A Redox-Based O₂ Sensor in Rat Pulmonary Vasculature

Stephen L. Archer, James Huang, Tim Henry, Douglas Peterson, E. Kenneth Weir

The effector mechanism of hypoxic pulmonary vasoconstriction (HPV) involves K⁺ channel inhibition with subsequent membrane depolarization. It remains uncertain how hypoxia modulates K⁺ channel activity. The similar effects of hypoxia and mitochondrial electron transport chain (ETC) inhibitors on metabolism and vascular tone suggest a common mechanism of action. ETC inhibitors and hypoxia may alter cell redox status by causing an accumulation of electron donors from the Krebs cycle and by decreasing the production of activated O₂ species (AOS) by the ETC. We hypothesized that this shift toward a more reduced redox state elicits vasoconstriction by inhibition of K⁺ channels. Pulmonary artery pressure and AOS, measured simultaneously using enhanced chemiluminescence, were studied in isolated perfused rat lungs during exposure to hypoxia, proximal ETC inhibitors (rotenone and antimycin A), and a distal ETC inhibitor (cyanide). Patch-clamp measurements of whole-cell K⁺ currents were made on freshly isolated rat pulmonary vascular smooth muscle cells during exposure to hypoxia and ETC inhibitors. Hypoxia, rotenone, and antimycin A decreased lung chemiluminescence (−62±12, −46±7, and −148±36 counts/0.1 s, respectively) and subsequently increased pulmonary artery pressure (+14±2, +13±3, and +21±3 mm Hg, respectively). These agents reversibly inhibited an outward, ATP-independent, K⁺ current in pulmonary vascular smooth muscle cells. Antimycin A and rotenone abolished subsequent HPV. In contrast, cyanide increased AOS and did not alter K⁺ currents or inhibit HPV. The initial effect of rotenone, antimycin A, and hypoxia was a change in redox status (evident as a decrease in production of AOS). This was associated with the reversible inhibition of an ATP-independent K⁺ channel and vasoconstriction. These findings are consistent with the existence of a redox-based O₂ sensor in the pulmonary vasculature. (Circ Res. 1993;73:1100-1112.)

KEY WORDS • cyanide • rotenone • antimycin A • K⁺ channels • chemiluminescence • hypoxic pulmonary vasoconstriction • redox • oxygen radicals • pyruvate • electron transport chain

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the passage of electrons from the Krebs cycle to the ETC should result in a direct accumulation of reduced high-energy electron donors, such as NADH. Second, lung levels of superoxide anion (O$_2^-$) and H$_2$O$_2$ (generated from O$_2^+$ by mitochondrial superoxide dismutase [SOD]) would fall, also favoring a shift in cytosolic redox balance to the reduced state.

How would modulation of a redox "O$_2$ sensor" cause membrane depolarization? We hypothesized that the redox changes caused by ETC inhibitors and hypoxia modulate K$^+$ channel activity. There is evidence for redox regulation of K$^+$ channel activity in several nonvascular systems. Recent data indicate that changes in cytosolic redox status (specifically the ratio of GSH to GSSG) modulate K$^+$ channel activity in pulmonary vascular smooth muscle cells. GSH decreases, whereas GSSG increases, the open probability of certain K$^+$ channels, including the Ca$^{2+}$-gated K$^+$ channel.

It is difficult to definitively identify a specific radical or peroxide by chemiluminescence in vivo, especially since dismutation of O$_2^-$ produced by the ETC accounts for most of the mitochondrial peroxide production. Consequently, we refer to a group of unstable O$_2$ metabolites (O$_2^-$, H$_2$O$_2$, and the hydroxyl radical) as AOS.

It is uncertain whether the changes in net cytosolic redox state associated with hypoxia and ETC inhibitors result from diminished AOS production, the accumulation of the reduced form of high-energy electron donors that are prevented from passing electrons to the cytochromes, or a combination of these effects. Changes in AOS production by the ETC and alterations in the redox status of sulfhydryl groups (eg, glutathione) and nucleotides (eg, NADH/NAD$^+$) may occur in parallel, although many unforeseen factors could alter these parameters separately. A schematic representation of the interaction between the AOS production by the ETC and net cytosolic redox status is provided in Fig 1.

Changes in pulmonary artery pressure and production of AOS caused by alveolar hypoxia, proximal ETC inhibitors (rotenone and antimycin A), and a distal ETC inhibitor (cyanide) were assessed in isolated Krebs-perfused rat lungs. The effects of hypoxia and ETC inhibitors on whole-cell K$^+$ currents were measured in freshly dispersed rat pulmonary artery smooth muscle cells using the whole-cell patch-clamp technique. The findings of the present study are consistent with the existence of a redox-based O$_2$ sensor, which varies tone by modulating K$^+$ channel activity in pulmonary vascular smooth muscle cells.

**Materials and Methods**

**Isolated Perfused Rat Lung**

Specific pathogen-free, adult, male Sprague-Dawley rats (Harlan, Madison, Wis) were anesthetized with Nembutal, as previously described. The lungs were suspended in a heated humidified chamber and perfused using a dual-lumen pulmonary artery catheter, which allowed simultaneous perfusion and pressure measurement. The perfusate was a Krebs/4% albumin solution containing 5 µg/mL of the cyclooxygenase inhibitor meclofenamate. Changes in perfusion pressure represent changes in pulmonary vascular resistance since flow was held constant (0.04 mL/kg weight per minute). The lungs were ventilated with normoxic gas (20% O$_2$ and 5% CO$_2$, with balance N$_2$; 60 times/min; positive end-expiratory pressure, 3 cm H$_2$O), and lung weight was monitored continuously.

Experiments consisted of four 24-minute periods. In each period, 10 minutes of normoxia was followed by bolus administration of angiotensin II (Ang II, 0.15 µg). Pressure was allowed to return to baseline over 8
minutes, and then hypoxic ventilation was initiated (2.5% O₂ for 6 minutes). The effects of the pharmacomechanical constrictor Ang II on pressure and chemiluminescence were compared with those of hypoxia and KCl, electromechanical constrictors. ETC inhibitors (rotenone and antimycin A or cyanide) were given during the normoxic portion of the third period, by which time all lungs were reactive to hypoxia (change in pulmonary artery pressure, >8 mm Hg). The doses of ETC inhibitors were chosen because they cause pulmonary vasoconstriction without producing pulmonary edema. Chemiluminescence and vascular reactivity were measured for an additional period after the ETC inhibitor, and then KCl (20 mmol) was given to evaluate the response to a depolarizing stimulus.

Measurement of Activated O₂ Species

The technique of measuring O₂ radicals in isolated lungs using enhanced chemiluminescence has been reported. Experiments were performed in the dark with the isolated organ housed in a black box within 2 mm of a foil-shielded 2-inch-diameter lucite rod. The lucite conveyed luminescence from the surface of the lung to a red-sensitive cooled photomultiplier tube (RCA C31034A) amplified at 1700 V using a high-voltage supply (EG & G, Princeton Applied Research). The signal was routed to a discriminator (EG & G, Princeton Applied Research) and displayed on a strip-chart recorder as counts per 0.1 second.

Protocol: Isolated Perfused Lungs

Inhibition of the proximal ETC (n=20). Inhibition of the proximal ETC (Fig 2) was accomplished by giving a bolus of rotenone (10⁻⁴ mol/L) or antimycin A (5×10⁻⁴ mol/L) into the pulmonary artery catheter. The effects of these drugs on pressure and production of AOS were studied in luminol- and lucigenin-treated lungs (n=5 per group).

Inhibition of the distal ETC (n=19). Inhibition of the distal ETC (Fig 2) was accomplished by giving incremental bolus doses of cyanide (1.5×10⁻⁵, 3.1×10⁻⁵, and 15.4×10⁻⁵ mol/L) into the pulmonary artery of lucigenin-treated lungs. Preliminary experiments showed that cyanide, unlike antimycin or rotenone, increased lung chemiluminescence. The mechanism of cyanide-induced AOS formation in control lungs (n=4) was evaluated by studying the effects of pretreatment with the iron chelator desferrioxamine (300 mg/40 mL, n=6) or SOD (4000 U/40 mL, n=4). To exclude the possibility that cyanide-induced chemiluminescence resulted from inhibition of lung SOD, SOD activity was measured in five more lungs. Lungs were homogenized, and the effect of the homogenate on cytochrome C reduction by superoxide anion, generated by xanthine and xanthine oxidase, was measured at 550 nm. The ability of lung homogenate to inhibit cytochrome C reduction was measured in control homogenate and after the addition of cyanide (10 to 100 μg).

Stimulation of the mitochondrial ETC (n=15). Pyruvate was given as a bolus into the pulmonary artery to determine whether a substrate for mitochondrial metabolism could increase production of AOS. The dose of pyruvate (1×10⁻³ mol/L) was chosen on the basis of preliminary experiments that found lower doses not to have a reproducible effect on chemiluminescence. Lungs received either pyruvate alone, pyruvate followed by antimycin A (1×10⁻³ mol/L), or antimycin A followed by pyruvate (n=5 per group).

Electrophysiology Experiments

Cell isolation. To compare the effects of metabolic inhibitors with those of hypoxia, whole-cell K⁺ currents were measured in isolated rat pulmonary artery smooth muscle cells. Rats were killed, and the second branch of either pulmonary artery was excised and placed in ice-cold modified Hanks' solution equilibrated with
100% O₂ containing (mmol/L) NaCl, 145; KCl, 5.0; MgCl₂, 1.0; CaCl₂, 0.05; HEPES, 10; and glucose, 10; pH 7.3. The vessel was dissected free of connective tissue, and single vascular smooth muscle cells were incubated using a modified procedure similar to that described previously. In brief, small pieces of arteries were incubated for 30 minutes at 37°C in Ca²⁺-free saline solution, after which the solution was replaced with a solution containing 50 μmol/L CaCl₂, 0.1% bovine albumin (Sigma), and 0.02% protease type XXIV (Sigma). The pieces of artery were incubated in the enzyme solution for 35 minutes at 36°C and then washed twice with 20 mL Ca²⁺-free solution at 37°C. Single cells were released by gentle trituration of the muscle pieces through a wide-bore fire-polished Pasteur pipette at room temperature (22°C). Subsequently, the cell suspension was centrifuged at 1000 rpm for 5 minutes, and the pellet was resuspended in a saline solution containing 10 μmol/L CaCl₂. Cells were stored at 4°C and patch-clamped on the same day.

Electrical recording. Whole-cell membrane currents were measured using standard patch-clamp techniques at 22°C. The voltage-clamp amplifier was an Axopatch 1D (Axon Instruments, Burlingame, Calif). The microelectrodes (outer diameter, 1.5 mm; World Precision Instruments, New Haven, Conn) were prepared on a Narishige pp-83 puller and Narishige heat forge type WF-83. The resistance of filled patch pipettes was approximately 3 MΩ. The pipette solution contained (mmol/L) KCl, 140; MgCl₂, 1.0; HEPES, 10; ATP (dipotassium salt), 5; phosphocreatine (disodium salt), 2; and EGTA, 5; pH 7.2. The bath solution contained (mmol/L) NaCl, 145; KCl, 5.4; MgCl₂, 1.0; CaCl₂, 1.5; HEPES, 10; and glucose, 10; pH 7.4. Current recordings were filtered at 2 KHz using a four-pole Bessel filter. Membrane current and potential were monitored on a digital storage oscilloscope (model v314, Hitachi, Tokyo, Japan) and were recorded at 2.5 KHz on a microcomputer (model 386SX, Zenith). Axopatch V.5.1 software and interface DMI were used in data acquisition. The series resistance was compensated (20% to 60%) using the series resistance compensation knobs of the Axopatch.

Electrophysiology protocol. In each experiment, the cell was held at −70 mV, and then stepwise changes in holding potential from −50 to +70 mV were made in 20-mV increments. Voltage steps were made at 10-second intervals with a pulse duration of 650 milliseconds. Initially, the whole-cell current was characterized by observing the effects of the K⁺ channel antagonists tetracyclammonium (TEA, 10 mmol/L) and 4-aminopyridine (4-AP, 5 mmol/L). In a separate series of experiments, cells were made hypoxic (PO₂, 48 mm Hg), and whole-cell current was observed, as previously described. Briefly, cells identified as myocytes by their characteristic elongated morphology were allowed to settle on the floor of a plastic perfusion chamber for 5 minutes. The custom-made chamber rested on a microscope stage so that the cells were readily accessible for micropuncture. The perfusion chamber has two wells or depressions (a proximal small pool for monitoring PO₂ and enhancing hypoxia and a 1-mL downstream pool for performing electrophysiological measurements). Perfusion of the cells was accomplished by gravity to minimize noise. The small well received flow from either normoxic (20% O₂ and 5% CO₂, with balance N₂; PO₂, 140 mm Hg) or hypoxic (0% O₂ and 5% CO₂, with balance N₂; PO₂, 68 mm Hg) reservoirs. PO₂ was monitored by a microelectrode (Microelectrode Inc, Londonderry, NH). During hypoxia, supplemental bubbling of the small well reduced the inflow PO₂ of the large well to 48 mm Hg.

The effects of antimycin A (1×10⁻⁵ mol/L), rotenone (1×10⁻⁷ mol/L), and cyanide (1×10⁻⁷ mol/L) (n=5 cells

### Table 1. Hypoxic Ventilation and Inhibitors of the Electron Transport Chain Reduce Luminol-Enhanced Lung Chemiluminescence and Cause Pulmonary Vasoconstriction

<table>
<thead>
<tr>
<th>Luminol</th>
<th>Hypoxia</th>
<th>Rotenone</th>
<th>Antimycin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Δ/basal CL, %</td>
<td>−62±12/268±35</td>
<td>−46±7/218±33</td>
<td>−148±36/394±67</td>
</tr>
<tr>
<td>Counts/0.1 s</td>
<td>−23%</td>
<td>−21%</td>
<td>−38%*</td>
</tr>
<tr>
<td>Time to ΔCL, s</td>
<td>14±2</td>
<td>110±10*</td>
<td>230±65*</td>
</tr>
<tr>
<td>ΔPAP, mm Hg</td>
<td>+14±2</td>
<td>+13±3</td>
<td>+21±3*</td>
</tr>
<tr>
<td>Time to δPAP, s</td>
<td>27±2</td>
<td>144±15*</td>
<td>299±69*</td>
</tr>
<tr>
<td>Lucigenin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Δ/basal CL, %</td>
<td>−4±2/130±30</td>
<td>−26±11/214±37</td>
<td>−33±5/140±41</td>
</tr>
<tr>
<td>Counts/0.1 s</td>
<td>−3%</td>
<td>−12%*</td>
<td>−24%*</td>
</tr>
<tr>
<td>δPAP, mm Hg</td>
<td>+13±4</td>
<td>+8±2</td>
<td>+9±2</td>
</tr>
</tbody>
</table>

Δ/basal CL indicates the change in chemiluminescence over the absolute chemiluminescence value that preceded the intervention. The time from the administration of the electron transport chain inhibitor or hypoxia to the onset of the change in chemiluminescence (time to ΔCL) and the earliest evidence of vasoconstriction (time to δPAP) are shown for luminol-treated lungs but were similar for lucigenin-treated lungs. Values are the mean±SEM.

*P<.05 vs values achieved with acute hypoxia.
each) on whole-cell current were measured. Current was measured at the peak and steady-state phases of the current tracings. Ten minutes after exposure, the drug was washed away, and current recovery was observed. In an additional five experiments, rotenone was given to cells after the administration of glibenclamide (1×10⁻⁵ mol/L), an inhibitor of the Kᵥ₅ channel. In these experiments, no ATP was added to the internal pipette solution. The appropriate vehicle (saline or 0.1% ethanol) was tested and had no effect on whole-cell current at these doses.

**Drugs**

Cyanide was obtained from Fischer, Pittsburgh, Pa. All other drugs were obtained from Sigma. Lucigenin (1×10⁻⁵ mol/L) was dissolved in saline, and luminol (7×10⁻⁵ mol/L) was dissolved in warm Krebs-albumin (4%) solution. Rotenone was dissolved in ethanol and then diluted in Krebs solution. Antimycin A was dissolved in HPB Moleculesol (Pharmatec Inc, Alachua, Fla). None of the solvents altered lung chemiluminescence or arterial pressure at the doses used.

**Statistics**

Values were expressed as mean±SEM. For comparison between two and three groups, Student’s t test and a factorial ANOVA were used, respectively. The Fisher least significant difference test was performed for post hoc comparisons using STATVIEW II (V4.0, Abacus Concepts). A value of P≤0.05 was considered statistically significant.

**Isolated Lungs**

Normoxic luminol chemiluminescence increased over the 72 minutes of perfusion before administering the ETC inhibitors (from 202±29 to 289±40 counts/0.1 s, P<0.05). Lucigenin-enhanced luminescence increased from 138±17 to 177±29 counts/0.1 s over the same period (P<0.05).

Hypoxia reduced luminol-enhanced chemiluminescence, and this decrease preceded the onset of HPV (Table 1, Figs 2 and 3). Lucigenin-enhanced chemiluminescence was not significantly reduced by hypoxia (Table 1, P=NS). Ang II and KCl had no effect on chemiluminescence (Fig 2). Normoxic and hypoxic perfusate O₂ tensions were 130±10 and 40±5 mm Hg, respectively.

**Rotenone and Antimycin A**

Rotenone and antimycin A (n=20) reduced lung chemiluminescence before causing vasoconstriction. The delay between the fall in chemiluminescence and rise in pressure was longer than with hypoxia (Table 1). Rotenone had a biphasic effect on luminol-enhanced chemiluminescence, causing a brief initial increase in chemiluminescence (+18±6%) followed by a larger decrease (−33±5% below baseline) (Figs 2 and 3). In contrast, antimycin A usually caused a monophasic decrease in chemiluminescence.

Antimycin A and rotenone also reduced lucigenin-enhanced chemiluminescence (−33±5 and −26±11 counts/0.1 s, respectively). As with luminol, the fall in chemiluminescence caused by antimycin A and rotenone was followed by a constriction (+9±2 and +8±2 mm Hg, respectively).

The magnitude of HPV was directly correlated with the size of the pressor response to rotenone (r=0.63, P=0.04) but not to antimycin A or cyanide. Rotenone and antimycin A completely eliminated subsequent HPV and reduced Ang II constriction (Fig 4). KCl-induced constriction was mildly reduced by antimycin A but not by rotenone (Fig 4).

Hypoxia caused a similar −33±12% decrease in chemiluminescence even after treatment with rotenone (−61±30 [before] and −66±27 [after] counts/0.1 s) (Fig 2). The fall in chemiluminescence caused by hypoxia was reduced after treatment with antimycin A (−62±12 [before] and −23±17 [after] counts/0.1 s), at least in part because basal chemiluminescence was somewhat depressed.

Lung weight was not increased by rotenone (change in weight, −0.2±0.1 g) or antimycin A (change in weight, −0.2±0.1 g). pH was not changed by rotenone (7.38±0.01 [before] and 7.38±0.02 [after]) or antimycin A (7.38±0.01 [before] and 7.36±0.02 [after]). Perfusate Po₂ was also unaffected by rotenone (124±3 [before]
and 119±3 (after) mm Hg) and antimycin A (111±5 [before] and 113±5 [after] mm Hg).

Bolus injection of pyruvate, a substrate for the electron transport chain, caused a brief increase in luminol-enhanced chemiluminescence in control lungs (+30±4%), which was reduced to 7±4% by pretreatment with antimycin A (P<.01). After pyruvate injection, administration of antimycin A lowered chemiluminescence and caused constriction to a degree that did not differ from the effects of antimycin A in the absence of pyruvate. Pyruvate did not decrease HPV or increase lung weight (−0.2±0.2 g).

Cyanide

Cyanide (n=14) caused dose-dependent pulmonary vasoconstriction (P<.05, Fig 5), which, unlike hypoxia, rotenone, and antimycin A, was preceded by a brief dose-dependent increase in chemiluminescence (Figs 2 and 5). The cyanide-induced increase in chemiluminescence was due to increased production of O₂⁻ rather than inhibition of lung SOD by cyanide. Cyanide (10 to 100 μmol/L) did not inhibit SOD activity in the isolated lung. SOD reduced both cyanide-induced increases in chemiluminescence and pulmonary artery pressure. Desferrioxamine inhibited the cyanide-induced chemiluminescence more effectively than did SOD but had less effect on cyanide-induced vasoconstriction (Table 2).

At doses of 1.5±10⁻⁵ mol/L, cyanide did not decrease subsequent hypoxic pressor responses (Fig 4), and the magnitude of cyanide-induced constriction was unrelated to the size of the hypoxic pressor response. SOD caused a small increase in normoxic perfusion pressure (+1.6±0.2 mm Hg, P<.05). In contrast to the effects of low-dose SOD on vascular tone, large doses of SOD reduced the responsiveness of cyanide-treated lungs to Ang II and hypoxia (Table 2). KCl constriction

FIG 5. Bar graph showing that cyanide causes a dose-dependent increase in normoxic chemiluminescence and pulmonary artery pressure (PAP). Values are mean±SEM of the change in normoxic PAP caused by incremental doses of cyanide. The rise in lucigenin-enhanced chemiluminescence (CL), which precedes vasoconstriction in each case, is shown on the right-sided y-axis. *P<.05 for PAP and CL values vs those caused by lower doses of cyanide.

Table 2. Effects of Superoxide Dismutase and Desferrioxamine on Cyanide-Induced Radical Formation and Vasoconstriction in the Isolated Lung

<table>
<thead>
<tr>
<th>Group</th>
<th>Cyanide Dose</th>
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<tbody>
<tr>
<td></td>
<td>1.5x10⁻⁵ mol/L</td>
<td>3.1x10⁻⁵ mol/L</td>
<td>15x10⁻⁵ mol/L</td>
<td></td>
</tr>
<tr>
<td>ΔPAP, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.1±2.0</td>
<td>10.3±1.6</td>
<td>16.4±1.8</td>
<td></td>
</tr>
<tr>
<td>SOD</td>
<td>1.9±1.0*</td>
<td>5.0±1.4*</td>
<td>5.1±1.1*</td>
<td></td>
</tr>
<tr>
<td>Desferrioxamine</td>
<td>2.8±0.8†</td>
<td>9.0±0.8</td>
<td>12.7±2.4</td>
<td></td>
</tr>
<tr>
<td>ΔCL, counts/0.1 s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>15.0±2.6</td>
<td>24.0±0.5</td>
<td>36.0±7.0</td>
<td></td>
</tr>
<tr>
<td>SOD</td>
<td>9.0±1.4†</td>
<td>9.0±1.7†</td>
<td>18.0±6.1†</td>
<td></td>
</tr>
<tr>
<td>Desferrioxamine</td>
<td>−3.0±3.0†</td>
<td>−5.0±2.0†</td>
<td>10.0±3.0†</td>
<td></td>
</tr>
</tbody>
</table>

ΔPAP and ΔCL indicate the change in mean pulmonary artery pressure and lucigenin-enhanced chemiluminescence, respectively; SOD, superoxide dismutase. Values are mean±SEM for desferrioxamine, n=6; for SOD and control, n=4 each. *P<.01 and †P<.05 vs control value.
following cyanide administration was similar whether lungs were pretreated with SOD (+13±1 mm Hg) or desferrioxamine (+14±3 mm Hg) or received no pretreatment (+15±1 mm Hg).

Cyanide did not prevent the acute decrease in chemiluminescence elicited by hypoxia. Before cyanide administration, hypoxia decreased lucigenin-enhanced chemiluminescence 9% (−5.3±0.3/58.3±5.9 counts/0.1 s); after the administration of cyanide, the percent fall in chemiluminescence increased to −18% (−6±1.7/34±2.4 counts/0.1 s). Although there was no acute decrease in chemiluminescence after the administration of cyanide, there was a gradual decline in chemiluminescence from 58±6 to 39±4 over the subsequent 96 minutes. This smooth, slow decline was unrelated to changes in normoxic basal tone.

Cyanide caused only mild alterations of pH and oxygenation in the lung: pH, 7.36±0.01 (before) and 7.34±0.02 (after); and PO₂, 122±4 (before) and 96±7 (after) mm Hg; P<.05.

Electrophysiology

Normoxic pulmonary artery smooth muscle cells displayed an outward K⁺ current that was reversibly inhibited by TEA and 4-AP (Fig 6). The initial and steady-state portions of the current were similarly inhibited. This current was also diminished by hypoxia (Fig 7). Rotenone and antimycin A reduced the outward K⁺ current in a time-dependent manner (Figs 8 and 9), with initial suppression of K⁺ currents evident within 2 to 3 minutes (Fig 9). The inhibition of the K⁺ current was reversible on removal of the ETC inhibitor (Fig 8). In contrast, cyanide had little effect on whole-cell K⁺ currents (data not shown). Glib-
enclamide had no effect on the rotenone-induced K\(^+\) current suppression (Fig 10). Addition of rotenone revealed the presence of two populations of currents. Rotenone suppressed an outward, low-noise, nonactivating component, revealing a more rapidly inactivating current (Fig 8A). Antimycin A, TEA, and 4-AP suppressed both components of the current.

Discussion

The effector mechanism of HPV involves inhibition of one or more K\(^+\) channels in the pulmonary vascular smooth muscle.\(^1,2\) Although this effector mechanism is fairly well characterized, little is known about the “sensor” that detects changes in O\(_2\) tension. ETC inhibitors were used to help us understand O\(_2\) sensing in the lung, since, like hypoxia, they cause pulmonary vasoconstriction and stimulate the carotid chemoreceptor.\(^9\) The present study demonstrates that pulmonary vasoconstriction in response to hypoxia and inhibitors of the proximal ETC is preceded by a change in lung redox status, which is manifest as a fall in production of AOS, and by inhibition of an outward K\(^+\) current in pulmonary vascular smooth muscle cells. We hypothesize that alveolar O\(_2\) tension modulates the activity of certain vascular smooth muscle K\(^+\) channels by altering vascular smooth muscle redox status, as previously suggested.\(^27\)

Analogies Between ETC Inhibitors and Hypoxia

Metabolic inhibitors have traditionally been used to evaluate the hemodynamic consequences of altering the phosphate potential (eg, the ratio of ATP to ADP plus inorganic phosphate). Lloyd\(^8\) reported that cyanide and 2,4-dinitrophenol caused pulmonary vasoconstriction. Rounds and McMurtry,\(^6\) noting the similar magnitude and time course of the pulmonary constriction in response to hypoxia and five metabolic inhibitors, proposed that depletion of a critical high-energy phosphate might account for HPV. They found that the magnitude of the pressor response to ETC inhibitors was predictive of the pressor response to hypoxia and noted that, once a lung had been exposed to an ETC inhibitor, it no longer constricted to hypoxia. These findings were confirmed in the present study.

However, there is evidence that the lung has a high tolerance to severe hypoxia, making high-energy phosphates poor candidates for a role as oxygen sensors. ATP, ATP/ADP, or lactate production in the lung does not change at physiological levels of hypoxia, as discussed in the introduction.\(^10,11\)

We have previously shown that production of AOS in the lung occurs in direct proportion to the inspired oxygen tension.\(^22\) In the present study, it is evident that many of the AOS, measured by chemiluminescence, derive from the proximal portion of the ETC, because chemiluminescence is markedly inhibited by rotenone or antimycin A (Fig 3, Table 1). Although the ETC is largely successful in conveying electrons to molecular oxygen without releasing AOS, there are several sites where radicals may be formed (Fig 1). Basal production of radicals and peroxides by the ETC is closely related to cytoplasmic redox status. Metabolic inhibitors shift the redox status of both mitochondria and cytosol toward the reduced state.\(^28\) It is uncertain whether the changes in cytosolic redox state result from diminished AOS production, accumulation of high-energy electron donors (eg, NADH, which remains reduced because it is prevented from passing electrons to the cytochromes), or a combination of these effects. Changes in AOS production by the ETC and alterations in the redox status of sulfhydryl groups (eg, glutathione) and nucleotides (eg, NADH/NAD\(^+\)) may occur in parallel. A schematic representation of the interaction between the AOS production by the ETC and net cytosolic redox status is provided in Fig 1.

The hypothesis that AOS production and lung redox status could serve as oxygen sensors is supported by the finding that rotenone and antimycin A, like alveolar hypoxia, decrease lung chemiluminescence before the onset of pulmonary vasoconstriction (Table 1, Fig 2). Although the interval from the administration of rotenone and antimycin A to the decrease in chemiluminescence was longer than for hypoxia (Table 1), the fall in chemiluminescence still preceded the increase in pulmonary artery pressure. The effect of rotenone and antimycin A on AOS production was not a nonspecific response to the elevation of pulmonary artery pressure or lung injury. Vasoconstrictors that do not alter ETC activity, Ang II and KCl, did not reduce chemiluminescence, and neither ETC inhibitor altered lung weight, perfusate pH, or Po\(_2\).

Indirect evidence that hypoxia and proximal ETC inhibitors share similar mechanisms of action is provided by the observation that pretreatment with rotenone or antimycin A obliterated subsequent HPV. Although KCl- and Ang II–induced pulmonary vasoconstriction were somewhat reduced by rotenone and antimycin A, the relatively greater inhibition of HPV is striking (Fig 4). In addition, the magnitude of the constrictor response to proximal ETC inhibition by rotenone was correlated with the magnitude of HPV but unrelated to the size of the Ang II– or KCl-induced constrictions.

Pyruvate, a substrate for the ETC, increased chemiluminescence. This increase in AOS appeared to be the result of pyruvate-promoting electron flow in the ETC,
Fig 8. Recordings and graphs showing that rotenone and antimycin A, but not cyanide, inhibit the whole-cell K⁺ current (IK) in pulmonary vascular smooth muscle cells. The columns illustrate (from top to bottom) control currents produced by step depolarizations, the maximal effect of the appropriate electron transport chain inhibitor, and the restoration of current with washout of the electron transport chain inhibitor. The bottom panel in each column represents the current-voltage relation, obtained at control and maximum current suppression. Current was measured at the peak (Ip) and steady-state (Iss) phases of the current tracings. Values are mean±SEM of five experiments. Em indicates membrane voltage. The current suppression caused by rotenone (A) and antimycin A (B) was significant (P<.05 at all voltages >−10 mV), whereas the effects of cyanide (KCN) (C) were not statistically significant (P=.08).
because the effect was blocked by antimycin A. The ability of antimycin A to decrease and pyruvate to increase the production of AOS illustrates that activity of the ETC can be rapidly modulated. The failure of pyruvate pretreatment to alter subsequent HPV probably relates to bolus administration of pyruvate with the return of chemiluminescence to a normal level before hypoxia.

Marshall et al\(^\text{29}\) have shown that doses of carbon monoxide that inhibit the respiratory cytochromes cause pulmonary vasoconstriction, even when oxygenation is maintained by ventilation with 10% O\(_2\). They interpret the persistence of vasoconstriction in the presence of O\(_2\) to indicate that there is not a specific O\(_2\) sensor involved in HPV and conclude that a change in energy state acts as a “functional” sensor. It is equally possible that inhibition of the ETC by carbon monoxide results in changes in lung redox status, much as occurs with rotenone and antimycin A.

**Cyanide Stimulates Production of O\(_2\) Radicals**

Cyanide causes pulmonary vasoconstriction by a mechanism distinct from that of HPV. Bovers and colleagues\(^\text{16,30}\) found that radical and peroxide generation occurs primarily in the proximal ETC. We confirm this observation with the demonstration that basal AOS production falls only when the proximal ETC is blocked. In contrast, cyanide-induced increases in pulmonary artery pressure were preceded by an increase in chemiluminescence and were inhibited by SOD. The combination of vasoconstriction and increased radical production is similar to what occurs during hypoxia rather than hypoxia.\(^\text{15}\) The effects of radicals on the pulmonary circulation are complicated. Low doses of radical cause vasodilatation,\(^\text{21}\) and high doses cause vasoconstriction and edema.\(^\text{24}\) Some of these effects are mediated by prostaglandins.\(^\text{31,32}\) In the present study, radical formation and vasoconstriction occurred despite cyclooxygenase blockade and were inhibited by SOD, suggesting a prostanoid-independent radical-induced constrictor mechanism.

Cyanide alone did not inhibit HPV (Fig 4). Only when lungs were pretreated with SOD was HPV blocked. The diminution of HPV may have resulted from the secondary production of H\(_2\)O\(_2\), which results from the dismutation of O\(_2^-\) by SOD. H\(_2\)O\(_2\) is known to diminish pulmonary vasoconstrictor responsiveness by a cyclooxygenase-independent mechanism.\(^\text{33-35}\)

**What Is the Link Between Sensor and Effector Mechanisms of HPV?**

The observation that agents inhibiting electron transport and decreasing lung AOS production mimic hypoxia is consistent with a previously described “redox” mechanism for regulation of pulmonary vascular tone.\(^\text{27}\) In this model, agents that shift the redox status toward an oxidized state (such as diamide and 4-butyl hydroperoxide) promote vasodilatation,\(^\text{35}\) whereas agents that cause a “reduced” state such as hypoxia (rotenone and antimycin A) promote vasoconstriction. The link between cellular redox state and membrane potential has been unclear.\(^\text{27}\) In the present study, a link between oxygen metabolism, production of AOS, and K\(^+\) channel activity is confirmed. This interconnected redox chain is supported by several recent studies in nonvascular cells\(^\text{17-19}\) showing that K\(^+\) channel activity can be modulated by changes in radical production and/or sulfhydryl oxidation. Increased production of intracellular radicals, elicited by radiation, increases an outward K\(^+\) current in tumor cells.\(^\text{19}\) In addition, the activity of certain K\(^+\) channels responds to changes in cellular glutathione redox status.\(^\text{18}\) We have recently shown that the whole-cell K\(^+\) current of pulmonary vascular smooth muscle cells is inhibited by GSH and enhanced by GSSG.\(^\text{20}\)

The whole-cell patch-clamp data show that hypoxia, rotenone, and antimycin A share the ability to reversibly inhibit the activation of a K\(^+\) current in isolated pulmonary artery smooth muscle cells (Fig 8). K\(^+\) current inhibition caused by rotenone and antimycin A is similar to that caused by hypoxia\(^1\) but is greater in magnitude and slower to develop. The relative specificity of rotenone and antimycin A as inhibitors of the ETC suggests.
that their inhibition of AOS production relates to their effects on the mitochondrial ETC. The temporal relation between the effects of the ETC inhibitor on AOS (onset in 1 to 2 minutes), K⁺ channel inhibition (onset in 2 minutes), and vasoconstriction (onset in 3 to 4 minutes) is consistent with the hypothesis that their redox properties explain their hemodynamic consequences. Furthermore, cyanide, which failed to inhibit AOS production, also did not inhibit whole-cell K⁺ current. These experiments suggest that changes in redox, occurring in association with changes in ETC activity, may alter K⁺ channel activity and vascular tone. Perhaps interference with electron shuttling by the ETC alters glutathione redox homeostasis and thus K⁺ channel activity in the pulmonary vasculature.

Whole-cell experiments do not permit determination of which channel(s) is inhibited by hypoxia and ETC inhibitors. Such a determination must await single-channel studies. The pharmacology and morphology of the whole currents (slow decay, low noise, and 4-AP sensitive) are consistent with an important role for the delayed rectifier in this response. The patch-clamp data argue against the possibility that the ETC inhibitors cause vasoconstriction by depleting ATP. Activation of KATP channels in pulmonary vascular smooth muscle has been shown to result in pulmonary vasodilatation with severe hypoxia. However, the experiments demonstrating K⁺ channel inhibition by antimycin A, rotenone, and hypoxia were conducted using an internal pipette solution replete with ATP. In experiments designed to directly study the role of the KATP channel, the effect of rotenone given to cells after administration of the KATP inhibitor glibenclamide was unaltered (Fig 10). These findings suggest that the relevant oxygen-sensitive K⁺ channel is not a KATP channel. Furthermore, removal of ATP from the pipette did not significantly increase basal K⁺ currents or the response to ETC inhibitors (data not shown), suggesting that KATP channels are not tonically active in pulmonary vascular smooth muscle cells.

Study Limitations

Chemiluminescence is a sensitive technique for measuring radicals and peroxides in isolated organs.
that correlates with other measures of redox status, such as reduction of nitro blue tetrazolium. Chemiluminescence measures the net production of radicals and peroxides in many pools simultaneously, not just those that relate to vascular tone and ion channel activity. Oxidation of the entire cell cytosol or membrane by AOS would not be a viable way of signaling physiological changes in $O_2$ tension. The potential for dissociation between chemiluminescence and vascular tone is illustrated by the example of dimethylsulfoxide (DMSO). Doses of DMSO of $>10^{-2}$ mol/L markedly reduce chemiluminescence without altering perfusion pressure or vascular reactivity (data not shown). It seems that in very high doses, DMSO inhibits chemiluminescence arising from lipid peroxidation, which is downstream from the “executive” AOS or redox couple that modulates $K^+$ channel gating. Doses of DMSO of $<10^{-3}$ mol/L had no effect on chemiluminescence.

We do not know if the “executive” AOS that alters vascular tone and $K^+$ channel function is superoxide anion, $H_2O_2$, or a related product. The observation that SOD causes only a small contraction suggests that $O_2^-$ may not be the most important AOS. This contention is supported by the finding that lucigenin-enhanced chemiluminescence, which is a sensitive indicator of $O_2^-$ production, is minimally decreased by hypoxia. In contrast, luminol-enhanced chemiluminescence (which is more sensitive to peroxides and hydroxyl radicals) is significantly depressed by hypoxia. It is also possible (as indicated in Fig 1) that the changes in chemiluminescence merely mirror the change in electron flux down the ETC and that the “executive” function is served by the reduced to oxidized ratio of ETC substrate(s) (eg, pyruvate and NADH).

Although metabolic inhibitors can impair endothelium-derived relaxing factor activity, the pulmonary vasculature is relatively resistant to their effects. Furthermore, inhibitors of nitric oxide synthesis, in contrast to rotenone and antimycin A, do not increase basal tone in the isolated perfused rat lungs. Finally, the inhibitory effects of ETC inhibitors on $K^+$ channels were demonstrated in an endothelium-free system.

The present study did not identify a specific redox couple that regulates a specific $K^+$ channel. Such studies will require measurement of the ratios of cytosolic GSH to GSSG and NADH to NAD and the use of single-channel recordings. The existence of a redox-based $O_2^-$ sensor does not exclude the possibility that there are other substances capable of modulating $K^+$ channels (such as cGMP or a $K^+$ channel-associated heme protein).

**Conclusion**

Proximal ETC inhibitors and hypoxia result in a sequence of events in the lung, beginning with a rapid change in lung redox state (decreased AOS production). This is followed by inhibition of an outward ATP-independent $K^+$ current in pulmonary vascular smooth muscle cells and vasoconstriction. These findings are consistent with a redox-based $O_2^-$ sensor in the pulmonary circulation.

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