Mitochondrial Reactive Oxygen Species Trigger Calcium Increases During Hypoxia in Pulmonary Arterial Myocytes

Gregory B. Waypa, Jeremy D. Marks, Mathew M. Mack, Chan Boriboun, Paul T. Mungai, Paul T. Schumacker

Abstract—We hypothesized that mitochondria function as the O₂ sensors underlying hypoxic pulmonary vasoconstriction by releasing reactive oxygen species (ROS) from complex III of the electron transport chain (ETC). We have previously found that antioxidants or inhibition of the proximal region of the ETC attenuates hypoxic pulmonary vasoconstriction in rat lungs and blocks hypoxia-induced contraction of isolated pulmonary arterial (PA) myocytes. To determine whether the hypoxia-induced increases in mitochondrial ROS act to trigger calcium increases, we measured changes in cytosolic calcium ([Ca²⁺]ᵢ) using fura 2-AM (fluorescence at 340/380 nm) during perfusion with hypoxic media (PO₂ 12 mm Hg). Hypoxia caused an increase in fura 2 fluorescence, indicating an increase in [Ca²⁺]ᵢ. In superfused PA myocytes, diphenyleneiodonium, rotenone, and myxothiazol, which inhibit the proximal region of the ETC, attenuated hypoxia-induced calcium increases. Antimycin A and cyanide, which inhibit the distal region of the ETC, failed to abolish hypoxia-induced [Ca²⁺]ᵢ increases. To test whether mitochondrial H₂O₂ is required to trigger [Ca²⁺]ᵢ increases, catalase was overexpressed in PA myocytes with the use of a recombinant adenovirus. Catalase overexpression attenuated hypoxia-induced increases in [Ca²⁺]ᵢ, suggesting that H₂O₂ acts upstream from calcium increases during hypoxia. These results support the conclusion that mitochondria function as O₂ sensors during hypoxia and demonstrate that ROS generated in the proximal region of the ETC act as second messengers to trigger calcium increases in PA myocytes during acute hypoxia. (Circ Res. 2002;91:719-726.)

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 as do rings of the pulmonary artery (PA),⁷,⁸ even when they are denuded of endothelium.⁹,¹⁰ Even isolated PA myocytes contract during hypoxia,¹¹ indicating that an O₂ sensor is intrinsic to those cells.

Although HPV has been well characterized, the underlying mechanism of O₂ sensing is not established. Mitochondria have long been known to generate reactive oxygen species (ROS),¹² although these oxidants have classically been viewed as toxic byproducts of the electron transport pathway, possibly contributing to the effects of aging.¹³ More recently, mitochondrial ROS have been implicated as intracellular signaling agents. We previously reported that mitochondria increase ROS generation during hypoxia and that site-specific inhibition of electron transport could attenuate hypoxia-induced ROS generation.¹⁴,¹⁵ In isolated buffered-perfused rat lungs and isolated PA myocytes, we found that inhibition of the mitochondrial electron transport chain (ETC) upstream from complex III attenuated HPV.¹⁶ By contrast, inhibition downstream from complex III either had no effect or augmented HPV.¹⁶ The likely explanation for these findings is that O₂⁻-dependent ROS production occurs within complex III. In the Q cycle, a free radical (ubisemiquinone) is normally generated during the electron transport process. This radical can potentially donate its unpaired electron to O₂, thereby generating superoxide. Our model suggests that the process of ROS generation from that site is amplified during hypoxia.

In support of this model, we found that antioxidants selectively abolish the HPV response in isolated lungs.¹⁶ However, that study did not test whether the calcium increases during hypoxia required ROS production. Accordingly, the present study sought to determine whether oxidant production from the mitochondria is responsible for triggering calcium increases and therefore myocyte contraction during HPV. This hypothesis was tested by measuring the effects of site-specific mitochondrial inhibitors and antioxidants on calcium signaling in primary cultured PA myocytes under hypoxic conditions.

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Materials and Methods

Pharmacological Agents

Fura 2-AM was obtained from Molecular Probes; diphenyleneiodonium (DPI) was obtained from CalBiochem; and angiotensin II, rotenone, myxothiazol, antimycin A, and cyanide were obtained from Sigma Chemical Co. The pharmacological agents were dissolved in dimethyl sulfoxide (100%) as a 1000× stock solution so that when it was added to the media, the dimethyl sulfoxide concentration was 0.1%. Stock solutions were stored at −20°C and thawed on the day of the experiment. Unless otherwise noted, pharmacological agents were incubated with the cells for 10 minutes before the start of hypoxia or the administration of angiotensin II, H$_2$O$_2$, or cyanide.

Pulmonary Microvessel Myocyte Isolation

Myocytes were isolated using a modification of the method of Marshall et al. As described previously, freshly excised rat heart and lungs were rinsed with PBS containing penicillin and streptomycin (1%). The right ventricle was cannulated, and the pulmonary vascularity was perfused (30 mL/min) with the PA cannula, growth medium 199 (M199, 30 mL containing HEPES (25 mmol/L) along with 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, the arteries were filled with the agarose and iron particles. The airways were filled via the trachea with M199 (15 mL) containing low-melting-point agarose (1%) without iron. The lungs were placed in cold PBS to cause the agarose to gel. After 10 minutes, the lobes were dissected free and finely minced in a Petri dish. Lung fragments were resuspended and washed (3 times) with PBS by use of a magnet to retain the iron-containing fragments. The iron-containing pieces were resuspended in M199 (25 mL) containing collagenase (80 U/mL) and incubated at 37°C for 30 to 60 minutes. To remove extravascular tissue, fragments were first drawn through a 15-gauge needle and subsequently through an 18-gauge needle. The iron-containing fragments then were washed (3 times) with M199 containing FBS (20%) and drained. The resulting fragments were placed in a Petri dish containing collagen-coated (0.01%) glass coverslips and resuspended in M199 containing FBS (10%). The Petri dishes were incubated at 37°C with CO$_2$ (5%) in air for 4 or 5 days, during which time the myocytes were observed to migrate and adhere to the coverslips. After 4 or 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 50% confluent. Cells were confirmed to be PA myocytes, as previously described.

Cytosolic Calcium Measurements

PA myocytes on coverslips were incubated at 37°C with fura 2-AM (5 μmol/L) for 1 hour and then incubated for an additional 15 minutes in medium without the dye to ensure that all of the intracellular dye had the ester moieties cleaved off. Coverslips were then placed in a low-volume glass-covered recording chamber (Warner) on the microscope and perfused (1 to 2 mL/min) with M199 bubbled with 21% O$_2$ /5% CO$_2$ /74% N$_2$ (normoxia). Cells were resuspended in M199 (25 mL) containing collagenase (80 U/mL) and incubated at 37°C for 30 to 60 minutes. To remove the iron-containing fragments then were washed (3 times) with M199 containing FBS (20%) and drained. The resultant fragments were placed in a Petri dish containing collagen-coated (0.01%) glass coverslips and resuspended in M199 containing FBS (10%). The Petri dishes were incubated at 37°C with CO$_2$ (5%) in air for 4 or 5 days, during which time the myocytes were observed to migrate and adhere to the coverslips. After 4 or 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 50% confluent. Cells were confirmed to be PA myocytes, as previously described.

Statistical Analysis

Changes in [Ca$^{2+}$]$_i$ were analyzed using a t test to evaluate significant differences between changes in hypoxia-induced fura 2 ratio in the absence and presence of the experimental agents. 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 $P<0.05$.20

Adenovirus Production

Recombinant adenoviruses containing either LacZ or catalase were obtained from Dr Jingxiang Bai (Mount Sinai School of Medicine, New York, NY). For adenovirus production, small aliquots of adenovirus were administered to low-passage 293 cells. Virus production was determined to have taken place when the cells displayed a cytopathic effect (CPE), ie, when the cells round up and begin to detach. When ~90% of the cells had detached because of CPE, all cells were harvested in their media and were then lysed by five rounds of freeze-thaw to release virus. Cell debris was spun down, and the supernatant was used to infect one 150-mm plate of 293 cells. When CPE was observed in these cells, the harvesting process was repeated, and the resulting supernatant was used to infect 20 to thirty 150-mm plates. Cells from this large-scale preparation were harvested, and the virus-containing supernatant was collected as described above in 10 mL complete medium. Infectious adenovirus was purified by two rounds of cesium chloride gradient ultracentrifugation, followed by dialysis against 10 mmol/L Tris pH 7.4, 1 mmol/L MgCl$_2$, and 10% glycerol. Total viral yield was determined by absorbance at 280, and infectious viral titer was determined by plaque assay. PA myocytes were infected with two plaque-forming units per cell in complete medium (M199 with FBS) for 18 hours and then changed to complete medium without virus. Experiments were performed 36 to 48 hours after the initial infection. Experiments were also performed as a blind study, in that the experimenter had no knowledge of which virus was used to treat the cells.

H$_2$O$_2$ Degradation in PA Myocytes

Overexpressing Catalase

To determine whether the catalase overexpressed in PA myocytes was functional, the rate of exogenous H$_2$O$_2$ degradation was determined using a xylene orange assay. Cells on coverslips were incubated in medium (2 mL) to which a bolus of H$_2$O$_2$ was added (700 μmol/L). The medium was sampled at 15-minute intervals for the measurement of H$_2$O$_2$. Samples (900 μL) of medium were incubated at 27°C for 45 minutes with 100 μL of a 10× xylene orange stock solution [10 μmol/L xylene orange, 2.5 μmol/L Fe(NH$_3$)$_2$(SO$_4$)$_2$ · 6H$_2$O, and 25 mmol/L H$_2$SO$_4$ (Sigma), after which absorbance was read at 590 nm, and the concentration of H$_2$O$_2$ was determined from a standard curve (0.1, 0.5, 1, 5, 10, 50, 100, 500, and 1000 μmol/L H$_2$O$_2$). The H$_2$O$_2$ concentration was determined by spectrophotometric analysis at 240 nm using an extinction coefficient of 43.6 (mol/L)$^{-1}$·cm$^{-1}$.

Statistical Analysis

Changes in [Ca$^{2+}$]$_i$ were analyzed using a t test to evaluate significant differences between changes in hypoxia-induced fura 2 ratio in the absence and presence of the experimental agents. 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 $P<0.05$.20
Results

Hypoxia Increases [Ca\textsuperscript{2+}] in PA Myocytes

Perfusion of the cells with medium containing angiotensin II (5 \mu mol/L) caused an increase in [Ca\textsuperscript{2+}], which returned to baseline levels within 10 minutes (data not shown). Angiotensin II was used to confirm the ability of these cells to increase [Ca\textsuperscript{2+}], in response to a vasoconstrictor and to prime the cells to respond to hypoxia. Figure 1A depicts a photomicrograph of fura 2 fluorescence (ratio of F340/F380) in isolated cultured PA myocytes during perfusion with normoxic medium (Po\textsubscript{2} 140 mm Hg). Perfusion with hypoxic medium (P O\textsubscript{2} 12 mm Hg) for 2 minutes resulted in an increase in fura 2 fluorescence compatible with an increase in [Ca\textsuperscript{2+}], (Figure 1B). Perfusion of isolated cultured PA myocytes with hypoxic medium caused an increase in [Ca\textsuperscript{2+}] from a baseline level of 165±8 to 290±29 nmol/L (Figure 1, bottom). This increase in [Ca\textsuperscript{2+}], was stable over at least 9 minutes of hypoxia. These results confirm the suitability of this system to measure hypoxia-induced increases in [Ca\textsuperscript{2+}], in isolated cultured PA myocytes.

Inhibition of Mitochondrial Electron Transport Upstream From Complex III Decreases the Hypoxic Response

To determine the requirement for electron transport upstream from complex III in hypoxia-induced calcium increases, the flavoprotein inhibitor DPI was added to the medium perfusing the PA myocytes. To determine whether our experimental drugs had nonspecific effects on [Ca\textsuperscript{2+}], we performed additional experiments measuring changes in [Ca\textsuperscript{2+}], in response to the vasoconstrictor angiotensin II and the ROS H\textsubscript{2}O\textsubscript{2}. DPI at 10 \mu mol/L, a dose previously shown to inhibit HPV,\textsuperscript{16} had no effect on an angiotensin II (5 \mu mol/L)– or an H\textsubscript{2}O\textsubscript{2} (50 \mu mol/L)–induced increase in [Ca\textsuperscript{2+}], (Figure 2A). However, DPI significantly decreased hypoxia-induced fura 2 fluorescence (0.02±0.02, peak ratio–baseline ratio) compared with control (0.20±0.03).

To clarify the role of the proximal region of the ETC, the complex I inhibitor rotenone was added to the medium during the incubation period with fura 2-AM. Rotenone at 5 \mu g/mL, a dose shown to irreversibly inhibit mitochondrial O\textsubscript{2} consumption,\textsuperscript{16} significantly decreased hypoxia-induced fura 2 fluorescence (0.06±0.01) compared with control (0.13±0.02) (Figure 2B) without affecting the response to angiotensin II or H\textsubscript{2}O\textsubscript{2}.

Myxothiazol inhibits complex III by blocking electron transfer to the Rieske iron-sulfur center in the bc\textsubscript{1} complex.\textsuperscript{21} Myxothiazol at 5 \mu g/mL, a dose shown to inhibit mitochondrial O\textsubscript{2} consumption and HPV,\textsuperscript{16} had no effect on the response to angiotensin II or H\textsubscript{2}O\textsubscript{2} (Figure 2C). However, myxothiazol significantly decreased hypoxia-induced fura 2 fluorescence (0.03±0.03) compared with control (0.20±0.03).

Inhibition of Mitochondrial Electron Transport Downstream From Complex III Does Not Alter the Hypoxic Response

Antimycin A inhibits the oxidation of cytochrome b\textsubscript{562} at complex III downstream from ubisemiquinone.\textsuperscript{21} Antimycin A at 1 \mu g/mL, a dose shown to inhibit mitochondrial O\textsubscript{2} consumption,\textsuperscript{16} had no effect on hypoxia-induced fura 2 fluorescence (0.17±0.01) compared with control (0.14±0.01) (Figure 3). In addition, antimycin A had no effect on the response to angiotensin II or H\textsubscript{2}O\textsubscript{2} (data not shown). Cyanide inhibits complex IV of the mitochondria. Cyanide at 10 \mu mol/L, a dose shown to inhibit mitochondrial O\textsubscript{2} consumption,\textsuperscript{16} had no effect on hypoxia-induced fura 2 fluorescence (0.10±0.01) compared with control (0.10±0.01) (Figure 3). Furthermore, cyanide had no effect on the angiotensin II or H\textsubscript{2}O\textsubscript{2} response (data not shown).
Increased ROS Mediate the Calcium Signal During Hypoxia

Having determined that the calcium response depends on a mitochondrial site downstream from complex I but upstream from cytochrome b562, we sought to determine whether increases in H₂O₂ are required for calcium increases during hypoxia. Catalase was overexpressed in PA myocytes by infection with a recombinant adenovirus 36 to 48 hours before the study. Expression levels were verified by Western blot analysis (Figure 4, inset). Control cells were infected with an identical virus containing the LacZ gene instead of the catalase insert. Compared with wild-type PA myocytes or PA myocytes that were infected with an adenovirus containing LacZ, PA myocytes infected with the catalase adenovirus displayed an increase in catalase expression. Inspection of cells expressing β-galactosidase indicated virtually 100% expression efficiency of the transgene (data not shown). To verify that catalase overexpression amplified H₂O₂ degradation, PA myocytes were incubated with exogenous H₂O₂ (700 μmol/L), and the concentration of H₂O₂ in the media bathing the cells was measured as a function of time (Figure 4). H₂O₂ was stable in medium (without cells), whereas wild-type and LacZ-transfected cells slowly degraded the H₂O₂ via their endogenous antioxidant machinery. PA myocytes overexpressing catalase degraded H₂O₂ significantly faster than did the wild-type or LacZ-transfected cells. Thus,
the adenovirus system resulted in the expression of the transgene with high efficiency at a level that could be adjusted by varying the titer of viral concentration. Catalase overexpression in cells had no effect on angiotensin II–induced fura 2 fluorescence (Figure 5) compared with wild-type and LacZ transfected cells. However, it did decrease H2O2-induced fura 2 fluorescence (0.10±0.05) compared with wild-type (0.27±0.07) and LacZ-transfected (0.29±0.10) cells. Catalase overexpression in cells also decreased hypoxia-induced increases in fura 2 fluorescence (0.06±0.004) compared with wild-type (0.11±0.02) and LacZ-expressing (0.10±0.01) cells.

Cyanide Mimics Hypoxia-Induced Calcium Increases
Cyanide has been shown to mimic HPV through normoxia by an as-yet-unknown mechanism. To determine whether cyanide mimics HPV by increasing ROS generation at complex III, we measured cyanide-induced [Ca2+]i during normoxia in PA myocytes (Figure 6). Cyanide at 10 μmol/L increased fura 2 fluorescence during normoxia (0.14±0.04). To determine whether inhibition of the mitochondrial ETC upstream from ubisemiquinone could block this response, cells were preincubated with myxothiazol. Myxothiazol (5 μg/mL) significantly attenuated cyanide-induced fura 2 fluorescence (0.04±0.02), suggesting that mitochondrial electron transport upstream from complex III is required for cyanide-induced [Ca2+]i increases. To determine whether increased H2O2 is required for cyanide-induced [Ca2+]i increases, PA myocytes overexpressing catalase were challenged with cyanide during normoxia. PA myocytes overexpressing catalase significantly decreased cyanide-induced fura 2 fluorescence increases (0.02±0.02), whereas PA myocytes expressing LacZ responded to cyanide in a manner similar to that in control cells (0.10±0.04). Thus, H2O2 generation is required for calcium increases in response to cyanide.

Discussion
Mitochondria Mediate Calcium Increases During Hypoxia
To determine whether mitochondria function as the O2 sensors mediating calcium increases during hypoxia, we measured hypoxia-induced fura 2 fluorescence in isolated PA myocytes. In cells loaded with fura 2, hypoxia induced an increase in [Ca2+]i, from a baseline of 165±8 to 290±28 nmol/L. This finding is in agreement with previously published results. Inhibitors of the proximal region of the mitochondrial ETC, including DPI, rotenone, and myxothiazol, attenuated the increase in calcium in response to hypoxia without affecting the response to angiotensin II or H2O2. By contrast, distal inhibitors, including antimycin A and cyanide, had no effect on hypoxia-induced calcium signaling. We conclude that 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 decrease the hypoxia-induced increase in fura 2 fluorescence.

These results are consistent with our previously reported observations in isolated buffer-perfused rat lungs. Recently, Leach et al reported a series of compatible observations in isolated PAs. They found that rotenone and myxothiazol...
Mitochondrial ROS-Induced Calcium Increases

Although H2O2 appears to act as a signaling messenger in the sequence leading to hypoxia-induced calcium increases, the details of that pathway are not fully understood. It has been suggested that hypoxia-induced calcium increases may involve 1 or more 4-aminopyridine (4-AP)-sensitive Kv channels. Inhibition of these channels during hypoxia would promote membrane depolarization, the opening of voltage-gated L-type calcium channels, and the subsequent intracellular calcium increases. However, other studies have questioned the requirement for Kv channels for this response. For example, HPV has been found to remain intact despite the inhibition of Kv channels with 4-AP. Furthermore, HPV was significantly blunted, but not completely abolished, in SWAP mice, which lack the Kv1.5 channel. This suggests that Kv channels may function to augment the HPV response rather than to trigger it directly.

An alternative explanation is that hypoxia causes the release of calcium from intracellular stores, a step that appears to be required for HPV. Release of intracellular stores could then cause inhibition of 4-AP-sensitive Kv channels, leading membrane depolarization. Such a mechanism would be consistent with a model of hypoxia-induced calcium increases in which hypoxia triggers the release of calcium from internal stores, resulting in Kv channel inhibition, membrane depolarization, the opening of voltage-gated L-type calcium channels, and subsequent contraction.

Possible sources of intracellular calcium during hypoxia include mitochondria, inositol 1,4,5-triphosphate (IP3)-sensitive sarcoplasmic reticulum (SR) stores, and ryanodine-sensitive SR stores. Oxidative stress has been shown to
promote mitochondrial depolarization, resulting in calcium efflux. However, our previous studies have demonstrated that these levels of hypoxia do not induce mitochondrial depolarization, which suggests that mitochondria are not the source of calcium in our experiments.\textsuperscript{16} Gelband and Gelband\textsuperscript{9} and Robertson et al\textsuperscript{35} found that hypoxic contraction of isolated PAs was inhibited by thapsigargin, which suggests that hypoxia triggers the release of calcium from IP$_{3}$-sensitive SR stores. Along these lines, exogenous H$_{2}$O$_{2}$ has been shown to activate phospholipase C in a time- and concentration-dependent manner.\textsuperscript{36} Therefore, it is possible that mitochondrial ROS could trigger intracellular calcium release by activating phospholipase C, which in turn would metabolize phosphatidylinositol 4,5-bisphosphate to IP$_{3}$, and diacylglycerol. IP$_{3}$ could then trigger the release of calcium from IP$_{3}$-sensitive intracellular stores. However, studies demonstrating this mechanism have not been published. Hypoxia does appear to trigger the release of calcium from ryanodine-sensitive SR stores.\textsuperscript{9,22,33,37–40} Recently, Wilson et al\textsuperscript{40} reported that hypoxia increases cyclic ADP-ribose accumulation in isolated rabbit PA and calcium release from ryanodine-sensitive SR stores. Richter et al\textsuperscript{41} reported that H$_{2}$O$_{2}$ could cause oxidation of mitochondrial pyridine nucleotides, resulting in increased ADP-ribose production, monoadenylylation of mitochondrial proteins, and calcium release. By these mechanisms, mitochondrial ROS could trigger calcium release from IP$_{3}$- and/or ryanodine-sensitive SR stores. The resulting increase in intracellular calcium could inhibit K$_{v}$ channels, and the increase in calcium resulting from membrane depolarization could trigger calmodulin-mediated activation of myosin light chain kinase, actin-myosin interaction, and contraction.

In the present study, we focused exclusively on the PA myocyte response to acute hypoxia. However, more prolonged hypoxia can result in the recruitment of additional components of HPV, including the endothelium, which can modulate HPV by releasing vasoconstricting substances that increase myofilament calcium sensitivity, or NO, which promotes relaxation. Recently, Leach et al\textsuperscript{23} demonstrated that glucose and glycolysis are required for the development of sustained HPV by supporting a hypoxia-induced endothelium-dependent calcium sensitization pathway. This suggests that an increase in [Ca$^{2+}$], is not the only requirement for the HPV response. Future studies are required to determine the mechanism of O$_{2}$ sensing underlying these endothelial responses to hypoxia during HPV.

In summary, our results demonstrate that hypoxia-induced calcium increases require mitochondrial electron transport proximal to the ubiquinone site but do not require the entire ETC to be functional. ROS generated by mitochondria appear to function as second messengers during hypoxia and act to trigger the calcium-signaling process responsible for the contraction of PA myocytes during hypoxia.

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