Model for Hypoxic Pulmonary Vasoconstriction Involving Mitochondrial Oxygen Sensing

Gregory B. Waypa, Navdeep S. Chandel, Paul T. Schumacker

Abstract—We tested whether mitochondria function as the O₂ sensor underlying hypoxic pulmonary vasoconstriction (HPV). In buffer-perfused rat lungs, rotenone, myxothiazol, and diphenyleneiodonium, which inhibit mitochondria in the proximal region of the electron transport chain (ETC), abolished HPV without attenuating the response to U46619. Cyanide and antimycin A inhibit electron transfer in the distal region of the ETC, but they did not abolish HPV. Cultured pulmonary artery (PA) myocytes contract in response to hypoxia or to U46619. The hypoxic response was abolished while the response to U46619 was maintained in mutant (ρ⁻) PA myocytes lacking a mitochondrial ETC. To test whether reactive oxygen species (ROS) derived from mitochondria act as signaling agents in HPV, the antioxidants pyrrolidinedithiocarbamate and ebselen and the Cu,Zn superoxide dismutase inhibitor diethyldithiocarbamate were used. These abolished HPV without affecting contraction to U46619, suggesting that ROS act as second messengers. In cultured PA myocytes, oxidation of intracellular 2’,7’-dichlorofluorescin diacetate (DCFH) dye increased under 2% O₂, indicating that myocytes increase their generation of H₂O₂ during hypoxia. This was attenuated by myxothiazol, implicating mitochondria as the source of increased ROS during HPV. These results indicate that mitochondrial ATP is not required for HPV, that mitochondria function as O₂ sensors during hypoxia, and that ROS generated in the proximal region of the ETC act as second messengers in the response. (Circ Res. 2001;88:1259-1266.)

Key Words: reactive oxygen species ■ hypoxia ■ redox signaling ■ pulmonary circulation ■ oxidants

Hypoxic pulmonary vasoconstriction (HPV) diverts blood flow away from the lung during fetal development and optimizes lung gas exchange after birth by enhancing the matching of blood flow and ventilation. Excised lungs retain the HPV response.1–6 Rings of pulmonary artery (PA) contract under low O₂ conditions7,8 even if denuded of endothelium.9,10 Even isolated PA myocytes contract during hypoxia,11 indicating that the O₂ sensor is intrinsic to those cells. Although HPV has been well characterized, the underlying mechanism of O₂ sensing is not established. Among the putative O₂ sensors that have been proposed, mitochondria have been discounted because the Kₘ of cytochrome oxidase for O₂ is too low to permit detection of physiological hypoxia.12 Moreover, inhibition of cytochrome oxidase with cyanide failed to abolish HPV.2 However, our studies have implicated mitochondria in the O₂ sensing underlying functional and transcriptional responses to hypoxia in other cells.13–15 Those data suggest that mitochondria generate reactive oxygen species (ROS) in response to low Pₒ₂, which constitutes an O₂-dependent signal.14,15 Oxidant signaling during hypoxia appears to originate at complex III, which could continue to function despite inhibition of complex IV with cyanide. The present study sought to determine whether mitochondria also function as the O₂ sensor during HPV and whether ROS generated by mitochondria function as second messengers in that response.

Materials and Methods

Isolated Perfused Lung
Lungs from Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, Ind.) were isolated as described previously.16 Lungs and heart were removed en bloc and the PA and left atrium were cannulated and perfused (8 mL/min) with a buffered salt solution containing BSA (0.5% wt/vol) and indomethacin (10 mg/L). Perfusion was maintained at 38°C, pH 7.4, and bubbled with 5% O₂, 5% CO₂, and 90% N₂. Lungs were ventilated with a humidified mixture of 21% O₂, 5% CO₂, and 74% N₂ (normoxia) at 54 breaths/min, tidal volume of 2 to 3 mL, and end-expiratory pressure of 3 cm H₂O. Left atrial and PA pressures were continuously recorded. All animals were housed and cared for under National Research Council guidelines for care and use of laboratory animals.

HPV in Isolated Lungs
Angiotensin II (10 nmol/L) was added to the perfusate, and HPV was induced by switching from normoxia to hypoxia (2% O₂, 5% CO₂, 93% N₂). Hypoxia-induced changes in pulmonary vascular impedance are represented as the change in PA pressure during constant flow, compared with normoxia, in cm H₂O. Two hypoxic challenges were averaged to define the baseline response before experimental intervention. The experimental agents were added to the reservoir and recirculated, after which two more hypoxic challenges were
administered and averaged. In the continued presence of the agents, the stable thromboxane A2 analogue U46619 (5 ng/mL) was added to the reservoir to determine the vasoconstrictor response to this receptor-mediated agonist.

PA Myocytes
PA microvessel myocytes were isolated after the method of Marshall et al.7 Myocytes were plated on collagen-coated coverslips and grown until 70% or 15% confluent, for 2′,7′-dichlorofluorescin diacetate (DCFH) or contraction studies, respectively. Mutant (p3) PA myocytes were generated from wild-type cells by incubation in ethidium bromide (25 ng/mL) for 2 weeks.11 This inhibits replication of mitochondrial DNA, which encodes critical subunits of the ETC.18 The absence of cytochrome oxidase subunit II was confirmed by polymerase chain reaction. Myocytes on coverslips were placed in a flow-through chamber on an inverted microscope and studied under controlled [O2] at 37° using Hoffman-modulation optics. Cell contraction was assessed from changes in cell length after 30 minutes and was expressed as percent decrease from the original length at t=0, [(original length)−(length at 30 minutes)]/(original length)×100).11

Measurement of ROS
ROS generation in PA myocytes was assessed using DCFH-DA (5 μmol/L, Molecular Probes). In the presence of H2O2, this probe is oxidized to 2′,7′-dichlorofluorescein (DCF), which was quantified using fluorescence imaging (excitation: 488 nm, emission: 535 nm) and reported as percent of initial values, after subtracting background (Universal Imaging).

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

Results
HPV in Isolated Perfused Lung
Figure 1A shows a representative PA pressure tracing for an isolated rat lung during alveolar hypoxia or in response to U46619. Hypoxia (2% O2, 5% CO2, 93% N2) increased PA pressure by 8.3±0.6 cm H2O (Hypoxia, Figure 1B). Administration of drug vehicle (0.1% DMSO) had no effect on the HPV response (7.2±0.6 cm H2O) compared with hypoxia. Addition of U46619 (5 ng/mL) increased PA pressure by 17.8±2.5 cm H2O, confirming the ability to respond to a receptor-mediated vasoconstrictor. Washout and replacement with fresh perfusate containing angiotensin II had no effect on HPV (8.3±0.5 cm H2O). These results confirm the suitability of this model for studies of HPV.

Mitochondria as O2 Sensors During HPV
To determine the requirement for electron transport in HPV, the flavoprotein inhibitor diphenyleneiodonium (DPI) was added to the isolated lung perfusate before hypoxia. DPI (10 μmol/L) blunted HPV compared with controls (6.6±0.6 cm H2O) without affecting the response to U46619 (11±3.8 cm H2O) (Figure 2A). In cultured PA myocytes, hypoxia elicited contraction compared with normoxia; this response was attenuated in the presence of DPI (Table 1).

To further clarify the mitochondrial ETC requirement for HPV, the inhibitor rotenone was added to the perfusate before hypoxia. Rotenone at 5 μg/mL inhibited HPV compared with controls (6.2±0.6 cm H2O) (Figure 2B), but it also abolished the response to U46619 (1.2±0.2 cm H2O). Rotenone at 50 ng/mL inhibited the rate of O2 uptake by isolated pulmonary cells by 78%, and it abolished HPV (8.0±1.0 cm H2O) (Figure 2C) without attenuating the response to U46619 (13.2±1.5 cm H2O). At 5 ng/mL, rotenone had no effect on HPV or the response to U46619 (Figure 2D). In cultured PA myocytes, rotenone at 50 ng/mL abolished the contractile response to hypoxia without affecting the response to U46619 (Table 1).

Myxothiazol inhibits complex III by blocking electron transfer to the Rieske iron-sulfur center in the bc1 complex.19 Myxothiazol at 50 ng/mL inhibited lung mitochondrial O2 uptake by 99%. It also attenuated HPV (1.6±0.2 cm H2O) compared with controls (12.2±0.8 cm H2O), without affecting the response to U46619 (17±0.6 cm H2O) (Figure 3A). These effects were reversed after washout with fresh perfusate (11.6±1.9 cm H2O). In cultured PA myocytes, myxothiazol attenuated the contractile response to hypoxia without affecting the response to U46619 (Table 1).

Antimycin A inhibits the oxidation of cytochrome b6f at complex III.19 Antimycin A (10 ng/mL) inhibited HPV but also attenuated the response to U46619 (not shown). At 1 ng/mL, antimycin A attenuated mitochondrial O2 uptake by 97% without inhibiting HPV (8.9±3.7 cm H2O) compared with controls (7.4±1.4 cm H2O) and without affecting the response to U46619 (13.8±2.5 cm H2O) (Figure 3B). In PA myocytes, antimycin A elicited contraction during normoxia (Table 1). Cyanide inhibits complex IV of mitochondria. Cyanide at 10 μmol/L inhibited mitochondrial O2 uptake by 89% and augmented HPV (10.2±0.8 cm H2O) compared with...
Figure 2. Changes in PA pressure during hypoxia in an isolated perfused lung. A, Hypoxia-induced response in the presence of DPI (10 μM). B, Hypoxia-induced response in the presence of rotenone (5 μg/mL). C, Hypoxia-induced response in the presence of rotenone (50 ng/mL). D, Hypoxia-induced response in the presence of rotenone (5 ng/mL). Data are mean±SE; n=4. *P<0.05 compared with Hypoxia.

Figure 3. Changes in PA pressure during hypoxia in an isolated perfused lung. A, Hypoxia-induced response in the presence of myxothiazol (50 ng/mL). B, Hypoxia-induced response in the presence of antimycin A (1 ng/mL). C, Hypoxia-induced response in the presence of cyanide (10 μmol/L). D, Hypoxia-induced response in the presence of DIDS (200 μmol/L). Data are mean±SE; n=4. *P<0.05 compared with Hypoxia.
controls (7.6±0.5 cm H₂O) without affecting the response to U46619 (14.8±1.2 cm H₂O) (Figure 3C).

To clarify the requirement for mitochondria in HPV, mutant ρ₀ cells were generated from wild-type rat PA myocytes. Contraction of ρ₀ cells under hypoxia (2% O₂) was attenuated compared with controls, while the response to U46619 was preserved (Table 1).

### Increased ROS Signaling in HPV

To determine whether increases in ROS are required for HPV, the thiol reductant pyrroliodindithiocarbamate (PDTC) was added before hypoxic challenge of isolated lungs. As an antioxidant, PDTC appears to enhance H₂O₂ clearance by reducing oxidized glutathione. PDTC (5 μmol/L) attenuated HPV to 2.6±0.7 cm H₂O compared with controls (7.3±0.8 cm H₂O) (Figure 4A), without affecting the response to U46619 (6.8±1.3 cm H₂O). The HPV response was restored (6.2±0.7 cm H₂O) after the PDTC was washed out and perfusate was replaced. At 10 μmol/L, PDTC also blocked HPV (2.1±0.5 cm H₂O) compared with controls (7.4±1.1 cm H₂O), without affecting the response to U46619 (9.2±1.2 cm H₂O) (Figure 4B). However, HPV was not restored (2.4±0.5 cm H₂O) after washout of this higher concentration. In cultured PA myocytes, PDTC inhibited the contractile response to hypoxia (Table 1).

Ebselen, a synthetic glutathione peroxidase, was added to the perfusate before hypoxic challenge to clarify the role of H₂O₂ in HPV. Ebselen (50 μmol/L) attenuated HPV (1.0±0.1 cm H₂O) compared with controls (6.4±0.4 cm H₂O) (Figure 4C), without altering the response to U46619 (8.1±1.1 cm H₂O). The HPV response was not restored after washout (1.4 cm H₂O). In cultured PA myocytes, ebselen also attenuated contraction during hypoxia (Table 1).

To clarify the relative importance of superoxide versus H₂O₂ in HPV, a cytosolic Cu,Zn superoxide dismutase (SOD) inhibitor, diethyldithiocarbamate (DDC), was added to the perfusate before hypoxic challenge. By inhibiting the formation of H₂O₂ in the cytosol, this should attenuate HPV if H₂O₂ is required for signaling, but could enhance the response if superoxide itself was involved. DDC (1 mmol/L) blunted HPV (2.1±0.5 cm H₂O) compared with controls (6.5±0.8 cm H₂O) (Figure 4D) without inhibiting the response to U46619 (18.3±0.8 cm H₂O). Inhibition of HPV by DDC was reversed after washout (6.2±0.5 cm H₂O). To test whether peroxide is sufficient to cause vasoconstriction, H₂O₂ (100 μmol/L) was added during normoxia. This increased PA pressure by 4.1±0.4 cm H₂O (Table 2). When administered to normoxic cultured PA myocytes, H₂O₂ also elicited contraction (Table 1).

### Mitochondrial Generation of ROS During Hypoxia

To determine the source of ROS during hypoxia, cultured PA myocytes were studied using DCFH dye at 37°C in a flow-through chamber. When the [O₂] bubbling the media was switched from 16% to 2% O₂, DCF fluorescence increased (Figure 5A). Return to 16% O₂ was associated with a return toward baseline in each case, which likely reflects leakage of oxidized dye from the cells. The response to hypoxia was repeated in the same field of cells before and after addition of myxothiazol. At 100 μmol/L, myxothiazol attenuated the increase in DCFH oxidation during hypoxia (Figure 5B).

Superoxide generated in the mitochondria would require an anion channel to reach the cytosol. If so, then an inhibitor of anion channels should attenuate cytosolic ROS signaling during hypoxia. To test this, we evaluated the anion channel inhibitor DIDS in perfused lungs. DIDS (200 μmol/L) inhibited HPV (2.8±0.7 cm H₂O) compared with hypoxia alone (9.4±0.7 cm H₂O) (Figure 3D), without affecting the response to U46619 (13.4±2.4 cm H₂O). HPV was restored after washout (8.4±1.1 cm H₂O). In PA myocytes, DIDS blunted contraction during hypoxia without affecting the response to U46619. DIDS also attenuated the contractile response to antimycin A seen during normoxia (Table 1).

### Alternative Sources of ROS

To test the involvement of NADPH oxidase in HPV, the inhibitor apocynin was added before hypoxia. Apocynin (3 mmol/L) abolished HPV compared with controls (6.3±0.7 cm H₂O), but it also abolished the response to U46619 (0.6±0.2 cm H₂O). At a lower concentration (300 μmol/L) that suppresses the respiratory burst of alveolar macrophages, apocynin failed to inhibit HPV (6.6±0.6 cm H₂O).
compared with controls (9.5 ± 1.0 cm H₂O), but did not inhibit the response to U46619 (12.7 ± 2.3 cm H₂O).

**Discussion**

**Role of Mitochondria as the O₂ Sensor in HPV**

We used parallel studies in intact lungs and in cultured PA myocytes to test whether mitochondria function as the O₂ sensor underlying HPV. In perfused lungs, inhibitors of the proximal region of the mitochondrial ETC including DPI, rotenone, and myxothiazol abrogated the hypoxia-induced increase in PA pressure without affecting the response to U46619. In PA myocytes, these inhibitors also abolished contraction during hypoxia. By contrast, more distal inhibitors of the ETC failed to abolish HPV. Cyanide augmented the HPV response, and antimycin A caused constriction of PA myocytes during normoxia. Thus, the response to hypoxia requires electron transport but does not require mitochondrial ATP because all of the inhibitors block oxidative phosphorylation, yet only the proximal inhibitors selectively abolish HPV.

To use a nonpharmacological approach, we generated mutant ρ⁰ cells from wild-type PA myocytes. These cells lack mitochondrial DNA, which encodes a number of essential subunits in the ETC complexes. These cells do not respire and depend on glycolytic ATP, yet they are morphologically indistinguishable from wild-type cells. We observed that ρ⁰ PA myocytes retain the contractile response to U46619 but fail to respond to hypoxia. These findings support the conclusion that mitochondria function as the O₂ sensor underlying HPV. Interestingly, we previously found that ρ⁰ cells...
Hep3B selectively lose the ability to activate the transcription factor HIF-1 during hypoxia, yet they retained the ability to respond to other stimuli such as cobalt chloride.\textsuperscript{15} Collectively, these observations suggest that a similar mitochondrial O$_2$ sensing mechanism may be responsible for HPV and for HIF-1–mediated transcriptional activation during hypoxia.

ROS as Second Messengers in HPV

Our results suggest that the mitochondrial ETC acts as an O$_2$ sensor during hypoxia by releasing ROS that function as signaling messengers (Figure 6). Superoxide generation is known to occur at the ubisemiquinone site of complex III via univalent electron transfer to O$_2$.\textsuperscript{22} The antioxidants PDTC, ebselen, and DDC blocked the response to hypoxia without affecting the U46619 response, suggesting that ROS and, in particular, H$_2$O$_2$ act as second messengers. The predicted increase in oxidant signaling during hypoxia was confirmed using the intracellular probe DCFH in cultured PA myocytes during hypoxia. Myxothiazol attenuated the increase in fluorescence during hypoxia, consistent with its expected inhibition of ubisemiquinone generation at complex III. These results further implicate ROS generated by the mitochondrial ETC in the signaling process.

Previous studies also implicate increased ROS generation in the response to hypoxia. Monaco et al\textsuperscript{23} showed that HPV was augmented when catalase was inhibited with aminotriazole. Weissmann et al\textsuperscript{5} observed that SOD and 4,5-dihydroxy-1,3-benzenedisulfonic acid (to accelerate H$_2$O$_2$ generation from superoxide) did not affect HPV, suggesting that H$_2$O$_2$, rather than superoxide, is involved. By contrast, nitroblue tetrazolium, which traps superoxide and prevents H$_2$O$_2$ formation, attenuated HPV. Finally, our data and previous studies show that H$_2$O$_2$ constricts the pulmonary circulation during normoxia.\textsuperscript{24} These findings are consistent with a role for increased H$_2$O$_2$ as a signaling molecule involved in HPV.

Mechanism of ROS Generation During Hypoxia

Our previous studies demonstrated that hypoxia affects cytochrome oxidase, causing it to cycle at a more reduced state.\textsuperscript{25} We had suggested that the increase in reduction state of that complex should cause a similar redox change at more proximal ETC sites, which might explain the increase in ROS generation at complex III during hypoxia.\textsuperscript{14} However, we later observed that hypoxia still increased ROS generation when electron transport at the distal end of complex III was inhibited by antimycin A.\textsuperscript{15} In the presence of an inhibitor, more proximal ETC complexes become fully reduced while those at more distal locations become oxidized. The observations that hypoxia augmented ROS signaling during antimycin A, and that antimycin A and cyanide failed to abolish the hypoxic constriction, suggest that the O$_2$-sensing site must be located upstream from the antimycin A inhibition site. Moreover, the O$_2$ sensor must still be able to function if the ETC chain is fully reduced.

During normoxia, ETC inhibitors acting at sites distal to ubisemiquinone (eg, cyanide, azide, or antimycin A) tend to augment ROS generation by increasing the reduction state of the ubiquinone pool.\textsuperscript{14,15,19,26} During hypoxia, our model suggests that the biophysical process for ROS generation from that site is amplified, even when the complex is fully reduced. By contrast, if the complex becomes fully oxidized by ETC inhibition at a more proximal site (eg, rotenone, DPI, or myxothiazol) then ROS generation and O$_2$ sensing are abolished by the lack of electrons. In accordance with this model (Figure 6), distal inhibitors of the ETC such as cyanide induced constriction during normoxia, through a mechanism that could be inhibited by ebselen (an antioxidant) or myxothiazol (a more proximal ETC inhibitor). Similarly, Rounds and McMurtry\textsuperscript{1} previously found that antimycin A elicits vasoconstriction in normoxic lungs, a response we reproduced (Table 2). Also, Archer et al\textsuperscript{2} found that cyanide induced vasoconstriction during normoxia and augmented...

Figure 5. Effect of hypoxia on DCFH oxidation in PA myocytes. A, DCF fluorescence intensity when [O$_2$] was decreased from 16% to 2%. Representative tracing; values are percent, relative to baseline. B, DCF fluorescence during 2% O$_2$ before and after addition of myxothiazol (100 ng/mL). Data are mean±SE; n=5, *P<0.005.

Figure 6. Model describing the mechanism of O$_2$ sensing by mitochondria underlying the HPV response. Boxes show sites of inhibition.
HPV without affecting the response to angiotensin II or KCl. Collectively, these observations are consistent with our proposed model, but the mechanism by which hypoxia amplifies ROS generation at complex III is not yet known.

An alternative explanation for the increase in ROS signaling during hypoxia involves the regulation of superoxide egress from mitochondria to cytosol. Superoxide would presumably require an anion channel to escape from the matrix. The inner membrane anion channel (IMAC) could conceivably function as that pathway. If the IMAC were an O2-sensitive channel that increased its conductance during hypoxia, the egress of superoxide from the matrix could increase even if the rate of mitochondrial superoxide generation were to decrease during hypoxia. This could explain why ROS signaling in the cytosol is increased during moderate hypoxia, and why DIDS, which inhibits mitochondrial IMAC, attenuated HPV and abolished PA myocyte contraction during hypoxia. It should be noted that DIDS is also a thiol-reactive compound; however, pretreatment with DIDS had no effect on H2O2-induced vasoconstriction in the lung (see online data supplement available at http://www.circresaha.org). In either case, it appears that superoxide enters the cytosol where it is dismutated to H2O2 by cytosolic Cu,Zn SOD. Inhibition of SOD by DDC abrogated the hypoxic responses, indicating that superoxide conversion to H2O2 is required for the response.

**Downstream Signaling in HPV**

Smooth muscle contraction during HPV requires an increase in cytosolic [Ca2+]. Although H2O2 appears to act as a signaling messenger in the sequence leading to calcium activation, the details of that pathway are not fully understood. One possibility is that potassium channels become functional as that pathway. If the IMAC were an O2-sensitive channel that increased its conductance during hypoxia, the egress of superoxide from the matrix could increase even if the rate of mitochondrial superoxide generation were to decrease during hypoxia. This could explain why ROS signaling in the cytosol is increased during moderate hypoxia, and why DIDS, which inhibits mitochondrial IMAC, attenuated HPV and abolished PA myocyte contraction during hypoxia. It should be noted that DIDS is also a thiol-reactive compound; however, pretreatment with DIDS had no effect on H2O2-induced vasoconstriction in the lung (see online data supplement available at http://www.circresaha.org). In either case, it appears that superoxide enters the cytosol where it is dismutated to H2O2 by cytosolic Cu,Zn SOD. Inhibition of SOD by DDC abrogated the hypoxic responses, indicating that superoxide conversion to H2O2 is required for the response.

In previous studies, the mitochondrial inhibitor rotenone attenuated HPV but also produced a transient increase in PA pressure immediately after administration.1 Rounds and McMurtry1 suggested that this was due to an inhibition of ATP production, whereas Archer et al2 suggested that a shift in the cytosolic redox status elicits contraction. We also observed a small transient increase in PA pressure on addition of rotenone, cyanide, or DPI. However, myxothiazol had no such effect, so not all ETC inhibitors elicited vasoconstriction. We suggest that the transient responses to some compounds reflect nonspecific effects on the pulmonary circulation. By contrast, their effects on the subsequent response to hypoxia reflect their influence on HPV.

**Alternative ROS Sources**

Marshall et al3 suggested that hypoxia accelerates ROS generation by a membrane-bound NADPH oxidase, based on their observation that DPI inhibited the oxidant signal and the contractile response to hypoxia. DPI inhibits a wide range of flavoproteins including NADPH oxidase, mitochondrial complex I,3,4 glutathione reductase, nitric oxide synthase, and prostaglandin synthetase.3,4 Therefore, the site of inhibition responsible for the attenuation of HPV is not known. It is conceivable that DPI abolished HPV by inhibiting mitochondrial complex I and abolished their chemiluminescence signal through a separate effect on NAD(P)H oxidase. Grimmer et al also found that DPI attenuated HPV without affecting the vascular response to U46619, consistent with our findings. To address the possible involvement of NADPH oxidase in HPV, we used apocynin, a selective inhibitor of the neutrophil form of this enzyme.1 At concentrations that preserved the response to U46619, apocynin failed to ablate either HPV or PA myocyte contraction during hypoxia. In homozygous knockout animals lacking the gp91phox subunit of the NADPH oxidase complex, Archer et al found that the response to hypoxia was preserved, further suggesting a lack of involvement of that system in HPV.

Collectively, these observations are consistent with our proposed model, but the mechanism by which hypoxia amplifies ROS generation at complex III is not yet known. An alternative explanation for the increase in ROS signaling during hypoxia involves the regulation of superoxide egress from mitochondria to cytosol. Superoxide would presumably require an anion channel to escape from the matrix. The inner membrane anion channel (IMAC) could conceivably function as that pathway. If the IMAC were an O2-sensitive channel that increased its conductance during hypoxia, the egress of superoxide from the matrix could increase even if the rate of mitochondrial superoxide generation were to decrease during hypoxia. This could explain why ROS signaling in the cytosol is increased during moderate hypoxia, and why DIDS, which inhibits mitochondrial IMAC, attenuated HPV and abolished PA myocyte contraction during hypoxia. It should be noted that DIDS is also a thiol-reactive compound; however, pretreatment with DIDS had no effect on H2O2-induced vasoconstriction in the lung (see online data supplement available at http://www.circresaha.org). In either case, it appears that superoxide enters the cytosol where it is dismutated to H2O2 by cytosolic Cu,Zn SOD. Inhibition of SOD by DDC abrogated the hypoxic responses, indicating that superoxide conversion to H2O2 is required for the response.

**Drug-Induced Contraction During Normoxia**

In previous studies, the mitochondrial inhibitor rotenone attenuated HPV but also produced a transient increase in PA pressure immediately after administration.1 Rounds and McMurtry1 suggested that this was due to an inhibition of ATP production, whereas Archer et al2 suggested that a shift in the cytosolic redox status elicits contraction. We also observed a small transient increase in PA pressure on addition of rotenone, cyanide, or DPI. However, myxothiazol had no such effect, so not all ETC inhibitors elicited vasoconstriction. We suggest that the transient responses to some compounds reflect nonspecific effects on the pulmonary circulation. By contrast, their effects on the subsequent response to hypoxia reflect their influence on HPV.

**Acknowledgments**

This research was supported by NIH Grants HL32646, HL35440, HL66315, and HL10405. The authors gratefully acknowledge the technical assistance of Carol Mathieu, Dr Ningfang Chen, and Dr Matthew Mack in these studies.

**References**


Online Supplementary Material

*Isolated perfused lung preparation.* Male or female Sprague-Dawley rats (250-400 g) were anesthetized with pentobarbital sodium (0.65 mg/kg; ip), and heparin (1000 U/kg) was administered intravenously. Lungs were isolated as described previously\(^1\). Briefly, the lungs and the heart were remove *en block* and the pulmonary artery (PA) and left atrium were cannulated and perfused with a buffered salt solution (BSS)(NaCl (117 mM), KCl (4 mM), NaHCO\(_3\) (18 mM), MgSO\(_4\) (0.76 mM), NaH\(_2\)PO\(_4\)•H\(_2\)O (1 mM), CaCl\(_2\) (1.21 mM), and glucose (1 g/L)) containing bovine serum albumin (0.5 % w/v) and indomethacin (10 mg/L). After flushing the pulmonary circulation with perfusate (100-200 ml), the lungs were perfused from a recirculating system (35 ml) at 8 ml/min. Perfusate was maintained at 38\(^\circ\) C, pH 7.4, and bubbled with 5 % O\(_2\), 5 % CO\(_2\), and 90 % N\(_2\). Left atrial pressure was maintained at 1 cm H\(_2\)O by elevating the venous outflow cannula. The lungs were ventilated with a small animal respirator using a humidified gas mixture of 21 % O\(_2\), 5 % CO\(_2\), and 74 % N\(_2\) (normoxia) at a rate of 54 breaths/min, a tidal volume of 2-3 ml and an end-expiratory pressure of 3 cm H\(_2\)O. Left atrial and PA pressures were continuously recorded on a multichannel strip chart recorder (Grass Instruments, Quincy, MA).

*HPV responses in isolated lungs.* Angiotensin II (10 nM) was added to the recirculating perfusate because isolated lungs perfused with BSS do not respond to hypoxia without such an agonist\(^2\). This typically increased PA pressure by 2 cm H\(_2\)O (data not shown). HPV was induced by switching ventilation from the normoxic gas to a hypoxia mixture containing 2 % O\(_2\), 5 % CO\(_2\), and 93 % N\(_2\) for 10 to 15 min during which time PA pressure increased. When the PA pressure had stabilized at the higher value, the lungs were returned to normoxia and the pressure in the PA returned to baseline.
Hypoxia-induced changes in pulmonary vascular impedance are represented as the change in PA pressure observed during hypoxic ventilation under constant flow conditions, compared with normoxia, in cm H$_2$O. Two hypoxic challenges were performed and averaged to define the baseline response prior to the experimental intervention, thus allowing each lung to act as its own control. The experimental agents then were added to the reservoir and allowed to recirculate for 5 - 10 min, after which two more hypoxic challenges were administered and averaged to evaluate the effects of the intervention. In the continued presence of the experimental agents, the stable thromboxane A$_2$ analog, U46619 (5 ng/ml) was then added to the reservoir to determine the effect on the ability to respond to a receptor-mediated vasoconstricting agonist. This provided a measure of the specificity of the intervention for the response to hypoxia.

*Pharmacological Agents.* Diphenyleneiodonium (DPI) was obtain from CalBiochem and rotenone, myxothiazol, antimycin A, cyanide, pyrrlloidinedithiocarbamate (PDTC), ebselen, diethylthiocarbmate (DDC), apocynin, and 4,4’-diiisothiocyanatostilbene-2,2’-disulphonic acid (DIDS) were obtained from Sigma. The pharmacological agents were dissolved in DMSO (100%) as a 1000x stock solution so that when added to the media the DMSO concentration was <0.1%. The stock solutions were stored at -20°C and thawed on the day of the experiment.

*Non-specific inhibition of PA smooth muscle cell contraction.* To determine whether the pharmacological agents that inhibited the HPV response in our study might also affect the response a receptor-mediated vasoconstrictor, studies were carried out using the stable thromboxane A$_2$ analog, U46619 (5 ng/ml) (Fig. 1-4 of the manuscript). Experiments were performed to assess the U46619 response before and after the addition of the experimental agents. Briefly, lungs were subjected to a challenge of U46619 and
the change in PA pressure was measured (Before; Table 1). The vasculature was then washed out with fresh perfusate until the PA pressure returned to baseline (approximately 10-min elapsed time), after which the experimental agents were administered to the reservoir. After 10 min, a second dose of U46619 was added to the reservoir and the change in PA pressure was measured (After, Table 1). None of the experimental agents blunted the U46619 vasoconstriction response except rotenone at 5 μg/ml (38.2 ± 27.5 % of the initial response). Myxothiazol (50 ng/ml), cyanide (10 μM), ebselen (50 μM), and DDC (1 μM) had no effect on the U46619 vasoconstriction response. DPI (10 μM), rotenone (50 ng/ml), antimycin A (1 ng/ml), PDTC (10 μM), and DIDS (200 μM) augmented the U46619 vasoconstriction response. These results indicate that the inhibition of HPV, observed with some of the compounds, is not a reflection of non-specific inhibition.

To confirm the ability of various pharmacological inhibitors to block electron transport, we measured the decrease in O2 uptake rates of mitochondria isolated from pulmonary cells. Briefly, mitochondria were isolated from lung tissue and studied in suspension in a magnetically stirred, water-jacketed (37 °C) Warburg respirometer (2 ml volume). A polarographic O2 electrode mounted in the flask was used to monitor the PO2 in the solution. Succinate (an electron source for Complex II) and ADP+ Pi were added to induce State 3 respiration. The mitochondrial inhibitors antimycin A (1 ng/ml), myxothiazol (50 ng/ml), and cyanide (10 μM) attenuated the rate of oxygen uptake by 97, 99, and 89%, respectively. Rotenone (50 ng/ml) attenuated the rate of oxygen uptake by 78% in isolated cell preparations. [N.B. A whole-cell suspension was used to assess the effects of rotenone because of a technical difficulty encountered using isolated
mitochondria with rotenone, which inhibits at Complex I. In the isolated mitochondrial preparations, electrons were fed into Complex II via succinate, bypassing that blockade. In the intact cell, the majority of electron flux passes through Complex I, so rotenone produces a significant decrease in O₂ uptake.] These data confirm that the concentrations of pharmacological agents used in this manuscript are sufficient to inhibit mitochondrial electron transport.

DIDS is a non-specific inhibitor of anion channels, but might also exhibit antioxidant activity. To determine whether the effects of DIDS were due to an antioxidant effect, the effects of this compound on the response to H₂O₂ was assessed in intact perfused lungs. Lungs were pretreated with DIDS (200 μM) for 10 min, after which H₂O₂ (100 μM) was administered into the perfusate during normoxic ventilation. Hydrogen peroxide increased PA pressure by 4.2±0.7 cm H₂O in the presence of DIDS, which was not different from the pressor response seen in the absence of DIDS (4.1±0.4 cm H₂O).

Pulmonary microvessel myocyte isolation. Myocytes were isolated using a modification of the method of Marshall et al. ³. Freshly excised rat heart and lungs were rinsed with phosphate-buffered saline (PBS) containing penicillin and streptomycin (1 %) (PBS). The right ventricle was cannulated and the pulmonary vasculature was flushed with PBS (30 ml). Using the PA cannula, M199 growth media (30 ml) containing HEPES (25 mM), and penicillin and streptomycin (1 %) plus low melting point agarose (0.5 %) and iron particles (0.5 %), was flushed through the pulmonary vasculature. The iron particles were too large to pass through the capillaries; therefore only the arteries contained iron particles. The airways were filled via the trachea with M199 (15 ml) containing low melting point agarose (1%) without iron. The lungs were plunged into
cold PBS to cause the agarose to gel. After 10 min, the lobes were dissected free and finely minced in a petri dish. The lung fragments were washed (3x) with PBS, using a magnet to retain the iron-containing fragments. The iron-containing pieces were resuspended in M199 media (25 ml) containing collagenase (80 U/ml) and incubated at 37° C for 30-60 min. To remove extravascular tissue, fragments were first drawn through a 15 ga needle, and subsequently through an 18 ga needle. The iron-containing fragments then were washed (3x) with M199 containing fetal bovine serum (FBS) (20%) and drained. The resulting fragments were placed in a petri dish and resuspended in M199 containing FBS (10%) (4 ml). The petri dishes were incubated at 37° C with CO₂ (5%) in air for 4 - 5 days, during which time the pulmonary myocytes were observed to migrate and adhere to the dish. After 4 - 5 days, the media and iron containing particles were transferred to a new dish containing fresh media. The adherent myocytes continued to propagate until the cells were 70% confluent. The myocytes were then replated on collagen-coated (0.01%) glass cover slips and grown in M199 with FBS (10%) until 70% confluent for measuring ROS generation or 15% confluent for measuring cell shortening.

*Immunocytochemistry.* To confirm that the cultured cells were indeed pulmonary arterial myocytes, isolated pulmonary smooth muscle cells and A549 cells (for comparison) were grown on coverslips in 6-well plates containing M199 with 10% FBS, penicillin and streptomycin. Before staining the cells, the medium was removed, the cells were washed with PBS twice, fixed with 100% methanol at -20°C for 10 min, and washed twice with PBS. The cells were then incubated with blocking buffer (5% FBS in PBS) for 1 hr, and incubated with 1:100 dilution of monoclonal anti-smooth muscle cell α-actin antibody (Sigma) in blocking buffer for 1 hr. Subsequently the cells were washed with PBS four times, and incubated with 1:100 dilution of FITC-labeled anti-
mouse antibody (Amersham) in blocking buffer for 45 minutes. Finally, cells were washed with PBS four times and mounted with permount medium (Fisher). The slides were air dried and examined with a fluorescence microscope (Supplementary Figure 1). Pulmonary smooth muscle cells stained for α smooth muscle actin, whereas A549 did not stain for α smooth muscle actin. These results show that the cultured cells were indeed pulmonary arterial myocytes.

*Mutant (ρ<sup>0</sup>) pulmonary artery myocytes.* These cells were generated from wild-type cultured myocytes by incubation for two weeks in M199 growth media containing ethidium bromide (25 ng/ml), pyruvate (1 mM) and uridine (50 μg/ml)<sup>4</sup>. Ethidium bromide inhibits replication of mitochondrial DNA, which encodes critical subunits of the electron transport system<sup>5</sup>. The ρ<sup>0</sup> cells depend on glycolytic ATP for survival and growth, but their morphology is indistinguishable from wild-type cells. The ρ<sup>0</sup> cells were selected from wild-type cells by incubation with rotenone (1 μg/ml) and antimycin A (1 μg/ml) for 2 hr. The absence of mitochondrial DNA was confirmed by PCR analysis. Total DNA (genomic and mitochondrial) was isolated from wild type and ρ<sup>0</sup> pulmonary arterial smooth muscle cells. This DNA was then used as a template to generate a PCR a product spanning bp 7094-7580 of cytochrome oxidase subunit II, a mitochondrial DNA-encoded gene, using GCTCCCTAGTACTTTATATTA as the 5’ primer and GAGCATTGGCCATAGAATAGA as the 3’ primer. Equal amounts of wild type and ρ<sup>0</sup> total DNA were subjected to PCR for 10, 20 and 30 cycles. The PCR products then were run on an 1% agarose gel and stained with ethidium bromide. In wild type cells with intact mitochondrial DNA, an increasing quantity of PCR product was observed at 10-30
cycles (Supplementary Figure 2). In \( \rho^6 \) cells there was little or no PCR product due to loss of mitochondrial DNA from ethidium bromide treatment.

*HPV responses in pulmonary myocytes.* Glass cover slips containing isolated pulmonary myocytes were placed in a stainless steel flow-through chamber on an inverted microscope and studied under controlled \( O_2 \) and temperature conditions. The chamber was sealed using thin-wafer gaskets to minimize any \( O_2 \) exchange between the chamber wall and perfusate. A water-jacketed glass equilibration column (37\(^\circ\) C) mounted above the microscope stage was used to equilibrate the perfusate to known \( O_2 \) tensions (\( PO_2 \)). The perfusate consisted of M199 media without serum. The gas used to control the \( PO_2 \) and \( PCO_2 \) of the perfusate was supplied by a precision mass flow controller. In previous studies, the \( PO_2 \) in the chamber was confirmed under conditions identical to those of the experiments using an optical phosphorescence quenching method (Oxyspot, Medical Systems Inc.).

*Myocyte contraction.* Contraction of pulmonary myocytes was assessed by measuring changes in cell length as described previously \(^6\). Myocytes on cover slips in the flow-through chamber were visualized using Hoffman Modulation phase contrast optics. Lengths of individual myocytes were measured from computer images (Supplementary Figure 3). Myocyte contraction at 30 min was expressed as percent decrease from the original length at \( t = 0 \), \( \{ [(\text{original length}) - (\text{length at 30 min})] / (\text{original length}) \} \times 100 \).

*Measurement of ROS.* ROS generation in pulmonary myocytes was assessed using the probe 2',7'-dichlorofluorescin diacetate (DCFH-DA, 5 \( \mu \text{M} \), Molecular Probes), which was continuously present in the perfusate. The diacetate form enters the cells where esterases cleave the acetate group, tending to trap the nonfluorescent 2',7'-
dichlorofluorescin (DCFH) intracellularly. In the presence of H₂O₂, this probe is oxidized to 2′,7′-dichlorofluorescein (DCF), which was quantified using fluorescence imaging (excitation: 488 nm, emission: 535 nm) in a system equipped with a 12 bit digital camera. Intensity values are reported as percent of initial values, after subtracting background (Metamorph, Universal Imaging, Inc.).

In steady state, increases in fluorescence occur when the rate of intracellular oxidation of the dye is accelerated. Chemical reduction of DCF to DCFH does not occur in cells, so decreases in fluorescence reflect lowered rate of oxidation of the dye, combined with a leakage of the oxidized dye from the cell. To confirm that the decrease in DCF fluorescence seen upon reoxygenation in Fig. 5 was not due to a loss of plasma membrane integrity, additional studies were carried out using Sytox Green dye (100 nM) to detect changes in membrane permeability. PA myocytes were studied during normoxia (15% O₂), during 30 min hypoxia (2% O₂), after return to normoxia, and after the plasma membrane was disrupted using digitonin (300 μM). No increase in nuclear staining was observed during hypoxia or after reoxygenation, but all cells demonstrated nuclear staining with Sytox Green after digitonin (data not shown). These findings suggest that loss of membrane integrity cannot explain the fall in intracellular DCF fluorescence observed at reoxygenation.

To confirm that the DCFH dye was sensitive to oxidation by H₂O₂, additional studies were carried out in PA myocytes superfused with normoxic (16% O₂) media in the presence of DCFH-DA (5 μM). After a steady level of intracellular fluorescence was reached, H₂O₂ was added to the media and the intracellular fluorescence increased significantly and progressively (data not shown). When the H₂O₂ – containing media was
replaced with fresh media containing DCFH without H₂O₂, intracellular fluorescence decreased toward baseline values. These findings confirm that DCFH oxidation is sensitive to H₂O₂, and that oxidized DCF can leak out of viable cells and explain the decrease in fluorescence seen at reoxygenation.

To confirm that the loss of DCF fluorescence upon reoxygenation was not due to cell death subsequent to the opening of the mitochondrial permeability transition pore, additional studies were carried out using tetraethylrhodamine methyl ester (TMRE) to assess mitochondrial membrane potential (ΔΨm). This cationic dye equilibrates across the mitochondrial inner membrane in accordance with the ΔΨm, and changes in fluorescence at these non-quenching concentrations can be used to detect changes in mitochondrial polarization. PA myocytes on collagen-coated cover slips were loaded with TMRE (100 nM) for 45 min at 37°C. The cells were then placed in a flow-through chamber on an inverted microscope and perfused with M199 media without serum containing TMRE (10 nM). Fluorescence images were obtained under baseline normoxic conditions (16% O₂, 5% CO₂) until a stable baseline was established. The PO₂ of the media was then lowered for 30 min (1% O₂, 5% CO₂), after which it was restored to normoxia for 30 min. Finally, to confirm sensitivity to changes in ΔΨm, the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, 10 μM) was added to dissipate the proton gradient across the inner membrane. Hypoxia did not induce a significant change in ΔΨm, nor did ΔΨm change after return to normoxia. However, a rapid, significant decrease in fluorescence was observed upon addition of FCCP (Fig. 4). These findings indicate that mitochondrial permeability transition pore opening did not occur during hypoxia or after return to normoxia.
Statistics. Isolated lung HPV responses were analyzed using a paired t-Test to evaluate significant differences between changes in the PA pressure before and after the administration of the experimental agents. Transient changes in PA pressure during normoxia after the administration of the experimental agents was analyzed using a t-Test comparing the changes in PA pressure with baseline or zero. A t-Test with Bonferroni correction was used to evaluate significant differences between hypoxia-induced PA myocyte contractions. Statistical significance was set at P<0.05.
Table 1. Effects of pharmacological agents on U46619 vasoconstriction response in intact lungs.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Δ PA Pressure Before (cm H₂O)</th>
<th>Δ PA Pressure After (cm H₂O)</th>
<th>Percent Change (After/Before)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (&lt;0.1 % DMSO)</td>
<td>20.7 ± 6.6</td>
<td>23.6 ± 3.4</td>
<td>137.7 ± 41.7 %</td>
</tr>
<tr>
<td>DPI (10 μM)</td>
<td>19.3 ± 2.3</td>
<td>45.3 ± 1.5</td>
<td>243.6 ± 38.7 %*</td>
</tr>
<tr>
<td>Rotenone (5 μg/ml)</td>
<td>31.8 ± 12.2</td>
<td>5.5 ± 0.8</td>
<td>38.2 ± 27.5 %</td>
</tr>
<tr>
<td>Rotenone (50 ng/ml)</td>
<td>15.7 ± 1.1</td>
<td>42.8 ± 2.7</td>
<td>273.0 ± 1.5 %*</td>
</tr>
<tr>
<td>Myxothiazol (50 ng/ml)</td>
<td>18.7 ± 5.4</td>
<td>34.7 ± 7.9</td>
<td>210.1 ± 49.3 %</td>
</tr>
<tr>
<td>Antimycin A (1 ng/ml)</td>
<td>15.0 ± 3.6</td>
<td>20.3 ± 3.2</td>
<td>145.0 ± 21.5 %*</td>
</tr>
<tr>
<td>Cyanide (10 μM)</td>
<td>7.7 ± 1.7</td>
<td>19.3 ± 6.6</td>
<td>281.8 ± 126.0 %</td>
</tr>
<tr>
<td>PDTC (10 μM)</td>
<td>8.5 ± 0.3</td>
<td>12.2 ± 0.2</td>
<td>143.5 ± 5.1 %*</td>
</tr>
<tr>
<td>Ebselen (50 μM)</td>
<td>17.0 ± 6.8</td>
<td>20.5 ± 7.4</td>
<td>123.5 ± 6.6 %</td>
</tr>
<tr>
<td>DDC (1 μM)</td>
<td>29.3 ± 4.6</td>
<td>32.0 ± 5.8</td>
<td>108.3 ± 2.7 %</td>
</tr>
<tr>
<td>DIDS (200 μM)</td>
<td>8.8 ± 1.7</td>
<td>12.3 ± 1.5</td>
<td>144.0 ± 11.7 %*</td>
</tr>
</tbody>
</table>

The U46619-induced change (Δ) in PA pressure before and after treatment with the pharmacological agents was measured in isolated, buffer perfused lungs. Percent change reflects the effect that the pharmacological agents had on the U46619 vasoconstriction response. Values are means ± SE; n=3 lungs. *P<0.05 change in PA pressure comparing Before and After (T-test for dependent variables).
References


Supplementary Figure Legends

**Figure 1.** Photomicrographs of isolated PA myocytes cells stained for smooth muscle cell α-actin. Similarly treated A549 cells are included for comparison.

**Figure 2.** The absence of mitochondrial DNA confirmed by PCR.

**Figure 3.** Bright field photomicrographs of isolated PA myocyte contraction. Myocytes were superfused with either Normoxic or Hypoxic media during observation. Arrows show fixed points of reference to indicate cell shortening.

**Figure 4.** TMRE fluorescence used to assess mitochondrial transmembrane potential in a representative experiment using PA myocytes. Cells were loaded with TMRE (100 nM) for 45 min, then superfused with 10 nM TMRE in media under controlled O₂ conditions in a flow-through chamber on an inverted microscope. Neither hypoxia nor return to normoxia were associated with significant changes in membrane potential, compared with the depolarization induced with FCCP (10 µM).
Figure 1 online
Figure 2 Online

<table>
<thead>
<tr>
<th></th>
<th>10 cycles</th>
<th>20 cycles</th>
<th>30 cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>ρ⁰</td>
<td>wt</td>
<td>ρ⁰</td>
</tr>
</tbody>
</table>

603 b.p. [Image]

310 b.p. [Image]