Oxygen-Sensitive Kv Channel Gene Transfer Confers Oxygen Responsiveness to Preterm Rabbit and Remodeled Human Ductus Arteriosus Implications for Infants With Patent Ductus Arteriosus

Bernard Thébaud, MD, PhD; Evangelos D. Michelakis, MD; Xi-Chen Wu, PhD; Rohit Moudgil, MSc; Michael Kuzyk, PhD; Jason R.B. Dyck, PhD; Gwyneth Harry, MSc; Kyoko Hashimoto, BSc; Alois Haromy, BSc; Ivan Rebeyka, MD; Stephen L. Archer, MD

Background—Oxygen (O₂)-sensitive K⁺ channels mediate acute O₂ sensing in many tissues. At birth, initial functional closure of the ductus arteriosus (DA) results from O₂-induced vasoconstriction. This mechanism often fails in premature infants, resulting in persistent DA, a common form of congenital heart disease. We hypothesized that the basis for impaired O₂ constriction in preterm DA is reduced expression and function of O₂-sensitive, voltage-gated (Kv) channels.

Methods and Results—Preterm rabbit DA rings have reduced O₂ constriction (even after inhibition of prostaglandin and nitric oxide synthases), and preterm DA smooth muscle cells (DASMCs) display reduced O₂-sensitive K⁺ current. This is associated with decreased mRNA and protein expression of certain O₂-sensitive Kv channels (Kv1.5 and Kv2.1) but equivalent expression of the L-type calcium channel. Transmural Kv1.5 or Kv2.1 gene transfer “rescues” the developmental deficiency, conferring O₂ responsiveness to preterm rabbit DAs. Targeted SMC Kv1.5 gene transfer also enhances O₂ constriction in human DAs.

Conclusions—These data demonstrate a central role for developmentally regulated DASMC O₂-sensitive Kv channels in the functional closure of the DA. Modulation of Kv channels may have therapeutic potential in diseases associated with impaired O₂ responsiveness, including persistent DA. (Circulation. 2004;110:1372-1379.)

Key Words: gene therapy ■ heart defects, congenital ■ oxygen sensing ■ viruses

In the hypoxic environment in utero, the ductus arteriosus (DA), a vital fetal artery that connects the pulmonary artery to the aorta, is widely patent and shunts more than half of the cardiac output of the right heart away from the nonventilated lung into the umbilicalplacental circulation, where gas exchange takes place.¹ Within minutes of birth, the increased P O₂ constricts the DA² and simultaneously dilates the pulmonary circulation.³ The response of the term DA to O₂ rarely fails; however, in humans, ~50% of preterm DAs do not close, despite adequate oxygenation.⁴ Failure of DA closure after birth complicates the hospital course of preterm infants. Patent DA is associated with increased incidence of chronic lung disease, intraventricular hemorrhage, and necrotizing enterocolitis.⁵ Both medical and surgical interventions to close the DA are associated with additional morbidity.⁶ The crucial role of endothelium-derived relaxing and constricting factors in regulating DA tone is well established.⁶ However, O₂ constricts the DA in the absence of endothelium,⁷ suggesting that the core of the O₂-sensing mechanism is intrinsic to the DA smooth muscle cell (DASMC). K⁺ channels in the vascular SMCs regulate vascular tone through modulation of the membrane potential (E_M).⁸ Closure of K⁺ channels leads to vasoconstriction by depolarizing E_M. This opens voltage-gated L-type calcium channels and increases extracellular calcium influx. In term rabbit⁹ and human¹⁰ DA, O₂-induced constriction is initiated by the inhibition of O₂- and 4-aminopyridine (4-AP)-sensitive, voltage-gated, K⁺ channels (Kv), including Kv1.5 and Kv2.1. We hypothesize that an important cause of impaired O₂ constriction in preterm rabbit DA is ionic immaturity, ie, reduced expression and function of O₂- and 4-AP-sensitive Kv channels.

Methods

The Animal Welfare and the Human Studies committees of the University of Alberta approved all procedures.
Rabbit DAs
New Zealand White rabbits were delivered by caesarian section at gestational day 26 (preterm) or 30 (term) as previously described. The DA was used within 5 minutes of harvest.

Tension Measurements in Isolated Preterm and Term DA Rings
The isolated DA was placed in an organ bath and equilibrated in hypoxic Krebs solution (P\textsubscript{O\textsubscript{2}} = 31±1 mm Hg, to mimic in utero conditions) as previously described. We compared O\textsubscript{2}–mediated DA constriction by exposing the DAs to increasing P\textsubscript{O\textsubscript{2}} concentrations. We also compared the contractile force between term and preterm DAs to several K\textsuperscript{+} channel antagonists (see online data supplement).

Whole-Cell Patch-Clamp
The effects of O\textsubscript{2} and K\textsuperscript{+} channel blockade on whole-cell K\textsuperscript{+} current (I\textsubscript{K}) and E\textsubscript{M} were measured in freshly dispersed DASMCs, using solutions and recording protocols as previously described. DASMCs were voltage-clamped at −70 mV, and currents were evoked by steps of 200-ms duration from −70 to +50 mV. The effects of O\textsubscript{2}, 4-AP (1 to 5 mmol/L), and iberiotoxin (IBTX, 100 nmol/L) on E\textsubscript{M} were recorded in current-clamp mode, and their effects on I\textsubscript{K} were studied in voltage-clamp mode using stepped depolarizations of 200 ms from −70 to +50 mV.

Confocal Microscopy
To qualitatively compare E\textsubscript{M} in intact term versus preterm DA rings, D\textsubscript{i}B\textsubscript{A}C\textsubscript{4} (20 μmol/L, bis-barbituric acid oxonol) fluorescent emission was measured using confocal microscopy (green fluorescent intensity increases with depolarization). D\textsubscript{i}B\textsubscript{A}C\textsubscript{4}-loaded intact DA rings were stimulated with multiphoton excitation at 740 nm, and emission was measured in the green range, 505 to 530 nm. The acute change in E\textsubscript{M} that occurred in response to 4-AP or a switch in P\textsubscript{O\textsubscript{2}} (30 to 120 mm Hg) was measured.
Laser Capture Microdissection and qRT-PCR

To quantify Kv mRNA in freshly harvested DA media, laser capture microdissection (LCM) was performed on frozen sections using the microdissector PixCell II (Arcturus Engineering), and the LCM specimens were analyzed by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) using validated primers for Kv1.5 and Kv2.1. Total RNA was extracted using RNeasy Mini Kit (Qiagen). Kv mRNA levels were expressed as $2^{\Delta\Delta C_{T}}$, which normalizes the Kv expression to that of a reporter (GAPDH) and a calibrator sample.

Immunoblotting

DAs were flash-frozen in liquid N$_2$ and homogenized in buffer containing an antiprotease cocktail (Sigma) before being run on 7.5% to 10% gels. Expression was quantified by use of densitometry.

Adenovirus Construct

Recombinant, replication-deficient adenoviral (Ad) vectors using a cytomegalovirus promoter (serotype 5, Ad5) and encoding genes for green fluorescent protein (Ad5-GFP), human Kv1.5 plus GFP (Ad5-GFP-Kv1.5), and rat Kv2.1 plus GFP (Ad5-GFP-Kv2.1) have been previously described to cause transmural, transgene expression. Total RNA was extracted using RNeasy Mini Kit (Qiagen). Kv mRNA levels were expressed as $2^{\Delta\Delta C_{T}}$, which normalizes the Kv expression to that of a reporter (GAPDH) and a calibrator sample.

Ionically Remodeled, Human-Term DAs

DAs were obtained from 2 term infants with hypoplastic left hearts during palliative surgery. The ionically remodeled human DA model is one in which 24 hours in tissue culture at a P O$_2$ of 100 mm Hg selectively impairs expression of certain Kv channels (Kv1.5, Kv2.1) and reduces O$_2$ and 4-AP, but not phenylephrine, constriction. Expression of other channels is unaltered or increased (Kv1.1, Kir2.1, TASK).

Ex Vivo Gene Transfer

Preterm rabbit DAs were incubated for 60 minutes with vehicle or a vector (Ad5-GFP, Ad5-GFP-Kv1.5, Ad5-GFP-Kv2.1). Human-term DAs were incubated with vehicle or Ad5-SM22a-Kv1.5myc (final viral concentration, 1.5x10$^9$ pfu/mL). The DAs were then incubated in tissue culture media in hypoxia (rabbit DAs, P O$_2$ 45 mm Hg) or normoxia (human DA, P O$_2$ 120 mm Hg). The effects of gene transfer on the O$_2$ and 4-AP vasoconstriction and gene expression were studied after 24 hours.

Statistics

Values are expressed as mean±SEM. Intergroup comparisons were performed with Student’s t test or a factorial, repeated-measures ANOVA, as appropriate. Fisher’s protected least significant difference test was used for post hoc comparisons. A probability value of $P<0.05$ was considered to be statistically significant. Unless otherwise stated, the sample size was $>5$.

Results

O$_2$-Induced Constriction Is Weak in Preterm DAs

In both term and preterm DAs, O$_2$-induced constriction increased in proportion to P O$_2$ (Figure 1, A and B). Although enhanced by N$^\circ$-nitro-L-arginine methyl ester (L-NAME) and...
meclofenamate (Figure 1B), O₂-induced constriction remained significantly weaker in preterm DA (Figure 1B).

Kv Channel Inhibition Causes Weak Constriction in Preterm DAs
Preterm DAs constricted less than term DAs to the Kv channel inhibitor 4-AP (Figure 1, C and D). 4-AP constriction was marginally increased by pretreatment with L-NAME and meclofenamate (Figure 1D, P/H11005 NS). Constriction in response to IBTX and glyburide, inhibitors of large-conductance calcium-sensitive K⁺ channels (BK Ca) and ATP-sensitive K⁺ channels (K ATP), respectively, was weak and was comparable in preterm and term DAs (Figure 1D).

Preterm DASMCs Display Less O₂-Sensitive Iₖ and Are Relatively Depolarized
In term DAs, O₂ and 4-AP significantly inhibited Iₖ (Figure 2, A and B), whereas IBTX induced minimal additional reduction in Iₖ. Thus, in term DASMCs, the majority of the Iₖ is Kv current, particularly at negative potentials. In contrast, in preterm DA, O₂ and 4-AP did not cause any decrease in Iₖ at potentials near resting Eₘ (Figure 2, A and B). Hypoxic Eₘ was depolarized in preterm versus term DASMCs whether Eₘ was estimated in DA rings using a potentiometric dye (Figure 3A) or measured directly using current-clamp technique (Figure 3B). O₂ and 4-AP significantly depolarized term but not preterm DAs, whereas IBTX had minimal effect on Eₘ (Figure 3).

Preterm DAs Express Less Kv1.5 and Kv2.1
qRT-PCR analysis of LCM samples revealed significantly reduced Kv1.5 mRNA in preterm versus term DASMCs (Figure 4, A–C). Kv1.5 expression increased with maturation in both the endothelium and SMCs. Likewise, immunoblotting showed decreased Kv1.5 and Kv2.1 protein expression in preterm compared with term DAs (Figure 4D). Expression of the L-type calcium channel, the downstream effector in this pathway, was unaltered in preterm DAs.

Kv Gene Transfer of O₂-Sensitive Kv Channels “Rescues” the Maturational Kv Deficiency
Infection with either Kv1.5 or Kv2.1 vectors increased channel expression and O₂- and 4-AP–induced constriction, compared with vehicle- or Ad5-GFP–treated DAs (Figure 5, A and B). qRT-PCR could reliably detect transgene expression because the probes were species-specific, and so human
Kv1.5 and rat Kv2.1 were uniquely identified in a rabbit background. The SM22α–promoted Kv1.5 virus increased Kv1.5 expression only in SMCs, as evidenced by SMC myc protein expression, which did not cross into the von Willebrand factor–positive endothelium (Figure 6A). Targeted SMC Kv transfer enhanced O₂ constriction in ionically remodeled human DAs (Figure 6B).

Discussion
This is the first study to demonstrate that decreased O₂ responsiveness of the preterm DA results from an immature Kv channel effector mechanism in the SMCs that is required for normal O₂ sensing. This mechanism may explain failure of DA closure in preterm infants.

Although all cells of aerobic organisms are sensitive to extreme hypoxia, certain cardiovascular and pulmonary cells display specialized O₂-sensing systems that are characterized by their rapid responses to physiological levels of hypoxia and the fact that they elicit homeostatic responses that optimize O₂ uptake, distribution, or delivery. Although the O₂ sensor systems vary among tissues, they generally include a sensor (often the mitochondria or a NADPH oxidase) that alters the production of a mediator (often a reactive O₂ species) in response to changes in P O₂. The mediator, in turn, alters the function of 1 or more effectors, which ultimately elicits the physiological response to altered P O₂. It is striking that in each of these O₂-sensitive tissues (the carotid body, pulmonary artery SMCs, neuroepithelial body, and adrenomedullary cell), the effector is a K⁺ channel.

The fetus has a unique O₂ sensor in the DA. In utero, the DA is tonically relaxed in its normal hypoxic environment (P O₂ 20 to 40 mm Hg). At birth, DA closure, crucial for postnatal adaptation, is initiated within minutes by an increase in P O₂. In full-term newborns, this mechanism rarely fails. However, in preterm infants, persistent DA is a common complication. The O₂-sensing system within the human-term DASMCs is composed of a sensor, the proximal electron transport chain of the mitochondria, which produces a mediator (H₂O₂) that inhibits redox-sensitive Kv channels promoting constriction.

This study first validated the model of the preterm DA as one with impaired O₂ constriction. Preterm DAs constrict less to O₂ than term DAs, even after NO and prostaglandin (PG) H synthase inhibition (Figure 1), confirming previous reports of developmental changes in DA O₂ responsiveness in several species. Furthermore, the increase in P O₂ at birth exerts a direct effect on the DASMCs, initiating constriction by inhibiting Kv channels and thereby causing membrane depolarization (Figures 2 and 3).

Next, because Kv channels constitute the final common step in the proposed O₂-sensor pathway of the DA (Figure 7),
we hypothesized that the weak O\textsubscript{2} response of the preterm DA resulted from immature expression of O\textsubscript{2}-sensitive Kv channels. Two complementary approaches confirm that the preterm rabbit DA is (compared with term DAs) deficient in specific O\textsubscript{2}-sensitive Kv channels, Kv1.5 and Kv2.1. Immunoblotting showed reduced Kv1.5 and Kv2.1 protein expression in preterm versus term DA (Figure 4). Second, qRT-PCR of LCM medial samples demonstrated decreased Kv1.5 mRNA expression in the preterm versus term DASMCs (Figure 4). Furthermore, gene transfer of Kv1.5 and Kv2.1 not only restored channel expression but also enhanced O\textsubscript{2} responsiveness in the preterm DA, providing further proof of concept for the importance of these Kv channels as O\textsubscript{2}-sensing effectors in the DA (Figure 6).

Although the rabbit DA data establish maturational changes in Kv1.5 and Kv2.1 as crucial to the rabbit DA O\textsubscript{2} response and suggest that the SMC is the crucial site for these O\textsubscript{2}-sensitive channels, they do not identify the cellular locus with certainty and do not directly address the relevance of these findings to the human DA. Because fresh, preterm human DA is virtually unobtainable, we assessed these issues in a previously characterized model, the ionically remodeled human DA.\textsuperscript{12} Although not identical to a preterm DA, similarities of this model with a preterm DA include the preferential loss of O\textsubscript{2} sensitivity and downregulation of certain Kv channels. Nonetheless, the relevance of this new model remains to be independently confirmed by others, particularly because it uses term DAs, which most likely have maturational changes in proteins other than ion channels. We demonstrate that selective DASMC gene transfer of the human Kv1.5 gene restores 4-AP and O\textsubscript{2}-induced constriction in human DA (Figures 5 and 6).

These findings do not diminish the importance of other maturational changes in DA biology that are known to occur. The importance of endothelium-derived mediators, such as PGs and endothelin-1 in the regulation of DA tone, are well established\textsuperscript{6} and clinically relevant.\textsuperscript{4} Inhibition of cyclooxygenase-1 and cyclooxygenase-2 by pharmacological\textsuperscript{13} and genetic\textsuperscript{32} manipulation causes failure of the DA to close. Likewise, in the fetal sheep, sensitivity to the vasodilators PGE\textsubscript{2} and PGI\textsubscript{2} decreases near term\textsuperscript{33} and PGI\textsubscript{2} synthase activity decreases,\textsuperscript{34} thereby permitting O\textsubscript{2} constriction. Endothelin (ET)-1 may promote DA closure at birth, although its role remains controversial. The DA synthesizes ET-1 as Po\textsubscript{2} increases, and ET-1 does constrict the DA in vitro\textsuperscript{35}; however, in vivo, ET-1 receptor blockade does not inhibit O\textsubscript{2} constriction.\textsuperscript{36} Furthermore, although ET-knockout mice have persistent but diminished acute O\textsubscript{2} constriction, their DAs close normally.\textsuperscript{37} Moreover, in human DAs, effective inhibition of the ET pathway does not inhibit O\textsubscript{2} constriction.\textsuperscript{10}
These data suggest that O2 responsiveness of the term DA is promoted by reduced synthesis and reduced responsiveness to endothelium-derived vasodilators and increased production of endothelium-derived vasoconstrictors. The relative importance of altered PG metabolism and O2-sensing immaturity as a cause of DA patency in preterm infants is unknown. PGs have a major role in dilating the DA in near-term rats, whereas NO has a minor role; the reverse is true in premature DAs, in which the NO pathway predominates. Accordingly, we show that PGH synthase and NO synthase inhibition enhanced O2 constriction more in preterm than in term DAs. Nonetheless, O2 constriction in the preterm DAs remained weaker, despite inhibition of PGH synthase and NO synthase (Figure 1). Responsiveness of the contractile apparatus itself may also play a role in the potential of the DA to constrict. The fetal rabbit DA is more sensitive to calcium than fetal rabbit aorta, pulmonary artery, and various nonvascular SMCs from adult animals. The precise mechanism of this is unknown, but the fetal rabbit DA expresses precociously the mature adult-specific vascular SM myosin heavy chain isoform SM2, which begins to be expressed in rabbit aorta and pulmonary artery only after birth. Likewise, maturational changes in calcium channels may account for differences in O2 constriction. The weaker constriction of preterm DA to 4-AP and O2 is disproportionately larger compared with the maturational change in phenylephrine-induced vasoconstriction (Figure 1, inset), indicating that the K^+ channel matura-
tion deficiency is not exclusively a result of an immature contractile apparatus. Thus, Kv and endothelium-derived modulators most likely act in concert to ensure adequate adaptation to extraterine life and survival of the newborn, because DA closure rarely fails in term infants.

We conclude that the Kv channel effector mechanism for O2 sensing is immature in the preterm DA (Figure 7). Modulation of expression and activity of O2-sensitive Kv channels may have therapeutic potential for persistent DA and other diseases associated with disordered O2 sensing.

Acknowledgments

Drs Thébaut and Dyck are supported by the Alberta Heritage Foundation for Medical Research (AHFMR) and the Canadian Institutes for Health Research (CIHR). Drs Michelakis and Archer are supported by the AHFMR, the Canadian Foundation for Innovation, and the Heart and Stroke Foundation of Canada and CIHR. Dr Archer is supported by NIH-R01-HL071115. We thank Dr Jeanne Nerbonne for the Kv2.1 cDNA.

References

9. Tristani-Firouzi M, Reeve HL, Tolarova S, et al. Oxygen-induced constriction of rabbit ductus arteriosus occurs via inhibition of a 4-amino-
11. Thebaud B, Michelakis E, Wu XC, et al. Sildenafil reverses O2 constriction of the rabbit ductus arteriosus by inhibiting type 5 phosphodi-
13. Archer SL, Gragasin FS, Wu X, et al. Endothelium-derived hyperpolarizing factor in human internal mammary artery is 11,12-
14. Pozeg ZI, Michelakis Ed, McMurtry MS, et al. In vivo gene transfer of the O2-sensitive potassium channel Kv1.5 reduces pulmonary hyper-

Figure 7. Proposed mechanism for impaired O2 constriction. Preterm DA has reduced O2 constriction because of decreased function/expression of O2-sensitive Kv channels, Kv1.5 and Kv2.1. O2 constriction can be enhanced by Kv1.5 and Kv2.1 gene transfer.

Bernard Thébaud, Evangelos D. Michelakis, Xi-Chen Wu, Rohit Moudgil, Michael Kuzyk, Jason R.B. Dyck, Gwyneth Harry, Kyoko Hashimoto, Alois Haromy, Ivan Rebeyka and Stephen L. Archer

Circulation. 2004;110:1372-1379; originally published online September 7, 2004;
doi: 10.1161/01.CIR.0000141292.28616.65

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/11/1372

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2004/09/13/01.CIR.0000141292.28616.65.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/