Diversity in Mitochondrial Function Explains Differences in Vascular Oxygen Sensing


Abstract—Renal arteries (RAs) dilate in response to hypoxia, whereas the pulmonary arteries (PAs) constrict. In the PA, O2 tension is detected by an unidentified redox sensor, which controls K+ channel function and thus smooth muscle cell (SMC) membrane potential and cytosolic calcium. Mitochondria are important regulators of cellular redox status and are candidate vascular O2 sensors. Mitochondria-derived activated oxygen species (AOS), like H2O2, can diffuse to the cytoplasm and cause vasodilatation by activating sarcolemmal K+ channels. We hypothesize that mitochondrial diversity between vascular beds explains the opposing responses to hypoxia in PAs versus RAs. The effects of hypoxia and proximal electron transport chain (pETC) inhibitors (rotenone and antimycin A) were compared in rat isolated arteries, vascular SMCs, and perfused organs. Hypoxia and pETC inhibitors decrease production of AOS and outward K+ current and constrict PAs while increasing AOS production and outward K+ current and dilating RAs. At baseline, lung mitochondria have lower respiratory rates and higher rates of AOS and H2O2 production. Similarly, production of AOS and H2O2 is greater in PA versus RA rings. SMC mitochondrial membrane potential is more depolarized in PAs versus RAs. These differences relate in part to the lower expression of proximal ETC components and greater expression of mitochondrial manganese superoxide dismutase in PAs versus RAs. Differential regulation of a tonically produced, mitochondria-derived, vasodilating factor, possibly H2O2, can explain the opposing effects of hypoxia on the PAs versus RAs. We conclude that the PA and RA have different mitochondria. (Circ Res. 2002;90:1307-1315.)

Key Words: rotenone ■ K+ channels ■ redox ■ pulmonary circulation ■ oxygen sensor

The normoxic pulmonary circulation is vasodilated and accommodates the entire cardiac output at much lower pressures than the systemic circulation. During hypoxia, the pulmonary arteries (PAs) constrict (hypoxic pulmonary vasoconstriction, HPV), whereas systemic arteries, such as renal arteries (RAs), dilate. The mechanism of this opposing control of tone between the two vascular beds is unknown. Although the response of each bed to hypoxia is significantly modulated by the endothelium, the mechanism for the opposing responses to hypoxia appear to lie within the vascular smooth muscle cells (SMCs). Hypoxia increases intracellular Ca2+ ([Ca2+]i) and contracts PASMCs; in contrast, isolated SMCs from systemic arteries display decreased [Ca2+]i and relax in response to hypoxia.1

Both the control of tone and the response of O2-sensitive tissues to hypoxia involve redox-sensitive mechanisms (for review, see Wolin2). Activated oxygen species (AOS) are now recognized as important mediators in vascular cellular signaling. Several kinases and sarcolemmal potassium channels (K+ channels) are redox-sensitive and modulated by AOS. Cysteine-rich K+ channels are inhibited when reduced and activated when oxidized, and oxidants and AOS, including hydrogen peroxide (H2O2), are important K+ channel openers.3 K+ channels contribute to regulation of vascular tone through their control of SMC membrane potential (Em). Closing of K+ channels depolarizes Em, which causes an increase in the open probability of the voltage-gated L-type Ca2+ channels, Ca2+ influx, and vasoconstriction. In contrast, K+ channel openers cause hyperpolarization and vasodilatation.4

The major source of AOS and peroxides in SMCs are cytochrome-based oxidases, such as nicotinamide adenine dinucleotide phosphate (NADPH) and NADH, and the electron transport chain (ETC) in mitochondria.5 Both vascular oxidases and the ETC produce AOS in proportion to PO2, and thus have been proposed as candidates for vascular oxygen sensors.5,6 Despite the low K50 for O2 of the mitochondrial cytochromes, lung mitochondria make AOS in direct proportion to PO2 over the physiological range.7

Most O2-sensitive systems consist of a sensor that produces a mediator in response to changes in PO2, which in turn alters the function of an effector. O2- and redox-sensitive K+ channels have been shown to be the effectors in O2 sensing in several tissues including the PA, the ductus arteriosus, the...
carotid body, the neuroepithelial body, and the fetal adrenomedullary cells. Inhibition of these O₂-sensitive K⁺ channels leads to depolarization, opening of L-type Ca²⁺ channels, Ca²⁺ influx, and vasoconstriction. The obligatory role of the L-type Ca²⁺ channel is suggested by the fact that blockers and agonists of this channel inhibit⁶ and enhance⁹ HPV, respectively. On the other hand there are reports suggesting that it is release of intracellular Ca²⁺¹, rather than influx of extracellular Ca²⁺, that initiates HPV.¹⁰ This controversy may relate to confusion regarding the pool of Ca²⁺ that initiates rather than sustains HPV and perhaps to the model used to study HPV (rings versus intact lungs).

There is growing consensus that the vascular O₂ sensors are redox based. A variety of sensors have been proposed, including NADPH oxidase,¹¹ NADH oxidase,¹² and the ETC.⁶ It is likely that there is a diversity of sensors among different O₂-sensitive tissues and species. The role of gp91phox-containing NADPH oxidase in O₂ sensing was recently challenged, at least in the lung¹³ and the carotid body,¹⁴ although the role of novel oxidases (NOX) in HPV has not been assessed. The role of mitochondria as O₂ sensors in the pulmonary circulation was proposed 15 years ago³ and is supported by several recent publications.⁶,¹⁵,¹⁶ Their role in O₂ sensing appears to be widespread, because ETC inhibitors mimic hypoxia in several other O₂-sensing organs, such as the carotid body¹⁷ and adrenal medullary cells.¹⁸ The acute hemodynamic effects of hypoxia and ETC inhibitors, unlike anoxia, do not relate to ATP depletion.¹⁹ Rather, we speculate that mitochondria alter vascular tone by regulating the production of diffusible redox mediators, such as AOS and peroxides. H₂O₂, with its long diffusion radius, can modify targets in the cytoplasm and membrane (guanylate cyclase and K⁺ channels), and thereby regulate tone.² In addition, mitochondria are now recognized as important regulators of intracellular Ca²⁺.²⁰

We hypothesized that the opposing responses of the pulmonary and systemic vascular beds to hypoxia are, at least in part, due to differences in mitochondria function. Although mitochondrial diversity has been shown among neurons²¹ and myocardial cells,²²,²³ and between organs (pigeon heart versus rat liver mitochondria²⁴) potential diversity of mitochondrial function and its link to tone has not been reported so far in the vasculature.

Materials and Methods

**Perfused Lung-Kidney Model**

In this model, the isolated lungs and kidneys are perfused in series with a shared, blood-free solution as previously described.²⁶ The flow rate was kept constant in the lung (28 mL/min) and the kidney (10.5 mL/min) using separate perfusion pumps. Changes in PA and RA pressure were recorded in response to hypoxia or drugs.

**Tissue Baths**

Resistance PA and RA rings (4 to 5th order), denuded of endothelium, were studied as previously described.²⁶

**Patch Clamping**

Whole-cell patch-clamp recordings were performed in freshly isolated PASMCS and RASMCs, as previously described.²⁷

**Chemiluminescence**

Lucigenin (10⁻⁷ mol/L) enhanced chemiluminescence, a measure of the production of AOS, was measured in vascular rings or isolated mitochondria using a Packard 1900CA Liquid Scintillation Analyzer as described.⁶ The amount of mitochondria studied was normalized for protein content. Both arteries and mitochondria were studied immediately after isolation.

**H₂O₂ Measurement**

H₂O₂ production in vascular rings and mitochondria at baseline and in response to hypoxia and ETC blockers was measured using the AmplexRed H₂O₂ assay kit (according to the manufacturer’s instructions) as well as DCFH-DA (2',7'-dichlorofluorescin diacetate) assay, both from Molecular Probes. Background as well as drug autofluorescence was subtracted. Significant autofluorescence was seen with 10 μmol/L (but not 1 μmol/L) aqueous sodium cyanide (see online Figure 2, which can be found in the online data supplement available at http://www.circresaha.org).

**Mitochondria Respiration**

Mitochondria isolation and respiration were studied using standard methodology.²⁸,²⁹ Lung and Kidney mitochondria were kept at 4°C and studied immediately. A protein assay (BioRad) was performed on the isolated mitochondria, and the pellets were diluted accordingly to achieve equal mitochondrial protein loading. O₂ consumption was measured with an O₂ electrode using a MacLab D/A converter (AD Instruments). The maximum change in the rate of respiration in response to the proximal ETC complex substrates (glutamate 10 mmol/L for complex I and succinate 2.5 mmol/L for complex II) or substrate plus inhibitor (rotenone 5 μmol/L, antimycin A 50 μmol/L) was then calculated.

**Mitochondrial Quality Control**

To determine whether the quality of mitochondria was similar between the lung and kidney preparations (ie, to exclude possible differential damage of the one versus the other preparation during isolation), we used 2 standard quality control techniques: the NADH-supported respiration in intact versus lysed mitochondria and measurement of the respiratory control ration (RCR).²⁸,²⁹

**Immunoblotting**

Immunoblotting of ETC proteins from isolated mitochondria and manganese superoxide dismutase (MnSOD) from isolated arteries was performed as previously described.²⁷

**Confocal Microscopy**

Mitochondrial membrane potential (ΔΨm) was studied in first passage cultured PASMCs and RASMC, using two different dyes that are widely used in studying ΔΨm: JC-1 and TMRM (tetramethylrhodamine methyl-ester perchlorate). Cells were loaded with either JC-1 (1 μmol/L) or TMRM (20 nmol/L) for 30 minutes (37°C). JC-1 is a cationic fluorescent dye that exhibits potential-dependent mitochondrial accumulation, in that it fluoresces green when uptaken in depolarized mitochondria, whereas it dimerizes and fluoresces red when uptaken in hyperpolarized mitochondria.³⁰,³¹ TMRM-loaded mitochondria exhibit stronger red fluorescence with hyperpolarization.³² ΔΨm was compared between PASMC and RASMC mitochondria at baseline and in response to hypoxia. All the imaging parameters were kept identical between the two preparations.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

**Rotenone Mimics Hypoxia Constricting the Pulmonary While Dilating the Renal Circulation**

Rotenone (5 μmol/L) and hypoxia both constrict the pulmonary, whereas they dilate the renal vascular bed (Figures 1A and 1B). This difference is independent of nitric oxide and
prostaglandins because the experiments were performed in the presence of inhibitors of the endothelial nitric oxide synthase, L-N^G-nitroarginine methylester (L-NAME, 5 \times 10^{-5} \text{mol/L}) and cyclooxygenase (meclofenamate, 1.7 \times 10^{-5} \text{mol/L}). In contrast, both vascular beds constrict to angiotensin II (5 \times 10^{-5} \text{mol/L}) and 4-aminopyridine (4-AP, 5 mmol/L), a Kv channel blocker. The order of hypoxia, rotenone, and 4-AP challenges varied.

Further evidence that the opposing response to hypoxia and rotenone is intrinsic to the SMCs and independent of the endothelium comes from tissue bath experiments. In endothelium-denuded vessels, rotenone (5 \mu mol/L) mimics hypoxia again, constricting PAs and dilating RAs, whereas 4-AP (5 mmol/L) constricts both RAs and PAs (Figure 1C).

Rotenone Mimics Hypoxia Inhibiting K^+ Current in PASMCs While Activating it in RASMCs
Whole-cell patch clamping in freshly isolated SMCs from resistance PAs and RAs shows that rotenone inhibits outward K^+ current (I_k) in PASMCs while it activates I_k in RASMCs (Figure 2). The effects of both hypoxia and rotenone were rapid and occurred within 5 minutes.

Production of AOS and H_2O_2 at Baseline and in Response to Hypoxia and ETC Inhibitors Is Differentially Regulated Between PAs and RAs
We measured baseline AOS production (during normoxia) as well as the effects of hypoxia and ETC inhibitors in isolated, denuded, resistance PA and RA rings using 3 independent
Figure 3. Differences between the PA and RA production of AOS. A, Lucigenin-enhanced chemiluminescence at normoxic baseline is much higher in denuded PA rings than in denuded RA rings (†P<0.001), even in the presence of the NAD(P)H oxidase inhibitor DPI (‡P<0.05). Both rotenone and hypoxia (PO₂ 40 mm Hg) decrease AOS in the PA (‡P<0.01) and increase AOS production in the RA (**P<0.05). B, PAs produce more H₂O₂ than RAs (‡P<0.01). Both hypoxia and antimycin A decrease H₂O₂ production in the PA (†P<0.05) but not the RA. C, PAs produce more H₂O₂ than RAs. Both rotenone and hypoxia decrease H₂O₂ production in the PA but not in the RA. Myxothiazol and cyanide do not alter H₂O₂ production in either vessel (‡P<0.05 compared with normoxic PAs; †P<0.01 compared with normoxic PAs).

Lucigenin-enhanced Chemiluminescence

Figure 3

Lung Mitochondria Are Less Active and Produce More AOS and H₂O₂ Than Kidney Mitochondria

The fact that the PAs have higher AOS than the RAs, even in the presence of an NADPH oxidase inhibitor, suggests that mitochondria might be the source of this differentially regulated production of AOS. Metabolically active mitochondria are known to produce less AOS than less active mitochondria. We isolated lung and kidney mitochondria and studied respiration driven by substrates that enter the ETC proximally at the complexes I and II (glutamate and succinate; for results with glutamate alone versus succinate and glutamate, see the online data supplement, online Figure 1). We show that lung mitochondria have significantly lower rates of respiration compared with kidney mitochondria (Figure 4A). Rotenone and antimycin A inhibit respiration in both lung and kidney mitochondria, although the lung mitochondria are more sensitive to both inhibitors than are kidney mitochondria. The percent inhibition in respiration by rotenone for the lung and kidney respectively is -87±6 and -53±5 and for antimycin A is -96±3 and -68±15, respectively. The purity of our preparations is shown by electron microscopy, where mostly intact mitochondria and a few lysosomes are seen (Figure 4B).

The mitochondria isolation process could theoretically have resulted in preferential damage of one preparation versus the other, complicating their comparison. We therefore used 2 different assays to ensure that the quality of the mitochondria is similar in both preparations. Both preparations have similar RCRs (Figure 4C). These RCRs are lower that the RCRs from heart mitochondria (>10 in the literature and 11.4±0.4, n=3 in our hands), which is expected because blood vessels are metabolically less efficient than the myocardium. They show, however, that the quality of our preparations is similar. This was also confirmed by another assay, the ratio of NADH-driven respiration in sonicated versus intact mitochondria. Intact good quality mitochondria are impermeable and do not respond to NADH, whereas damaged “leaky” mitochondria (sonicated) respond to NADH with increased respiration. The ratio of NADH-supported respiration in sonicated mitochondria is significantly lower than that in intact mitochondria (Figure 4D).
cated/intact mitochondria is similar between the lung and kidney (Figure 4C).

We measured AOS and H$_2$O$_2$ production in lung and kidney mitochondria preparations with similar amounts of mitochondria, based on protein assays, and under identical conditions (Figures 5A and 5B). Lung mitochondria produce more AOS and H$_2$O$_2$ than the kidney mitochondria at baseline. The same proximal ETC substrate (glutamate/succinate) was used in both assays. The near complete elimination of the signal in the AmplexRed assay by catalase (10,000 U) confirms the specificity of the assay for H$_2$O$_2$ (Figure 5B). Antimycin A significantly inhibits the lung AOS production, but it does not alter the kidney mitochondria AOS production (Figure 5A). In contrast, the distal ETC inhibitor cyanide (10 µmol/L) does not alter AOS production in either preparation, confirming previous reports. These data are consistent with complexes I and III being the major sites of AOS production within the ETC.

We then measured the expression of ETC complexes I and III (where most of the production of AOS occurs) in PAs and RAs using immunoblotting. Although the ETC complexes consist of several subunits each, the commercially available subunits that we studied suggest that complexes I and III are expressed more in the RAs than the PAs (Figure 5C). Similar loading of the gels was confirmed by the Ponceau staining. In addition, using the same immunoblots (see online Figure 3B), we show that PAs express much more MnSOD, suggesting that the lower expression of proximal ETC complexes in this vessel is not a nonspecific finding or artifact of loading.

Figure 4. PA mitochondria are less active than RA mitochondria. A, Baseline respiration is greater in isolated kidney vs lung mitochondria. Rotenone and antimycin A inhibit respiration significantly in both organs (†P<0.01, *P<0.05). Substrates = glutamate (10 mmol/L) + succinate (2.5 mmol/L). B, Mitochondrial preparations are >90% pure as seen by electron microscopy. A few lysosomes are seen. D, Quality assessment by 2 assays (respiratory control ratio and the ratio of NADH-supported respiration in sonicated/intact mitochondria) shows that the condition of mitochondria from the lung and kidney is similar.

Figure 5. Lung mitochondria produce more AOS than kidney mitochondria, perhaps due to differences in the expression of ETC complexes I and III. A, Lucigenin-enhanced chemiluminescence is higher at baseline in the lung vs kidney mitochondria (*P<0.05). Antimycin, but not cyanide, decrease AOS production in the lung but not in the kidney (†P<0.05). B, Catalase-sensitive production of H$_2$O$_2$ is higher in the lung vs kidney mitochondria at baseline (*P<0.05). Glutamate + succinate were used as substrates in both A and B. C, Expression of complex I and III (immunoblotting) is less in the PA.
PASMC Mitochondria Are More Depolarized Than RASMC Mitochondria

We studied mitochondrial function within cultured PASMCs and RASMCs (first passage) loaded with 2 different dyes that are widely used in the measurement of Δψm, JC-1 and TMRM, using confocal microscopy (Figure 6).

With JC-1, mitochondria from RASMCs (n=44) have significantly higher Δψm than mitochondria from PASMCs (n=28) under identical loading and imaging conditions (Figure 6A). Extensive filamentous networks of mitochondria throughout the cytoplasm are shown in the representative pictures in Figure 6A. When the SMCs were superfused with a hypoxic solution, the PASMC mitochondria (n=30) hyperpolarized (decreased green/red ratio), whereas the RASMC mitochondria (n=40) depolarized.

In agreement with the JC-1 data, TMRM-loaded mitochondria from RASMCs (n=33) have significantly higher Δψm compared with mitochondria from PASMC, loaded and imaged under identical conditions (Figure 6B).

Discussion

We report that physiologically significant mitochondrial diversity exists between the renal and the pulmonary circulations. This diversity exists both at baseline and in response to ETC inhibitors and hypoxia. We show that mitochondrial function is different not only between the PAs and RAs but between the lung and kidney as well, perhaps reflecting differences in the overall redox environment of the two organs. We propose a model by which this diversity might at least in part explain the differences of the two circulations in both their redox state and the response to hypoxia. Our data are supported by a multitude of techniques, including perfused organs, tissue baths, patch clamping, measurement of AOS by 3 different techniques, mitochondrial respiration, immunoblotting, and dynamic confocal imaging using 2 different Δψm-sensitive dyes.

Baseline Differences Between the PA/Lung and RA/Kidney Mitochondria

Under identical experimental conditions, lung mitochondria have slower respiratory rates (Figure 4A) than kidney mitochondria. Quality control studies, showed that there was no differential damage during the isolation procedure (Figures 4B and 4C). Direct imaging of vascular SMCs showed that PASMC are more depolarized than RASMC mitochondria (Figures 6A and 6B). In addition, and perhaps related to these differences, lung mitochondria produce more AOS and H2O2 than kidney mitochondria at baseline (Figure 4A and 4B). Although antimycin inhibited respiration in both preparations (Figure 4A), the AOS production in the lung is sensitive to antimycin, whereas the kidney AOS production is antimycin-insensitive (Figure 5A). Early work by Boveris and Chance24 showed similar differences in the antimycin sensitivity between the pigeon heart and rat liver mitochondria H2O2 production. This suggests that the regulation of AOS production within the pETC might be different among organs or vessels.

Redox Potential and the AOS Production Are Different in the PAs Compared With the RAs

We used 3 different techniques and showed that freshly isolated PAs have higher AOS and H2O2 production at baseline compared with the RAs (Figure 3). We also showed that hypoxia and proximal, but not distal ETC inhibitors, significantly decrease AOS production in the PA. Several groups have suggested that proximal ETC function is important in HPV.15,16 However, these authors reported that AOS were increased by hypoxia and proximal ETC inhibition, whereas most groups find hypoxia decreases AOS.6,7,36,37 A
strength of our study is that AOS were measured in both mitochondria and in vessels using 3 different assays. The results of these assays were all concordant. In addition, we studied AOS production is freshly isolated arteries, which may be more physiological than the studies of AOS in cultured PASMCs, as performed by others. Even after inhibiting NADPH oxidase, there is residual AOS production, which is greater in the PA than the RA (Figure 3A), consistent with the concept that mitochondrial diversity exists between PAs and RAs, beyond any difference in NADPH oxidase that may exist.

Interestingly, although lucigenin-enhanced chemiluminescence was increased in RAs in response to hypoxia and proximal ETC inhibitors, the amount of H2 O2 production was not altered. This might be due to lower levels of MnSOD in the RASMCs. MnSOD catalyzes the formation of H2O2 from superoxide, the major AOS detected by chemiluminescence. Indeed, we showed that both mRNA and expressed protein for MnSOD are significantly lower in the RAs (online data supplement results and online Figure 3).

The differences in AOS production in PAs versus RAs parallel the differences in AOS production in lung versus kidney mitochondria. This might be related to the fact that there might be a more generalized difference in the redox environment between the 2 organs that affects their vasculature as well. In vivo, the lungs and the resistance PAs are exposed to alveolar oxygen levels, which are higher than those in arterial blood perfusing the rest of the organs like the kidneys. This might have induced redox differences. In fact, we show that the levels of reduced glutathione (GSH) in the lungs in normoxia are much higher than those in the kidney (online Figure 3). The findings that the lungs have a more oxidized redox potential than the kidneys and their importance are discussed in the online data supplement.

ETC Function Is Inversely Related to AOS Production: Lessons From Human and Animal Disease Models

The difference between PA and RA mitochondria is analogous to the comparison of mitochondria from healthy subjects versus patients with ETC complex I deficiency. Fibroblasts from complex I–deficient patients have increased mitochondrial-derived AOS production when compared with controls. In addition, their fibroblasts have impaired mitochondrial function, as reflected by depolarized ΔΨm. Furthermore, superoxide-induced MnSOD is upregulated in these patients. The normoxic PASMCs, like the fibroblasts from complex I deficiency patients, have (compared with the kidney) decreased levels of complex I (Figure 5C), decreased mitochondrial respiration (Figure 4A), and depolarized ΔΨm (Figure 6A). Lung mitochondria also make more superoxide and H2O2 than the kidney mitochondria (Figures 5A and 5B). The higher oxidative stress of PAs is associated with an apparently homeostatic induction of MnSOD expression and elevated GSH levels (online Figure 3).

How do these differences in ETC function and AOS production relate to the opposing response of the PA and RA to hypoxia? Important clues come from the striking similarities in the effects of ETC inhibitors and hypoxia discussed subsequently.

Proximal ETC Inhibitors Mimic the Opposing Effects of Hypoxia in PAs Versus RAs

We show that proximal ETC inhibitors and hypoxia inhibit PASMC I k and constrict the PAs, whereas they activate RASMC I k and dilate the renal circulation (Figures 1 to 2). These inhibitors are the only class of drugs, to our knowledge, that mimic hypoxia in both circulations. Other important modulators of HPV do not mimic hypoxia in that they constrict both the pulmonary and systemic circulations. For example, endothelin-1 constricts both PAs and RASMCs and inhibits Kv currents depolarizing both the PASMCs and RASMCs. Similarly, the Kv blocker 4-AP constricts both vessels (Figure 1). This suggests that the mechanism of the opposing effects of hypoxia on the 2 circulations is intrinsic to the mitochondrial ETC, upstream from the Kv channels. Furthermore, because these opposing effects persist in the absence of endothelium (Figures 1A through 1C) and are present in isolated SMCs (Figures 2 and 6), it suggests that they are intrinsic to the vascular SMCs and not the endothelium.

Vascular SMC Mitochondrial ETC Is an O2 Sensor

Our findings are in agreement with several recent studies that suggest a central role of mitochondria ETC and redox mediators in HPV, as previously proposed. Based on these studies, a potential mechanism for vascular O2 sensing includes (Figure 7) an O2 sensor (mitochondrial ETC or vascular oxidases) that regulates the production of a diffusible redox factor in proportion to O2 (oxygen radicals and peroxides, collectively known as AOS). Of the various species, H2O2 is an attractive candidate mediator because it is more stable than oxygen radicals, such as superoxide, it is freely diffusible, and it can affect several cellular mechanisms.

Figure 7. Proposed sensors, mediators, and effectors in vascular O2 sensing.
involved in tone control, including O₂-sensitive K⁺ channels (effectors; for review, see Archer at al⁴⁵).

Our study supports many of the known features of vascular O₂ sensing: the high level of H₂O₂ production at baseline in the PAs could explain the relatively vasodilated (low-pressure) state of the pulmonary, compared with the systemic, circulation. Tonically produced AOS/H₂O₂ from the proximal ETC can diffuse to the cytoplasm and cause K⁺ channel activation, SCM hyperpolarization, decreased opening of the voltage-gated Ca²⁺ channels and decreased [Ca²⁺]l levels and vasodilatation (Figure 7). During acute hypoxia, the tonic production of these vasodilators (AOS/H₂O₂) decreases (Figure 3). Thus, promoting Iₕ inhibition (Figure 2) and vasoconstriction (Figure 1), ie, HPV. In contrast, a hypoxia-induced increase in AOS (Figure 3A) would increase Iₕ and promote RA vasodilatation.

We acknowledge that more than one O₂-sensing mechanism might take place in one or both circulations at the same time. Thus, novel vascular oxidases could also contribute to redox signaling.

The concept of mitochondrial diversity in the vasculature is new and requires further study as it is likely relevant to other aspects of vascular function. In addition to its role in vascular O₂ sensing, the diversity concept may have implications for aspects of vascular function. In addition to its role in vascular redox signaling.

References


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Mitochondrial Diversity in the Vasculature
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Methods

The rats were euthanized with pentobarbital overdose before the lungs and kidneys were removed. All protocols were approved by the University of Alberta animal ethics committee.

The perfused lung-kidney model. In this model the isolated lungs and kidneys are perfused in series with a shared, blood-free solution as previously described. ¹ Under anesthesia (pentobarbital, 50mg/kg, IP) the abdomen was opened and dual lumen cannulas were advanced into the right RA and PA. The pulmonary circuit drained into a heated reservoir via a left atrial cannula. The lungs were suspended by a tracheal cannula and ventilated. The right kidney was removed and placed in a heated Petri dish. The flow rate was kept constant in the lung (28ml/min) and the kidney (10.5ml/min) using separate perfusion pumps. Changes in PA and RA pressure were recorded in response to hypoxia (PO₂ 40mmHg, PCO₂ 40mmHg, pH 7.4) or drugs. Hypoxia was induced by ventilating the lungs with a hypoxic gas (2.5% O₂, 5% CO₂, balance N₂).

Tissue baths. Resistance PA and RA rings (4-5th order), denuded of endothelium, were studied in 4-mL tissue baths as previously described. ² Rings were constricted with phenylephrine (10⁻⁵M) but failed to relax to Acetylcholine (Ach, 10⁻⁷M). This confirmed their denudation since in the absence of functional endothelium, Ach either fails to relax or causes a slight vasoconstriction. Optimal resting tension, defined as the tension at which maximal constriction to KCl 80mM occurred, was 0.8g for the PA and 1g for the RA.
**Patch clamping:** Whole cell patch-clamp recordings were performed as previously described 3. The pipette solution contained (in mM): KCl 140, MgCl₂ 1.0, HEPES 10, EGTA 5, Glucose 10, pH 7.2. The chamber containing the cells was perfused (2 ml/min) with a solution containing (in mM) NaCl 145, KCl 5.4, MgCl₂ 1.0, CaCl₂ 1.5, HEPES 10, glucose 10, pH 7.4 (extracellular solution). Cells were voltage-clamped at a holding potential of -70 mV and currents were evoked by steps from -130 mV to +50mV, as shown in Figure 2. The normoxic and hypoxic solution perfusate reservoirs were bubbled with 20% and 0% O₂, respectively (plus 3.5% CO₂/balance N₂), producing normoxic and hypoxic PO₂ s in the cell chamber of 120 and 40 mm Hg, respectively. PCO₂ was 40 mm Hg, and pH was 7.40 to 7.41 under both conditions. Drugs were dissolved in the normoxic solution. The pH of 4-aminopyridine (4-AP) was adjusted to 7.4 before dissolved in the normoxic solution.

Pipette resistances ranged from 1 to 4 M after Sylgard coating and fire-polishing, with series resistance typically compensated by 90% to 95%. Data were recorded and analyzed using pCLAMP 6.02 software (Axon Instruments, Foster City, CA). Whole-cell capacitance was measured using the automated function of the Axopatch amplifier. Whole-cell currents for each cell were divided by the cell's capacitance, giving a measure of current density (in picoamperes per picofarad).

**Chemiluminescence:** Lucigenin (10⁻⁵M) enhanced chemiluminescence, a measure of the production of AOS, was measured using a Packard 1900CA Liquid Scintillation Analyzer as described 4. Vascular rings or isolated mitochondria were placed in a 2 ml heated cuvette (37°C) and photon counts were displayed at 0.1 second intervals. An equal weight of resistance RA and PA rings was used in each
experiment. The amount of mitochondria studied was normalized for protein content. Both arteries and mitochondria were studied immediately after isolation. Background luminescence was subtracted from the recorded values and readings after 3 minutes of hypoxia or drugs were reported. The preparation was made hypoxic via bubbling with a hypoxic (PO2~40mmHg) versus normoxic (PO2~120mmHg) solution (balanced CO2 in each case). Careful attention was made to preserve physiological pH and temperature.

H2O2 measurement.

The AmplexRed™ H2O2 assay (Molecular Probes, Eugene, Oregon): The assay is based on the detection of H2O2 using 10-acetyl-3,7-dihydroxyphenoxazine (AmplexRed reagent), which in the presence of horseradish peroxidase, reacts with H2O2 with a 1:1 stoichiometry to produce the highly fluorescent resorufin-1 (absorption and fluorescence emission maxima of approximately 563nm and 587nm). Sections of vascular tissue (1-3mg) were incubated in vented 2ml capped Eppendorf tubes in 0.5ml of Krebs’ buffer (119mM NaCl, 3.52mM KCl, 1.17mM MgSO4, 1.18mM KH2PO4, 3.2mM CaCl2, pH 7.4) plus 0.5ml of AmplexRed reagent. Hypoxic (PO2~40mm Hg) or normoxic (PO2~120mm Hg) gas was bubbled through the incubation medium. Similar experiments were performed with freshly isolated lung and kidney mitochondria, using the isolation media described below.

The DCF assay: Production of H2O2 by isolated RA or PAs was measured by oxidation of 10µM 2’,7’-dichlorofluorescein diacetate (DCF) (Molecular Probes, Eugene OR) in Krebs buffer at 37°C over
a sixty minute interval. Aqueous sodium cyanide was used at 1µM concentrations, myxothiazol 100ng/ml (in ethanol) and rotenone at 10µM (in DMSO) (Sigma Aldrich, Oakville ON.) Drugs were tested under normoxia. Fluorescence resulting from oxidation of DCF was detected using a spectrofluorometer, (Spectra Max Gemini XS, Molecular Probes, Eugene OR) with excitation at 495nm and emission at 530nm. Background was corrected for by running controls containing the appropriate drug. Relative Fluorescence (RF) was calculated as follows (FU=fluorescence units):

Sample fluorescence (S)= sample FU\textsubscript{60min} – sample FU\textsubscript{0min}

Control fluorescence (C)  control FU\textsubscript{60min}-control FU\textsubscript{0min}

RF = S - C/Tissue wt (mg)

**Mitochondria isolation and respiration** were studied using standard methodology. Tissue was homogenized in 15ml of isolation medium (250mM Mannitol, 5mM HEPES pH 7.2, 0.5mM EGTA, 0.1% fatty acid free BSA) in a homogenizer at 4°C. Debris was precipitated by two rounds of centrifugation at 600xg for 5min at 4°C in a Sorvall superspeed centrifuge (DuPont Instruments New Town CT USA). The mitochondrial fraction was recovered from the supernatant by centrifugation at 10,000xg, washed twice, and the pellet resuspended in 250mM sucrose with 0.1% BSA. Mitochondria were kept at 4°C and studied within 3 hours from isolation. A protein assay (BioRad Hercules, CA USA) was performed on the isolated mitochondria and the pellets were diluted accordingly to achieve equal mitochondrial protein loading. Mitochondria were placed in an air-tight 2ml chamber in a heated water bath with a tight control of temperature at 37°C. The chamber was stirred with a magnetic bar.
O₂ consumption was measured with an O₂ electrode using a MacLab D/A converter (AD Instruments, Mountain View, CA). A buffer (1.5ml) was added containing (mM) KCl (45), potassium acetate (25), EGTA (0.1), MgCl₂ (1) and HEPES (10) (pH7.2) and respiration was measured for 10 minutes. The maximum change in the rate of respiration in response to the proximal ETC complex substrates (glutamate 10mM and succinate 2.5mM or substrate plus inhibitor (rotenone 5µM, antimycin A 50µM) was then calculated.

We used succinate and glutamate together because we observed a significant additive effect in mitochondrial respiration. This cocktail is commonly used in the studies of the proximal electron transport chain function as in the classic paper by Boveris and Chance. Because the respiratory rates of these mitochondrial are lower than other metabolically more active tissues (heart, liver) this increase was helpful for our experiments. We have observed however that whether we use succinate alone or glutamate alone, the kidney mitochondria have higher respiratory rates than the lung mitochondria (online supplement figure 1).

**Mitochondrial quality control:** To determine whether the quality of mitochondria was similar between the lung and kidney preparations we used two standard quality control techniques, NADH-supported respiration in intact versus lysed mitochondria and measurement of the respiratory control ration (RCR). Mitochondrial integrity was assessed by calculating the ratio of NADH (10mM)-supported respiration by sonicated versus untreated extracts. Sonication was performed in 5 second bursts x7. Respiratory control ratio (RCR), a sensitive index of mitochondrial integrity, was
determined by calculating the ratio of state 4 (200µM ADP) to state 3 respiration (substrate). The buffer contained 300mM sucrose, 1mM EGTA, 5mM MOPS pH 7.2, 5mM KH₂PO₄, 0.1% fatty acid free BSA.

**Immunoblotting.** Immunoblotting of proteins from isolated mitochondria was performed as previously described. Monoclonal antibodies against MnSOD and the nuclear encoded ETC subunits of Complex I (NADH ubiquinol oxidoreductase, 39 KDa subunit) and Complex III (ubiquinol cytochrome c oxidoreductase, subunit 1) were used (Molecular Probes, Eugene, OR). The supplier's recommended antibody dilutions were followed.

**RT-PCR.** Total RNA was isolated from homogenized rat PA and RAs using a QIAGEN RNeasy mini Kit (Missisauga, ON, Canada) and 2 µgs of RNA was reverse transcribed using QIAGEN Omniscript reverse transcriptase as described. The primer for MnSOD was designed based on rat cloned sequences from GeneBank. The PCR product was sequenced and confirmed the specificity of the product.

**Confocal microscopy.** Mitochondrial membrane potential (Δψm) was studied in first passage cultured PASMC and RASM, loaded with either JC-1 (1µM) or TMRM (20nM) for 30 minutes (37ºC). JC-1 is a cationic fluorescent dye that exhibits potential-dependent mitochondrial accumulation and is considered superior to several other dyes used in the measurement of Δψm. Within depolarized mitochondria JC-1 fluoresces in the green spectra, whereas within hyperpolarized mitochondria, the dye dimerizes and fluoresces in the red spectra. The green/red fluorescence ratio is therefore used to
estimate $\Delta \psi_m$ and thus mitochondrial function. TMRM-loaded mitochondria exhibit stronger red fluorescence the more hyperpolarized they are.\textsuperscript{11}

Confocal images were obtained within 30 minutes using an Axiovert 100M inverted confocal microscope (Carl Zeiss Canada, North York, Ontario). A 488nm Argon laser was used for excitations and the resultant red and green fluorescence was quantified using LP 560nm and BP 505-530nm filters respectively. A custom-made perfusion chamber on the stage of the microscope allowed for perfusion of the cells with normoxic versus hypoxic solutions (see patch-clamping section above). $\Delta \psi_m$ was compared between PA and RASMC mitochondria at baseline and in response to hypoxia. All the imaging parameters (including laser power and gains) were kept identical between the two preparations.

**HPLC.** A previously published method was used to measure glutathione (GSH) levels on homogenized normoxic rat lung and kidney.\textsuperscript{12}

**Statistics:** Values are expressed as the mean±SEM. Intergroup differences were measured using a repeated measures ANOVA with post-hoc analysis using Fisher’s PLSD test (Statview 4.02, Abacus Concepts). A p<0.05 was considered statistically significant. All drugs are from Sigma unless stated otherwise.
Online supplement Results/Discussion

The PAs have more oxidized redox potential compared to the RAs at baseline.

The data presented in figure 3 of the manuscript suggest that the RAs are in a more reduced state than the PAs at baseline. We confirmed this by comparing glutathione levels between the 2 arteries at baseline, using HPLC (online suppl. figure 3). Glutathione is a major antioxidant and it is also needed for the conversion of \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) by catalase. The higher levels of glutathione in the PAs are an expected compensation with the higher levels of AOS, including \( \text{H}_2\text{O}_2 \), in this vessel, compared to the RAs. Similarly, the mitochondria-specific MnSOD dismutates superoxide to \( \text{H}_2\text{O}_2 \) and it is known to be induced by high superoxide levels. \(^{13}\) The higher levels of superoxide in the PA (figure 3A) are in agreement with our finding that the levels of MnSOD expression is much higher in the PA versus the RA (online suppl. Figure 3B). The fact that the mRNA levels for MnSOD are slightly lower in the PAs than the RAs (online suppl. Figure 3C), suggests that MnSOD protein expression might be induced by the higher levels of superoxide in the PA at a post-transcriptional level.

Online Supplement Figure legends

Figure 1

The respiratory rates are higher in the kidney compared to the lung mitochondria whether glutamate is used as substrate alone or in combination with succinate.
Figure 2

Using the DCF technique we show that in contrast to the rest of the drugs (and their solvents) used, 10µM (but not 1µM) aqueous solution of sodium cyanide has very high auto-fluorescence, preventing its use in our experiments at this dose.

Figure 3

A. GSH levels are higher in the lung versus the kidney. (†p<0.001).

B. mRNA levels for MnSOD are higher in the RA than the PA.

C. Conversely, MnSOD protein levels (immunoblotting) are much higher in the PA (representative trace + normalized data).

References


10. Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A. JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett*. 1997;411:77-82.


Drug auto-fluorescence

FU

Buffer  Rotenone  Myxothiazol  Cyanide 1μM  Cyanide 10μM

P < 0.001