O₂ Sensing in the Human Ductus Arteriosus

Regulation of Voltage-Gated K⁺ Channels in Smooth Muscle Cells by a Mitochondrial Redox Sensor

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Abstract—Functional closure of the human ductus arteriosus (DA) is initiated within minutes of birth by O₂ constriction. It occurs by an incompletely understood mechanism that is intrinsic to the DA smooth muscle cell (DASMC). We hypothesized that O₂ alters the function of an O₂ sensor (the mitochondrial electron transport chain, ETC) thereby increasing production of a diffusible redox-mediator (H₂O₂), thus triggering an effector mechanism (inhibition of DASMC voltage-gated K⁺ channels, Kv). O₂ constriction was evaluated in 26 human DAs (12 female, aged 9 ± 2 days) studied in their normal hypoxic state or after normoxic tissue culture. In fresh, hypoxic DAs, 4-aminopyridine (4-AP), a Kv inhibitor, and O₂ cause similar constriction and K⁺ current inhibition (Iₖ). Tissue culture for 72 hours, particularly in normoxia, causes ion remodeling, characterized by decreased O₂ and 4-AP constriction in DA rings and reduced O₂- and 4-AP-sensitive Iₖ in DASMCs. Remodeled DAMSCs are depolarized and express less O₂-sensitive channels (including Kv2.1, Kv1.5, Kv9.3, Kv4.3, and BKα₂). Kv2.1 adenoviral gene-transfer significantly reverses ion remodeling, partially restoring both the electrophysiological and tone responses to 4-AP and O₂. In fresh DASMCs, ETC inhibitors (rotenone and antimycin) mimic hypoxia, increasing Iₖ and reversing constriction to O₂ but not phenylephrine. O₂ increases, whereas hypoxia and ETC inhibitors decrease H₂O₂ production by altering mitochondrial membrane potential (ΔΨm). H₂O₂, like O₂, inhibits Iₖ and depolarizes DASMCs. We conclude that O₂ controls human DA tone by modulating the function of the mitochondrial ETC thereby varying ΔΨm and the production of H₂O₂, which regulates DASMC Kv channel activity and DA tone. (Circ Res. 2002;91:478-486.)

Key Words: Kv2.1 ■ Kv1.5 ■ mitochondrial membrane potential ■ redox ■ hydrogen peroxide

In the fetus, the ductus arteriosus (DA) is tonically relaxed by its hypoxic environment. This allows venous blood to bypass the nonventilated lungs. At birth, simultaneous pulmonary vasodilatation and DA vasoconstriction direct right ventricular blood flow into the pulmonary circulation. Functional closure of the DA (due to vasoconstriction) precedes anatomical closure (due to cell proliferation) by days and is crucial to the newborn’s transition to an air-breathing organism. The DA constrictor response to O₂, although modulated by the endothelium (reinforced by endothelin [ET] and inhibited by vasodilatory prostanoioids and nitric oxide [NO]), is intrinsic to the DA smooth muscle cell (DASMC). O₂ constriction persists ex vivo, after endothelial denudation, and despite inhibition of prostaglandin H synthase (PGHS), nitric oxide synthase (NOS, and ET-A receptors. We have recently provided pharmacological evidence that O₂-induced constriction of the human DA involves inhibition of voltage-gated potassium channels (Kv) in DASMCs. As in most arteries, this Kv channel inhibition leads to SMC depolarization, opening of the voltage-gated L-type Ca²⁺ channels, influx of Ca²⁺, and vasoconstriction. However, the molecular identity of the relevant DASMC Kv channels, as well as the mechanism of O₂ sensing in this important human vessel, remain unknown.

Vascular O₂ sensing systems consist of a sensor, the function of which is altered by changes in P O₂, a mediator, produced by this sensor, and an effector, which alters vascular tone in response to the mediator. Many aspects of the O₂ sensor-effector pathway are conserved among O₂-sensitive mammalian tissues, the pulmonary artery (PA), the DA, the adenomedullary cells, the neuroepithelial body, and the carotid body. In each tissue, K⁺ channels have been implicated in the effector mechanism (see review), whereas the O₂ sensor has been proposed to involve a change in redox state, as determined by mitochondria or NADPH oxidase. Attention has focused on the mitochondria because electron transport chain (ETC) inhibitors mimic hypoxia, constricting the PA and activating the carotid body. Mitochondria respond to

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changes in PO2 by altering their respiration and production of reactive O2 species (ROS).4,5 This changes cellular redox potential and alters the function of many redox-sensitive genes, second messenger systems, and O2-sensitive K+ channels in the membrane, before any depletion of ATP. Because K+ channels control SMC membrane potential (Em), and thus tone, in most vascular beds, O2-sensitive K+ channels are attractive candidate effectors in vascular redox-based O2-sensing systems. O2-sensitive K+ channels include homo- and heterotetramers composed of the several Kv α-subunits (Kv1.2,5 Kv1.5,7 Kv2.1,5–8 Kv3.1b, Kv4.3,9 and Kv9.3) and the calcium-sensitive K+ channels, BKCa.10,11

In rabbit DAs, O2, and oxidants such as H2O2,12,13 inhibit DASMC Kv current and depolarize Em, leading to vasoconstriction. We now directly assess the hypothesis that O2 constrains the human DA via inhibition of DASMC Kv channels. We also hypothesize that the channels are under the control of an O2 sensor within the mitochondrial ETC and speculate that the sensor and effector are linked by mitochondrial-derived ROS, specifically H2O2.

Materials and Methods

DA Rings

The human studies committee of the University of Alberta approved the use of discarded DAs, excised from 26 neonates with hypoplastic left heart syndrome during the Norwood procedure (aged 9 ± 2 days, 12 female, arterial PO2 ~40 mm Hg). DAs were transported in iced saline, maintained in hypoxia, and used within 1 hour. Ring tension was recorded as previously described2 (see the expanded Materials and Methods section, which can be found in the online data supplement available at http://www.circresaha.org).

Whole-Cell Patch Clamping Technique

Current and voltage clamp technique, SMC isolation, and pipette solutions were performed as previously described (see online data supplement).14 The response to O2, ETC inhibitors, and the membrane permeable H2O2 analog, t-butyl hydrogen peroxide, were compared between DASMCs enzymatically dispersed from fresh DAs versus those maintained in tissue culture for 72 hours (either under normoxic or hypoxic conditions).

DASMC Em was measured both using patch clamp technique in current clamp mode and noninvasively with DiBAC4(3) (20 μmol/L, bis-barbituric acid oxonol), a potentiometric dye that increases green fluorescence on depolarization (see online data supplement).

Laser capture microdissection and immunofluorescence were performed as described in the online data supplement.

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify human Kv channel mRNA, relative to GAPDH. The qRT-PCR methodology and equations for calculating the relative copy number of Kv channel mRNA, normalized to GAPDH, 2(-ΔΔCt) are described in the online data supplement.

Measurement of ROS by Chemiluminescence

Lucigenin-enhanced chemiluminescence was measured from DA rings, as previously described (see online data supplement).15,16

H2O2 Assay

H2O2 was measured using a specific, fluorometric AmplexRed assay, as previously described16 (see online data supplement and a calibration curve in online Figure 1).

Mitochondrial membrane potential (ΔΨm) was measured using two independent and well-validated probes JC-1 and TMRM (tetramethyl rhodamine methyl ester), as previously described.16 These well-characterized, cationic fluorescent dyes exhibit potential-dependent mitochondrial accumulation. JC-1 dimerizes and fluoresces red in hyperpolarized mitochondria versus green in depolarized mitochondria (high versus low ΔΨm, respectively), whereas TMRM accumulates and fluoresces red in proportion to ΔΨm16 (see online supplement).

Ex Vivo Gene Transfer

Adenoviruses (serotype 5, Ad5) carrying genes for rat Kv2.1 and green fluorescent protein (GFP) (GFP, Ad5-GFP-Kv2.1) or GFP alone (Ad5-GFP), each under a CMV promoter, were constructed using the Adeasy-1 system as previously described.17 DAs were incubated for 12 hours with either vehicle or viruses. The rings were then incubated in tissue culture media under normoxic (PO2 ~120 mm Hg) or hypoxic (PO2 ~45 mm Hg) conditions for ~60 hours. The effects of gene transfer on vasoconstriction to O2 and 4-AP, whole-cell electrophysiology (Ik and Em), and gene expression were studied in fresh DAs and DAs exposed to normoxic or hypoxic culture.

Statistics and Drugs

For detailed information regarding statistics and drugs, please see the online data supplement.

Results

Kv Channels Control Tone

Fresh human DA rings were studied in hypoxia to mimic conditions in utero, except when otherwise specified. The Kv channel inhibitor 4-AP (1 to 10 mmol/L) significantly constrains the human DA in a dose-dependent manner (Figure 1A); iberiotoxin (IBTx, 200 nmol/L, a BKCa blocker) does not. The magnitude of O2 and 4-AP constriction are strongly correlated (Figure 1B). Although both 4-AP and IBTx significantly inhibit whole-cell K+ current (Ik; Figures 1C and 1D), IBTx causes minimal inhibition of Ik at potentials close to the resting Em (Figure 1E). In contrast, O2 and 4-AP cause similar, significant decreases in Ik at ~30 mV (Figure 1E).

mRNA for Kv1.5 and Kv2.1 is expressed in the media and SMCs of DA, selectively extracted with laser capture microdissection (LCM) (Figure 2A). This technique is important because the DA is composed of many cell types and LCM allows specific selection of the media and thus preferential measurement of channel expression in SMCs. Furthermore, immunofluorescent colocalization shows that Kv2.1 (Figure 2B) and Kv1.5 (not shown) protein is expressed in SM α-actin positive DASMC. This is also confirmed by conventional immunohistochemistry shown in online Figure 2.

Proximal ETC Is the O2 Sensor

Both rotenone, a complex I ETC inhibitor, and antimycin, a complex III inhibitor, relax the O2-preconstricted DA, mimicking hypoxia (Figures 3A and 3B). In contrast, cyanide does not dilate the DA (Figures 3A and 3B). Whereas either rotenone or antimycin alone only partially relax the normoxic DA, pretreatment with both inhibitors completely eliminates O2 constriction (Figure 4A). ETC inhibitor relaxation is not due to DA damage or nonspecific suppression of tone, because phenylephrine constriction is unaltered by rotenone and antimycin A (Figure 4A). Further support for the contention that O2 and rotenone target the same mechanism is the strong, direct correlation between the magnitude of O2 constriction and rotenone-relaxation (Figure 3C).

In fresh human DASMCs, O2 rapidly and reversibly inhibits Ik (Figures 3D through 3F). Rosetone mimics hypoxia, precisely

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restoring the current that was rapidly suppressed by \( \text{O}_2 \) (within 5 minutes). In contrast, cyanide (a complex IV blocker, 10 \( \mu \text{mol/L} \)) does not alter \( I_K \) (data not shown). These data provide strong evidence that the mechanism by which both proximal ETC inhibitors and hypoxia relax the DA is DASMC Kv activation.

**A New Model of Ionic Remodeling: DAs in Tissue Culture**

The effects of \( \text{O}_2 \) and rotenone on tone (Figure 4A), \( K^+ \) channel function (Figures 4B and 5A through 5C), and expression (Figure 5E) were studied in DAs kept in normoxic versus hypoxic tissue culture for 72 hours. The chronically normoxic DA rings retain the ability to constrict to phenylephrine, whereas they loose both the \( \text{O}_2 \) constriction and the rotenone-relaxation responses (Figure 4A). SMCs from chronically normoxic DAs have a significantly decreased current density, compared with the freshly isolated cells studied under identical conditions (Figure 4B). In addition, when these DASMC are returned to hypoxic conditions, their \( I_K \) is unresponsive to abrupt increase in \( P_{\text{O}_2} \), 4-AP, or rotenone (Figure 4B). The loss of \( \text{O}_2 \) and ETC sensitivity in chronically normoxic DA is associated with basal, hypoxic \( E_M \) depolarization and loss of the ability to depolarize in response to rapid increases in \( P_{\text{O}_2} \) or 4-AP, whereas the response to KCl is preserved (Figures 5A through 5D).

**Figure 1.** Kv channels control DA tone. A, Freshly harvested human DAs, studied in hypoxia, constrict to 4-AP but not IBTx. B, There is a strong direct correlation between constriction in response to 4-AP and \( \text{O}_2 \). C, Mean current density-voltage plots and representative traces showing that the \( I_K \) of the freshly isolated, hypoxic DASMC is extremely sensitive to 4-AP. \(*P < 0.01 \) for dose effect. D, Iberiotoxin (IBTx) inhibits current compared with the control value obtained after 4-AP washout. \(*P < 0.05 \) IBTx inhibits current but only at potentials positive to 0 mV. E, 4-AP and \( \text{O}_2 \) cause similar large inhibition of \( I_K \) at negative potential near resting \( E_M \), whereas iberiotoxin only inhibits current at positive potential; \(*P < 0.05 \) (derived from C and D)

**Figure 2.** DASMCs express Kv channels. A, Using LCM, a circumferential ring of media was selectively removed from a human DA. Presence of Kv2.1 and Kv1.5 \( \alpha \)-subunit proteins and SM \( \alpha \)-actin was confirmed by performing qRT-PCR on the isolated ring of media. Expression of Kv1.5 in the DASMC is further confirmed by immunocolocalization (B) in which the SMCs in the media of an intact human term DA are stained for \( \alpha \)-SM actin (green staining) and Kv1.5 (red). Colocalization results in the combined image showing yellow SMCs. Nuclei are stained with Hoechst 33342. Note the SMCs are separated by a significant amount of unstained myxoid tissue.
findings of impaired membrane responses to O2 using both whole-cell current clamp and potentiometric dyes in both DASMCs and DA rings excludes the theoretical possibility that this could be an artifact, related to enzymatic dispersion of cells or loss of cell-cell connection.

**Ionic Remodeling Results From Downregulation of O2-Sensitive Kv Channels**

DAs were divided in thirds and both the function and expression of K+ channels and ETC complexes were compared in fresh hypoxic DAs versus DAs cultured in chronic normoxia or chronic hypoxia. In chronic normoxia, mRNA for Kv1.5 and Kv2.1 (measured using qRT-PCR) and Kv4.3, Kv9.3 and BKCa (measured using conventional RT-PCR; online Figure 3) is downregulated, relative to the housekeeping gene GAPDH (Figure 5E). There is no decrease in Kv1.1, Kir2.1, or TASK expression, measured using conventional RT-PCR, suggesting that the downregulation of these O2-sensitive channels is somewhat specific (see online Figure 3). Although there was also a trend to lower Kv1.5 and Kv2.1 mRNA in chronic normoxia versus chronic hypoxia (Figure 5E), this was not statistically significant. Expression of selected subunits from ETC complexes I-IV is unaltered by chronic normoxia (online Figure 4).

**Kv2.1 Gene Restoration in the Iionically Remodeled DA**

If the depressed Kv expression in this model is both real and important, it would follow that restoring Kv expression would be sufficient to restore the missing O2 responsiveness of...
chronically normoxic DASMCs. Indeed, Kv2.1 gene therapy does partially restore O₂ responsiveness (Figure 6). DAs were divided into 4 pieces. One piece was studied immediately upon arrival in the laboratory under hypoxia, whereas the others were exposed to normoxia for 12 hours in the presence of vehicle, Ad5-GFP, or Ad5-GFP-Kv2.1. This was followed by 60 hours of normoxic incubation to allow gene and protein expression. Of the 6 human DAs infected, successful gene transfer, as measured by GFP fluorescence, was confirmed in 4 (Figures 6A and 6B). The Kv2.1 transgene was derived from rat and, using the species specificity of the qRT-PCR probe, we were able to show that expression of rat Kv2.1 mRNA occurred exclusively in Ad5-GFP-Kv2.1-infected DAs (Figure 6C). The transgene yielded functional channels, indicated by the fact that DASMCs isolated from Ad5-GFP-Kv2.1 rings had a larger Kv current compared with the DASMCs from the noninfected DAs (Figure 6B). Kv2.1 gene transfer, significantly restores the ability of the normoxic DA to respond to O₂ and 4-AP (Figures 6D and 6E).

Mitochondria-Derived ROS Are the Redox Mediators of Normoxic DA Constriction

Inhibitors of the proximal ETC and authentic hypoxia rapidly depolarize ΔΨm in DASMCs in primary, hypoxic culture (Figures 7A through 7D). Conversely, cyanide 10 μmol/L does not rapidly alter ΔΨm (Figure 7C). To address the concern that cyanide should (at some dose) be effective in collapsing ΔΨm, we also assessed its effects on a cardiac

Figure 5. Long-term exposure to O₂ causes ionic remodeling. In these experiments, Eₓ was measured using DiBAC₄. A, Confocal microscopy of intact DA rings reveals depolarization (more green) in the half of the ring exposed to O₂ for 72 hours than in the half incubated in hypoxia. Blue stain (Hoechst 33342) marks the nuclei. B, Similar findings are noted in DASMCs grown in normoxic vs hypoxic primary culture. Figure 6. Rat Kv2.1 gene transfer partially restores O₂ and 4-AP sensitivity in ionic remodeled human DAs. A, Ex vivo gene transfer is confirmed by confocal microscopy showing GFP fluorescence only in the infected half of an intact DA ring. B, DASMCs from the infected DA show increased Kv current. C, Using a qRT-PCR specific for rat Kv2.1, it is confirmed that only the rings infected with the rat Kv2.1 virus express the transgene (n=4). **P<0.01 compared with all other groups. D and E, Kv2.1 gene transfer significantly restores the responsiveness of the chronically normoxic DA ring to both 4-AP and O₂. D, Constrictor responses to O₂ and 4-AP are expressed as a percentage of the maximal phenylephrine constriction noted in the same rings. Note that infection with a virus containing only GFP is without effect (n=4).
HL-1 cell line. These experiments showed that 10 μmol/L CN readily depolarizes ΔΨm in HL-1 cells, suggesting diversity in mitochondria between vascular versus cardiac cells (online Figure 5). Thus, ΔΨm in DASMCs is much more sensitive to rotenone than to cyanide, consistent with its lack of electrophysiological and hemodynamic effects.

Lucigenin-enhanced chemiluminescence (n=2) and H2O2 production (n=5) are increased within minutes by raising Po2 from 40 to 100 mm Hg in freshly isolated, human DA rings (Figures 8A and 8B). Rotenone decreases H2O2 production (Figures 8A and 8B). t-butyl-H2O2 mimics the effects of O2 on DASMC electrophysiology. It inhibits IK, depolarizes freshly dispersed DASMCs, and both these effects are lost after exposure to chronic normoxia (Figures 8C and 8D).

Discussion

This is the first comprehensive study of the mechanism of O2 sensing in the human DA. There are 3 major findings of this study. First, Kv channels are the effectors of O2 constriction (Figures 1 and 2). Inhibiting these channels, whether by 4-AP or O2 leads to vasoconstriction. Second, the proximal mitochondrial ETC serves as the O2 sensor (Figures 3, 4A, and 7). Inhibition of the complex I or III inhibits O2 constriction. Third, consistent with data in rabbit DA,13 the mediator linking the sensor and effector in the human DA appears to be a ROS (H2O2) (Figure 8). The ability of hypoxia and proximal ETC inhibitors to depolarize ΔΨm (Figure 7) provides a probable mechanism by which hypoxia alters ROS production. In addition, a new model of ionic remodeling is
introduced that is useful in understanding the relative contributions of Kv channels and mitochondria to O\textsubscript{2} sensing in the human DA (Figures 4 through 6). Together, these findings suggest that ROS produced in the proximal ETC in response to O\textsubscript{2} could be redox mediators that link the mitochondrial sensor to the Kv effector and thus control tone, as illustrated schematically in Figure 8D.

Roulet and Coburn first demonstrated that O\textsubscript{2}-induced DA constriction is associated with membrane depolarization.\textsuperscript{19} Although the K\textsuperscript{+} channel effector mechanism is widely conserved among O\textsubscript{2} sensitive tissues, the type of K\textsuperscript{+} channel and downstream response to channel inhibition may vary among species, between tissues, and with maturation.\textsuperscript{3} Therefore, we have focused our studies on the mechanism of O\textsubscript{2} constriction in term human DA. In addition, special effort was made to ensure that all DAs were maintained in their normal hypoxic environment, except when they were intentionally exposed to acute or chronic normoxia. Despite the congenital heart disease, these DAs have normal responses to O\textsubscript{2} with similar thresholds for onset of O\textsubscript{2} constriction and maximal O\textsubscript{2} responses,\textsuperscript{2} as in the literature.\textsuperscript{1}

In the human DA, O\textsubscript{2} and 4-AP cause nonadditive vasoconstriction.\textsuperscript{2} Nifedipine, an inhibitor of L-type Ca\textsuperscript{2+} channels, blocks both 4-AP and O\textsubscript{2} constriction,\textsuperscript{1,2} consistent with an obligatory and coordinated role for Kv and Ca\textsuperscript{2+} channels in O\textsubscript{2} constriction. The present study confirms the ability of Kv, but not BK\textsubscript{Ca}, channel blockers to constrict the human DA and demonstrates a strong correlation between the magnitude of 4-AP constriction and O\textsubscript{2} constriction (Figure 1A and 1B). The basis for these physiological observations is now directly identified. The DASMCs express several O\textsubscript{2}- and 4-AP-sensitive K\textsuperscript{+} channels, which are involved in hypoxic pulmonary vasoconstriction (Kv1.5 and Kv2.1)\textsuperscript{7} and O\textsubscript{2} responses in the carotid body\textsuperscript{9} (Figure 2 and online supplement Figure 3).

A new model was developed to further understand the role of Kv channels in O\textsubscript{2} constriction. In this model, designed to mimic the conditions in the first days after birth, exposure to tissue culture conditions ex vivo (particularly at normoxic P\textsubscript{O\textsubscript{2}}), inhibited DASMC I\textsubscript{K}, (Figure 4B) and downregulated several O\textsubscript{2}-sensitive Kv channels, including Kv1.5 and Kv2.1 (Figure 5E). The results shown in the online data supplement demonstrate that expression of other O\textsubscript{2}-sensitive Kv channels, Kv4.3,\textsuperscript{9} Kv9.3,\textsuperscript{4} and BK\textsubscript{Ca} channels,\textsuperscript{10,11,20} also decrease in this model. However, the lack of effect of iberiotoxin on tension suggests that the role for BK\textsubscript{Ca} channels may be less important in O\textsubscript{2} response in the DA than in the carotid body. The role of Kv4.3 is somewhat less likely as these channels generate a rapidly inactivating current, unlike the slow non-inactivating O\textsubscript{2}-sensitive current in DASMCs.\textsuperscript{21}

The loss of these Kv channels is associated with membrane depolarization (Figures 5A through 5C) and impaired ability of the membrane to further depolarize to either O\textsubscript{2} or 4-AP (Figure 5D), even hours after the DASMCs are returned to a hypoxic environment. These changes in the electrical properties of the DA are termed ionic remodeling. Similar ionic remodeling and loss of O\textsubscript{2} responsiveness has also been reported in other O\textsubscript{2}-sensitive tissues. Loss of acute hypoxic pulmonary vasoconstriction, which occurs with chronic hypoxia, is also associated with downregulation of Kv2.1 and Kv1.5 expression and loss of the O\textsubscript{2}-sensitive portion of I\textsubscript{K}.\textsuperscript{15,17}

The ionic remodeling model further highlights the importance of the Kv channels to O\textsubscript{2}-induced constriction. The reduction in K\textsuperscript{+} current density is associated with loss of sensitivity of current and tone to O\textsubscript{2} and 4-AP (Figure 4). Furthermore, the ability of rotenone to increase I\textsubscript{K} is lost in this model (Figure 4B). Coupled with the qRT-PCR evidence for loss of O\textsubscript{2}-sensitive channels, but not ETC complexes (online Figure 4), these data suggest that the loss of O\textsubscript{2} constriction is primarily due to modifications of the Kv effector, rather than the mitochondrial sensor. Consistent with this interpretation, restoration of Kv2.1 expression by ex vivo gene transfer significantly restores the constriction to both 4-AP and O\textsubscript{2} (Figure 6). The fact that restoration of O\textsubscript{2} and 4-AP constriction is incomplete after Kv2.1 restoration may relate to the fact that other downregulated O\textsubscript{2}- and 4-AP-sensitive channels (eg, Kv1.5, Kv9.3, and Kv4.3) were not replaced. Alternatively, alterations in function of other components of the contractile apparatus may be abnormal.

Although the pulmonary artery and the DA are contiguous, their response to O\textsubscript{2} is reversed. Hypoxia causes pulmonary vasoconstriction versus DA relaxation. It is intriguing that similar Kv channels are present in both arteries.\textsuperscript{2,7} The fact that the Kv inhibitor 4-AP constricts both arteries suggests that the Kv channel setting E\textsubscript{M} in the 2 tissues may be similar.\textsuperscript{1,7} By extension, this implies that their differential O\textsubscript{2} response may relate to differences in either the O\textsubscript{2} sensor or the response of the K\textsuperscript{+} channels to the redox messenger produced by a shared sensor. In human DAs, it appears that O\textsubscript{2}-sensitive Kv channels are the effectors and the mitochondrial ETC is the O\textsubscript{2} sensor, very similar to the pulmonary artery.\textsuperscript{7} What differs, as is discussed subsequently, is the response to ROS.

What is the evidence that Kv function and vascular tone is regulated by the mitochondria? First, rotenone and antimycin mimic hypoxia better than any other class of drugs, suggesting a role for mitochondria in vascular O\textsubscript{2} sensing.\textsuperscript{5,13,15,22,23} Proximal ETC inhibition causes pulmonary vasoconstriction,\textsuperscript{5} systemic dilatation,\textsuperscript{24} carotid body activation,\textsuperscript{25} and DA vasodilatation (Figure 3). Rotenone and antimycin, but not cyanide, selectively inhibit O\textsubscript{2} constriction in the human DA and reverse O\textsubscript{2}-induced inhibition of I\textsubscript{K} in DASMCs (Figures 3D through 3F). Indeed, the effects of rotenone and antimycin are additive, and the combination completely prevents O\textsubscript{2} constriction, without preventing phenylephrine constriction (Figure 4A). In addition, the more a DA constricts to O\textsubscript{2} the more relaxation occurs in response to rotenone, but not to cyanide (Figure 3C). These findings are a mirror image of those in the pulmonary circulation where ETC inhibitors, also mimicking hypoxia, cause pulmonary vasoconstriction and inhibition of I\textsubscript{K}.\textsuperscript{5} However, in both the pulmonary circulation and DA, hypoxia, and ETC inhibitors decrease ROS production (Figure 8),\textsuperscript{26} suggesting the difference between the vessels relates to the response of their K\textsuperscript{+} channels to H\textsubscript{2}O\textsubscript{2} and ROS. Indeed, DASMCs depolarize in response to H\textsubscript{2}O\textsubscript{2} (Figure 8), the opposite of the response seen in pulmonary artery SMCs.\textsuperscript{12}

The link between the mitochondria, Kv channels, and tone is through a redox mediator, rather than ATP levels (see
Figure 8D). Although mitochondria have been dismissed as poor candidates for O$_2$ sensing because the Km of their cytochromes may be too low for modulation by physiological levels of hypoxia, they exhibit a reversible inhibition of respiration during prolonged hypoxia due to inhibition of cytochrome c oxidase. Mitochondria are increasingly recognized to be involved in intracellular Ca$^{2+}$ control and in redox signaling, in part, because they are important sources of ROS. DeltaPsi$m$, a major determinant of cellular redox potential, changes rapidly over a physiological range of P0$_2$ values in type I carotid body cells, depolarizing in response to hypoxia and metabolic inhibitors. Similarly, hypoxia, rotenone, and antimycin depolarize DeltaPsi$m$ in human DASMCs within 1 to 2 minutes (Figure 7) and this is associated with impaired ROS production (Figures 8A and 8B). Our confocal images also show that the mitochondria in DASMCs, far from being remote from the plasma membrane, form a ubiquitous, filamentous network that permeates the cytosol and is thus positioned to signal changes in P0$_2$ to all cellular compartments, including the plasma membrane (Figure 7).

We propose that the increase in the H$_2$O$_2$ levels that occurs with normoxia at the time of birth inhibits DASMC Kv current, causing depolarization and vasoconstriction. Indeed, in human DAs, H$_2$O$_2$ production increases as P0$_2$ rises (Figures 8A and 8B) and H$_2$O$_2$ inhibits IK, causing membrane depolarization (Figures 8C and 8D). Likewise, in rabbit DASMCs, intracellular administration of physiological doses of H$_2$O$_2$ decreases IK, and these effects are inhibited by catalase. The molecular basis for the differential electrophysiological response to H$_2$O$_2$ in the pulmonary artery SMC (activation) versus the DASMC (inhibition) is unknown. Possible explanations include tissue-specific differences in Kv channel heterotrimer composition, alpha-subunit splice variants, sulfhydryl redox state of key channel amino acids, or beta-subunit expression.

Several questions remain to be answered. First, why is the distal ETC not as involved in O$_2$ sensing as the proximal ETC? In the pulmonary circulation, rotenone and antimycin mimick hypoxia (decrease IK, cause constriction, and inhibit additional hypoxic constriction); cyanide does not. Cyanide’s lack of effect is not due to inadequate dosage. This dose of cyanide, administered under similar circumstances, depolarizes DeltaPsi$m$ in HL-1 cells (online Figure 5) and large doses of cyanide depolarize DeltaPsi$m$ in DASMCs (Figure 7C). Rather, we believe that because the majority of ROS production occurs at complex I and III, inhibitors of this portion of the pathway are most effective in interrupting the production of ROS signaling molecules and thus impair O$_2$ sensing (Figure 8D).

Second, although there is growing agreement that mitochondria or a vascular NADPH oxidase are vascular redox O$_2$ sensors, there is debate as to whether hypoxia and ETC inhibitors decrease or increase AOS production. We recently have found concordant depression of vascular generation of ROS and H$_2$O$_2$ by hypoxia using three independent techniques: dichlorofluorescein, lucigenin, and Amplex Red.

The initial constriction of the DA is required for the remodeling and ultimately closure of the DA. Thus, understanding the mechanism of O$_2$ constriction has implications for the common clinical problem of patent DA in premature infants. Perhaps DAs in premature infants fail to constrict to normoxia because their DASMCs are “deficient” in Kv channels. Restoration of Kv2.1 or Kv1.5 expression, whether accomplished by gene transfer, as in this study, or by other means, might have therapeutic potential. Augmenting Kv2.1 or Kv1.5 expression in premature DAs might restore its ability to constrict to O$_2$ and thus enter a normal remodeling phase, leading to closure.

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References


O₂ Sensing in the Human Ductus Arteriosus: Regulation of Voltage-Gated K⁺ Channels in Smooth Muscle Cells by a Mitochondrial Redox Sensor

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Methods

**Laser Capture Microdissection.** LCM utilizes a microscope platform\(^1\) combined with a low energy infrared laser to melt a plastic capture film onto the flash frozen human ductus arteriosus\(^1\). The PixCell II LCM System (Arcturus Engineering, Mountain View, CA) is used to selectively remove specific tissue layers from the DA wall. The machine settings were as follows: slice thickness 7\(\mu\)M, power 45mW, duration 1.5ms. The freshly section ductus was mounted without a coverslip on a DNA-free microscope slide. The tissue was dehydrated using the HistoGene LCM Frozen Section Staining Kit and then the dissected section was placed on a thermoplastic membrane pre-mounted on optically transparent caps. After visual confirmation of the adequacy of the dissection by examining the tissue in the cap, the specimen was placed in RNA-later\(^\circledR\) for subsequent qRT-PCR.

**Real-Time Polymerase Chain Reaction:** Total RNA was extracted using RNeasy Mini Kit (Qiagen, Mississauga, Canada) and quantified with UV spectrophotometry. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify human \(K_v\) channel mRNA and expression of rat \(K_v2.1\) mRNA transgene. The TaqMan One-Step RT-PCR Master Mix reagent kit was used (Applied Biosystems, Foster City, CA). The reaction used 50ng RNA in 50\(\mu\)l using the relevant primer (500nM), and TaqMan probe (200nM), as designed by us. The assay was performed using an ABI PRISM 7700
Sequence Detector System (Applied Biosystems). Reverse transcription proceeded for 30 min at 48°C. AmpliTaq Gold activation occurred for 10min at 95°C. Subsequently, 40 cycles of PCR were performed. Each cycle consisted of 15 seconds of denaturing (at 95°C) and 1 minute of annealing and extension (at 60°C). $2^{\Delta\Delta Ct}$ is a ratio of the expression of the Kv channel to glyceraldehyde dehydrogenase (GAPDH).

$2^{\Delta\Delta Ct}$ calculation: $2^{\Delta\Delta Ct}$ is a conversion factor allowing the amount of Kv mRNA to be expressed in terms of copy number relative to the calibrator (the sample with the least amount of Kv mRNA) and normalised to expression of a housekeeping gene, GAPDH.

Ct=threshold cycle for target amplification

The $\Delta Ct$ $\text{Kv2.1}$ was computed for each sample (Equation 1). The largest $\Delta Ct$ (indicating the smallest amount of Kv2.1) is defined as the calibrator.

**Equation (1)** $\Delta Ct \text{Kv2.1} = Ct \text{Kv2.1} - Ct \text{GAPDH}$

Then the relative copy number $\Delta \Delta Ct$ is calculated for each sample using formula 2

**Equation (2)** $\Delta \Delta Ct = \text{calibrator} - \Delta Ct \text{Kv2.1}$

In the case of the sample that is experimentally selected as the calibrator (the lowest expressing sample), Equation 2 yields a value of 0 (subtracting the value from itself). By expressing the $\Delta \Delta Ct$ as an exponent of 2, the copy number in the calibrator becomes $2^0=1$, allowing easy expression of the larger amounts of Kv mRNA in other specimens relative to this value.
Conventional PCR was performed on DAs, as previously described.

Lucigenin enhanced chemiluminescence: This assay is sensitive for ROS but does not differentiate amongst the redox species. DA rings were loaded with lucigenin 5 μM and placed in a 2 ml heated cuvette at 37°C. Counts were recorded using a Packard 1900CA Liquid Scintillation Analyzer.

Amplex Red Assay: An independent, specific measure of the candidate redox mediator, H$_2$O$_2$, was made using the one-step, fluorometric Amplex Red (Molecular Probes, Eugene, OR) assay. The assay detects H$_2$O$_2$ using 10-acetyl-3,7-dihydroxyphenoxazone (Amplex Red reagent) a stable probe that, in the presence of horseradish peroxidase (HRP), reacts with H$_2$O$_2$ with a 1:1 stoichiometry to produce the highly fluorescent substance, resorufin. The assay was calibrated (Figure 1) and found to be linear over the range of 0.25 to 5.0μM. Freshly harvested DA rings were placed in a normoxic or a hypoxic solution containing AmplexRed and, after 20 minutes, fluorescence was recorded (excitation 530nm, emission 590nm), see Figure 1.

Ex-vivo gene transfer: Adenoviruses (serotype 5) were constructed using the Adeasy-1 system as previously described. A recombinant adenovirus encoding GFP and Kv2.1 was prepared using Adeasy-1, an adenoviral backbone vector, which contains adenovirus
(serotype 5) genomic DNA with E1 and E3 deleted. A 2609 bp cDNA fragment encoding the open reading frame of the rat Kv2.1 channel was excised from its original pBK-CMV plasmid (kindly provided by Dr. K Takimoto, University of Pittsburgh) using restriction endonucleases Not I and Sal I and ligated into the Not I and Sal I sites of pAdTrack-CMV, which contains a kanamycin resistance gene and two cytomegalovirus (CMV) promoters (one promoter located upstream of GFP and the other upstream of Kv2.1). When pAdTrack-CMV and Adeasy-1 are co-transformed into BJ5183 cells, the Kv2.1 gene undergoes homologous recombination with the adenoviral backbone resulting in a plasmid that contains the Kv2.1 and GFP genes. The resultant pAdTrack-CMV Kv2.1 construct was linearised with a Pme I restriction endonuclease digest, transformed together with supercoiled adenoviral vector Adeasy-1 into BJ5183 cells and plated on LB plates containing kanamycin. Subsequent colonies were isolated and the plasmid DNA was purified using plasmid purifying columns (Qiagen). The plasmid containing Kv2.1 cDNA within the adenoviral DNA was selected, amplified, purified, linearised and transfected into HEK 293 cells using LipofectAMINE reagent. Five days after transfection, plates that demonstrated complete cell lysis were collected and analyzed for Kv2.1 cDNA using PCR. Upon confirmation of the Kv2.1 cDNA in the viral genome, multiple rounds of Ad5Kv2.1 replication were performed in HEK 293 cells. The resulting virus carrying GFP and Kv2.1 cDNA was isolated, precipitated and concentrated by discontinuous CsCl gradient. The final viral titer obtained for Ad5-GFP-Kv2.1 was 1.5 X
10⁹ pfu/ml. 24 hours incubation with recombinant replication deficient adenovirus (Ad5-GFP-Kv2.1) achieved 90% infection rates in Chinese hamster ovary cells (CHO) grown and kept under hypoxia.

Vectors carried the genes for rat Kv2.1 and green fluorescent protein (GFP) or GFP alone, each under a CMV promoter. Twelve hours incubation with recombinant replication deficient adenovirus (Ad5-GFP-Kv2.1) transduced 90% of Chinese hamster ovary cells and generated a functional Kv2.1 current.⁹ DA were incubated for 12 hours with either vehicle (normal saline), Ad5-GFP or Ad5-GFP-Kv2.1, the virus was then washed off and the vessels were kept in either a normoxic (PO₂~120 mmHg) or hypoxic (PO₂ ~45mmHg) incubator for 60 hours (total incubation 72 hours). Successfully infected vessels were selected based on their green fluorescence (excitation 488nm, detection 505-530nm). The finding that GFP fluorescence is at a much lower intensity than DiBAC₄ fluorescence allowed measurement of Eₘ in infected rings by increasing the threshold for detection of green emission.

**Conventional whole cell patch clamping technique:** Current and voltage clamp technique, SMC isolation and pipette solutions were performed as previously described¹⁰.¹² Cells were voltage clamped at a holding potential of -70 mV and currents were evoked by 10 mV steps to +50mV using test pulses of 200 ms duration. Data were recorded and
analyzed using pCLAMP 6.02 software (Axon Instruments, Foster City, CA). Current density was calculated (pA/pF) and plotted against voltage.

$E_m$ was measured in the current clamp mode in DASMCs dispersed from DA rings cultured for 72-96 hours in normoxia or hypoxia. The response to the membrane permeable H$_2$O$_2$ analog, tert-butyl hydrogen peroxide (Sigma Aldrich, St. Louis, MO; 100μM) was compared between these tissues.

**DA Rings** The human studies committee of the University of Alberta approved the use of discarded DAs, excised from 24 neonates with hypoplastic left heart syndrome during the Norwood procedure (age 2±1 days, arterial PO$_2$~40 mmHg). DAs were received in the laboratory within 1 hour and were transported in iced saline. On receipt, the DAs were immediately suspended in hypoxic tissue baths, as previously described. Optimal passive ring tension was determined to be 1 g, as defined by the maximal constriction to 80 mM KCl. Tension was recorded using a force transducer connected to a MacLab A-D convertor (AD Instruments, Toronto, Canada). Tension was recorded from rings bubbled with 95% N$_2$ and 5% CO$_2$ in Krebs’ solution, to mimic in utero conditions (PO$_2$ 40 mmHg, pH 7.4, pCO$_2$ 40-50 mmHg). Normoxia was created by bubbling with 20%O$_2$ and 5% CO$_2$ (balance N$_2$) resulting in PO$_2$ 150 mmHg, pH 7.4, and PCO$_2$ 40 mmHg. Optimal resting tension was previously found to be 1000 mg. To exclude the contribution of several endothelial-derived vasoactive substances, the Krebs’ solution also contained
meclofenamate 17μM to inhibit PGHS and L-NG-nitroarginine-methylester (L-NAME) 10⁻⁵ M to inhibit NOS.

Mitochondrial membrane potential (JC-1) and tetramethyl-rhodamine methyl ester (TMRM): JC-1 ¹⁴ and TMRM ¹⁵ are both well validated dye that have been demonstrated to measure ΔΨm in cell. First-passage cultured DASMC were loaded with either JC-1 (1μM) or TMRM (20nM) for 30 minutes (37°C) at 37°C. Confocal images were obtained within 30 minutes using a Zeiss Axiovert 100M inverted microscope equipped with an Apochromat 40x1.2 water-corrected objective. Settings (amplifier gain, pin hole size and filter set) were kept constant between experiments.

JC-1, a cationic fluorescent dye that exhibits potential-dependent mitochondrial accumulation is used extensively to study ΔΨm ¹⁴,¹⁶,¹⁷. JC-1 fluoresces in the red spectra in hyperpolarized mitochondria (high ΔΨm) and in the green spectra in depolarized mitochondria (low ΔΨm). KCl (40mM) was used to clamp the plasmalemmal membrane potential in some experiments. This did not alter JC-1 fluorescence (not shown), confirming the specificity of this potentiometric dye for the ΔΨm and not the plasmalemmal Eₘ. TMRM binds the inner and outer aspects of the inner mitochondrial membrane and accumulates in the mitochondria at greater quantities than predicted by the Nernst equation ¹⁸. TMRM fluorescence red in proportion to ΔΨm. ¹⁵,¹⁸⁻²⁰ A 488nm Argon
laser was used for excitation and the resultant red/green fluorescence was quantified in channels 1 and 2 using LP 560nm and BP 505-530nm filters, respectively. 8-bit images were collected with a laser loiter time of 4.48µs/pixel. Pinholes yielded optical slices of 1µm. Results are expressed as the ratio of red/green channel fluorescence.

**DASMC** $E_m$ was measured with DiBAC$_4$(3) (20µM, bis-barbituric acid oxonol). DiBAC$_4$(3) a potentiometric dye that increases fluorescence upon depolarization. DiBAC$_4$ loaded cells or intact DA rings were stimulated with multi-photon excitation at 740 nm and emission was measured in the green range, 505-530nm. DASMCs were studied after culture in normoxic or hypoxic environment for 4-5 days. The acute change in $E_m$ that occurred in response to 4-AP (10mM), KCL (80mM) or a switch in PO$_2$ from 40 to 120mmHg was measured.

**Statistics** Values were expressed as the mean±SEM. Intergroup differences were assessed by Student’s paired t-test or an ANOVA (factorial or repeated measures) as appropriate. Post hoc analysis used a Fisher’s probable least significant difference test (Statview 4.02, Abacus Concepts). A $P<0.05$ was considered statistically significant. Regression analysis was also performed using Statview.
Drugs: All drugs were dissolved in saline and were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Antibodies to ETC complexes were purchased from Molecular Probes (Eugene, OR). The antibodies were directed against NADH ubiquinol oxidoreductases (Complex I, alpha complex, ~39 kDa), succinate-ubiquinol oxidoreductase (Complex II flavoprotein, ~70 kDa), ubiquinol-cytochrome c oxidoreductase (Core 2 subunit, Complex III ~45kDA) and cytochrome c oxidase (COX chain I, ~40kDa). Kv channel antibodies were from Alomone (Jerusalem, Israel, respectively. MnSOD antibody was from Upstate Biotech (New York, Lake Placid, New York).

ONLINE FIGURES

Online figure 1

A calibration curve for the AmplexRed assay is shown.

Online figure 2

Immunohistochemistry shows the presence of Kv2.1 and Kv1.5 α-subunit proteins (brown staining) in DASMCs. DASMCs also stain positive for α-SM actin. Note the SMCs are separated by a significant amount of unstained myxoid tissue. Nuclei are counterstained blue.
On line figure 3

Conventional RT-PCR was performed on individual ducti as previously described. 13 Although expression of several channels is unaltered, there is also less Kv9.3 and BKCa channel mRNA and less eNOS mRNA in chronic normoxic DAs, despite a similar expression of GAPDH.

F=Fresh, H= chronic hypoxic culture 96 hours, N=chronic hypoxic culture 96 hours

On line figure 4

Expression of subunits of ETC complexes I-IV in DA rings was not altered by 72 hours culture in O2 versus expression in fresh hypoxic DAs (Hyp).

Online figure 5

TMRM (red) images the mitochondria and measures ΔΨm (more red=more negative value for ΔΨm. Note that mitochondria in a contacting, HL-1 cardiac myocyte cell line are much more sensitive to the depolarizing effects of cyanide than are mitochondria in DASMCs. The relative insensitivity of DASM C mitochondria probably reflects diversity on the types of mitochondria (and complexes) between these tissues, consistent with recent reports of mitochondrial diversity 21.
References for on-line


Online 1

\[ Y = 1.308 + 385.493 \times X \]
\[ R^2 = .997 \]
### ETC Complex

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**Culture**

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