B). Finally, hypoxia significantly reduced RoGFP targeted to the mitochondrial matrix from normoxic baseline (48.7±3.3% to 21.7±2.3% oxidized; Figure 5C), as we observed in the PASMCs. To examine the effect of the hypoxia-induced increase in ROS signaling on cytosolic calcium ([Ca$^{2+}$]$_i$) signaling during hypoxia, PASMCs and SASMCs expressing YC2.3 were compared. Hypoxia significantly increased the YC2.3 FRET ratio in PASMCs by 15 minutes (2.31±0.03 to 2.62±0.05; Figure 5D), whereas it significantly decreased the ratio in SASMCs within 30 minutes (2.29±0.04 to 2.05±0.08; Figure 5D). Taken together, these results indicate that hypoxia induces a similar pattern of redox modulation in SASMCs and PASMCs, and that there are cell type–specific downstream effectors that respond to this pattern of redox modulation.

Discussion

In this study, we genetically targeted a reporter of redox state to subcellular compartments. The expression of this sensor in vascular smooth muscle cells reveals important differences in protein thiol redox state between the cytosol, IMS and the mitochondrial matrix. Under normoxic conditions we found that the cytosol is the most reduced and the mitochondrial matrix is the most highly oxidized. The intermembrane space demonstrates an intermediate level of protein thiol oxidation.

We previously used a FRET redox sensor (HSP-FRET) to detect increases in oxidant stress in the cytosol of cultured PASMCs during hypoxia.$^7$ Those measurements provided evidence of an increase in ROS signaling during hypoxia, but slow refolding of the HSP-FRET protein after removal of the oxidant stress hindered our ability to calibrate its redox state during the experiment. Moreover, attempts to target its expression to the mitochondria were unsuccessful, impeding its usefulness for assessing subcellular redox signaling. By contrast, the ratiometric nature of RoGFP, and its successful targeting to distinct subcellular compartments, make it a useful probe for the molecular dissection of redox changes in response to hypoxia. The high level of basal oxidation detected by RoGFP in the matrix is consistent with a large literature identifying that compartment as the primary site of ROS production in the cell.$^{20}$ During hypoxia, the matrix undergoes a reductive shift. The decrease in oxidation during hypoxia suggests that ROS generation in the matrix arises from the nonspecific, [O$_2$]-dependent generation of superoxide at a number of sites, such that a decrease in O$_2$ availability decreases ROS production in a nonspecific manner. In contrast to the mitochondrial matrix, the cytosol and the IMS demonstrate an oxidative shift during hypoxia. The increase in oxidant stress in the IMS and the cytosol occurs despite the decrease in [O$_2$], and therefore appears to reflect a regulated increase in the generation of ROS in response to physiological hypoxia. Nevertheless, the oxidant signal we detect is subtle and it produces a relatively small change in protein thiol oxidation state.

To our knowledge, this is the first report comparing redox conditions in the cytosol, IMS and matrix compartments, and their responses to hypoxia. If mitochondria are the source of the oxidant signaling in the IMS and cytosol during hypoxia, the most likely site(s) of ROS generation could be within the IMS, at the outer membrane, or at the outer surface of the inner mitochondrial membrane. Importantly, the site of ROS generation cannot be in the matrix because hypoxia decreased oxidant stress in that compartment. We cannot exclude the possibility that ROS generation originates in the cytosol...
during hypoxia and diffuses into the IMS, because the oxidation response in these 2 compartments developed simultaneously. Weissmann et al recently demonstrated a role for nonphagocytic NADPH oxidase function during the acute phase of HPV, and suggested that NADPH oxidase may contribute to the generation of ROS during hypoxia. It is therefore possible that NADPH oxidase, or an alternative system, contributes to the hypoxia-induced ROS generation that affects both the cytosol and IMS. We favor the conclusion that the mitochondrial electron transport chain is the principal source of hypoxia-induced ROS signals, based on previous studies using mitochondrial inhibitors, mitochondria-deficient \(^{\rho^0}\) cells, and RNAi suppression of the electron transport chain.\(^6\)–\(^8\),\(^22\)–\(^28\) Interestingly, Rathore et al recently reported that hypoxia increases mitochondria-derived ROS in PASMCs, which leads to the activation of NAD(P)H oxidase through PKC\(_\alpha\) activation, suggesting that both mitochondria and cytosolic oxidant systems may contribute to the overall response.\(^24\)

Our findings conflict directly with the model of HPV by Michelakis, Archer, and Weir and colleagues, who find that hypoxia-induced decreases in mitochondrial ROS generation lead to redox-dependent alterations in \(K_v\) channel activation in the plasma membrane, thereby triggering depolarization and subsequent contraction.\(^2\),\(^4\),\(^29\)–\(^32\) One possible explanation for this conflict relates to their use of lucigenin chemiluminescence to assess oxidant stress. Our data reveal that redox responses can differ markedly among subcellular compartments. The unknown distribution of lucigenin between the intracellular and extracellular spaces, and its possible accumulation in mitochondria, cytosol, sarcoplasmic reticulum or other intracellular compartments, makes it difficult to interpret data obtained when the luminescence responses from all these compartments are recorded as a single signal. Our data demonstrate that, during hypoxia, oxidation increases slightly in some compartments, whereas it decreases significantly in others. Therefore, it is likely that the lucigenin signal reports a decrease during hypoxia because the collective oxidant stress across all of these compartments undergoes a net decrease. However, that combined signal would not detect important increases in oxidant stress in specific compartments where oxidant signaling acts as a critical mediator of cellular responses.
For PASMCs, hypoxia causes an increase in $[\text{Ca}^{2+}]_i$, and vasoconstriction\(^{33-44}\). However, for SASMCS, the result is a decrease in $[\text{Ca}^{2+}]_i$, and vasodilation.\(^{45,46}\). Michelakis et al. reported that ROS generation increases during hypoxia in systemic arterial tissue, but decreases in pulmonary arteries, leading them to conclude that tissue-specific differences in mitochondrial function explain their opposite responses.\(^4\) Those findings were contradicted by Mehta et al., who detected a decrease in hypoxia-induced ROS generation in both PASMCs and coronary artery smooth muscle cells.\(^5\) Our comparison of PASMCs and SASMCS revealed that both cell types exhibit increases in cytosolic ROS signaling during hypoxia, yet the functional responses were dissimilar, with PASMCs exhibiting an increase in $[\text{Ca}^{2+}]_i$, whereas SASMCS showed a decrease (Figure 5). Our results suggest that the differing physiological responses to hypoxia in the two types of vascular smooth muscle must therefore be mediated by cell-specific expression of downstream signaling pathways, allowing the same upstream oxidant signal to activate different tissue-specific responses to a hypoxic stimulus. This conclusion is supported by a growing number of studies that implicate hypoxia-induced increases in ROS signaling in mediating diverse tissue-specific responses across a wide range of cell types.\(^6-8,27,47-54\)

The ability of cytosolic catalase to attenuate the hypoxia-induced oxidation of the Cyto-RoGFP sensors is consistent with previous studies.\(^7,8\). However, the overexpression of mitochondrial catalase had no effect on the hypoxia-induced decrease in oxidation of Mito-RoGFP (Figure 4B). One possibility is that Mito-RoGFP is oxidized primarily by superoxide in the matrix. If so, then overexpression of mitochondrial catalase might not affect Mito-RoGFP oxidation because catalase degrades H$$_2$O$$_2$ but not superoxide. Alternatively, ROS production in the matrix may be largely absent during hypoxia. If so, then the hypoxia-induced decrease in Mito-RoGFP oxidation would reflect the rate at which the protein dithiols are rereduced by matrix reductases. If matrix H$$_2$O$$_2$ formation is minimal during hypoxia, then the rate of Mito-RoGFP oxidation would be minimal and mitochondrial catalase overexpression would not affect its redox state. The fact that cytosolic catalase overexpression attenuated the hypoxia-induced increase in cytosolic oxidant signaling underscores the importance of H$$_2$O$$_2$ signaling in that response.

In summary, these results are consistent with a model for HPV in which hypoxia augments oxidant signaling by H$$_2$O$$_2$ in the cytosol and IMS, whereas it decreases oxidant stress in the mitochondrial matrix (Figure 6). The cytosolic ROS signal triggers an increase in cytosolic calcium, which mediates PASMC contraction. Smooth muscle cells from systemic arteries demonstrate a similar pattern of oxidant signaling, indicating that the differences between PASMCs and SASMCS in terms of their response to hypoxia cannot be attributed to differences in mitochondrial function.

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### Disclosures

None.

### References


Novelty and Significance

What Is Known?

- The mitochondria of pulmonary arterial smooth muscle cells function as the O2 sensor underlying HPV by triggering a change in ROS signaling.
- Conflicting reports from various groups have shown that mitochondrial ROS signaling either increases or decreases in response to hypoxia.
- Attempts to resolve the question of whether ROS levels increase or decrease during hypoxia have been hindered by the technical limitations of the tools used to assess changes in oxidant stress.

What New Information Does This Article Contribute?

- A novel, redox-sensitive, ratiometric fluorescent protein sensor, RoGFP, was used to assess the effects of hypoxia on redox signaling in cultured pulmonary artery smooth muscle cells.
- For the first time, genetic targeting sequences were used to express RoGFP in the cytosol, the mitochondrial matrix, and the mitochondrial intermembrane space, allowing assessment of oxidant signaling in distinct subcellular compartments.
- The results are consistent with a model in which hypoxia induces the release of ROS from the outer surface of the inner mitochondrial membrane, thereby eliciting an increase in oxidant signaling in the intermembrane space. This ROS signal subsequently diffuses into the cytosol, where it elicits an increase in Ca2+, thereby activating HPV.

In the lung, alveolar hypoxia triggers constriction of small pulmonary arteries in a response known as hypoxic pulmonary vasoconstriction (HPV). Although this response has been studied extensively, the underlying mechanism of cellular oxygen sensing is not established and the signaling pathways that link the O2 sensor to the contractile response have been a focus of debate.

Our model proposes that hypoxia triggers the release of reactive oxygen species (ROS) signals from the outer surface of the inner mitochondrial membrane; these oxidants initially affect the intermembrane space and subsequently enter the cytosol, where they trigger increases in cytosolic calcium. To test this, we measured redox status in the mitochondrial matrix, the intermembrane space, and the cytosol of pulmonary artery myocytes during hypoxia, using targeted expression of a thiol redox-sensitive fluorescent protein sensor, RoGFP. Consistent with the model, we detected increases in ROS signaling in the intermembrane space and the cytosol of pulmonary artery myocytes during hypoxia, whereas oxidant stress in the mitochondrial matrix decreased. The increase in cytosolic ROS signaling is then required for the subsequent increase in ionized calcium. These findings reveal that hypoxia activates compartmentalized subcellular redox signaling, and that increases in cytosolic ROS are required for activating HPV.
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