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

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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. Within minutes of birth, the increased Po2 constricts the DA and simultaneously dilates the pulmonary circulation. The response of the term DA to O₂ rarely fails; however, in humans, ~50% of preterm DAs do not close, despite adequate oxygenation. 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. Both medical and surgical interventions to close the DA are associated with additional morbidity. The crucial role of endothelium-derived relaxing and constricting factors in regulating DA tone is well established. However, O₂ constricts the DA in the absence of endotheli-
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 O₂/H₁₁₀₀₅ 31/H₁₁₀₀₆ 1 mm Hg, to mimic in utero conditions) as previously described. We compared O₂-mediated DA constriction by exposing the DAs to increasing P O₂ concentrations. We also compared the contractile force between term and preterm DAs to several K⁺ channel antagonists (see online data supplement).

Whole-Cell Patch-Clamp
The effects of O₂ and K⁺ channel blocker on whole-cell K⁺ current (I_K) and E_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 O₂, 4-AP (1 to 5 mmol/L), and iberiotoxin (IBTX, 100 nmol/L) on E_M were recorded in current-clamp mode, and their effects on I_K were studied in voltage-clamp mode using stepped depolarizations of 200 ms from −70 to +50 mV.

Confocal Microscopy
To qualitatively compare E_M in intact term versus preterm DA rings, DiBAC₄ (20 µmol/L, bis-barbituric acid oxonol) fluorescent emission was measured using confocal microscopy (green fluorescent intensity increases with depolarization). DiBAC₄-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_M that occurred in response to 4-AP or a switch in P O₂ (30 to 120 mm Hg) was measured.

Figure 1. Reduced O₂ and K⁺ channel blocker–induced constriction in preterm vs term DA rings. Representative tracings and mean data showing decreased O₂–induced (A, B) and 4-AP–induced (C, D) constriction in preterm vs term DAs. In preterm DAs, augmentation of O₂–induced constriction by L-NAME and meclofenamate (Meclo) was significantly greater than in term DAs, indicating a greater role of these vasodilator pathways in preterm DAs. Nonetheless, greater O₂ constriction in term DAs persists despite inhibition of PGH and nitric oxide synthases.

Figure 2. Response of I_K to O₂ and K⁺ channel blocker in preterm and term DASMCs. Representative patch–clamp recordings and mean current density–voltage plots show significant O₂–induced (A) and 4-AP–induced (B) inhibition of whole-cell I_K in term rabbit DASMCs. Conversely, O₂ (A) and Kv channel blocker 4-AP (B) induced only a small decrease in I_K in preterm DASMCs. Upper and lower insets show subtraction analysis identifying magnitude of current inhibited by O₂ and 4-AP, respectively.
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 $2^{-\Delta\Delta Ct}$, 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 $2^{-\Delta\Delta Ct}$, 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-SM22α-Kv1.5myc (final viral concentration, $10^9$ 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$^\bullet$-nitro-L-arginine methyl ester (L-NAME) and
meclomenamate (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 meclomenamate (Figure 1D, \( P = \text{NS} \)). 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 \( I_K \) and Are Relatively Depolarized

In term DAs, \( O_2 \) and 4-AP significantly inhibited \( I_K \) (Figure 2, A and B), whereas IBTX induced minimal additional reduction in \( I_K \). Thus, in term DASMCs, the majority of the \( I_K \) is Kv current, particularly at negative potentials. In contrast, in preterm DA, \( O_2 \) and 4-AP did not cause any decrease in \( I_K \) at potentials near resting \( E_m \) (Figure 2, A and B). Hypoxic \( E_m \) was depolarized in preterm versus term DASMCs whether \( E_m \) was estimated in DA rings using a potentiometric dye (Figure 3A) or measured directly using current-clamp technique (Figure 3B). \( O_2 \) and 4-AP significantly depolarized term but not preterm DAs, whereas IBTX had minimal effect on \( E_m \) (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 \( O_2 \)- 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,13,16 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.17–20 Although the O2 sensor systems vary among tissues, they generally include a sensor (often the mitochondria or a NADPH oxidase12,21–24) that alters the production of a mediator (often a reactive O2 species12–23) in response to changes in Po2.17 The mediator, in turn, alters the function of 1 or more effectors, which ultimately elicits the physiological response to altered Po2. 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.17,18,20,25

The fetus has a unique O2 sensor in the DA. In utero, the DA is tonically relaxed in its normal hypoxic environment (Po2 20 to 40 mm Hg).1,2 At birth, DA closure, crucial for postnatal adaptation, is initiated within minutes by an increase in Po2. In full-term newborns, this mechanism rarely fails. However, in preterm infants, persistent DA is a common complication.4,5,26 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.12

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.27–29 Furthermore, the increase in Po2 at birth exerts a direct effect on the DASMCs, initiating constriction by inhibiting Kv channels and thereby causing membrane depolarization (Figures 2 and 3).7,9,10,12,30

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 O₂ response of the preterm DA resulted from immature expression of O₂-sensitive Kv channels. Two complementary approaches confirm that the preterm rabbit DA is (compared with term DAs) deficient in specific O₂-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 O₂ responsiveness in the preterm DA, providing further proof of concept for the importance of these Kv channels as O₂-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 O₂ response and suggest that the SMC is the crucial site for these O₂-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. Although not identical to a preterm DA, similarities of this model with a preterm DA include the preferential loss of O₂ 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 DASM C gene transfer of the human Kv1.5 gene restores 4-AP and O₂-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 and clinically relevant. Inhibition of cyclooxygenase-1 and cyclooxygenase-2 by pharmacological and genetic manipulation causes failure of the DA to close. Likewise, in the fetal sheep, sensitivity to the vasodilators PGE₂ and PGI₂ decreases near term and PGI₂ synthase activity decreases, thereby permitting O₂ constriction. Endothelin (ET)-1 may promote DA closure at birth, although its role remains controversial. The DA synthesizes ET-1 as Po₂ increases, and ET-1 does constrict the DA in vitro; however, in vivo, ET-1-receptor blockade does not inhibit O₂ constriction. Furthermore, although ET-knockout mice have persistent but diminished acute O₂ constriction, their DAs close normally. Moreover, in human DAs, effective inhibition of the ET pathway does not inhibit O₂ constriction.
These data suggest that $O_2$ responsiveness of the term DA is promoted by reduced synthesis and reduced responsiveness to endothelium-derived vasoconstrictors and increased production of endothelium-derived vasoconstrictors. The relative importance of altered PG metabolism and $O_2$-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 $O_2$ constriction more in preterm than in term DAs. Nonetheless, $O_2$ 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 $O_2$ constriction. The weaker constriction of preterm DA to 4-AP and $O_2$ is disproportionately larger compared with the maturational change in phenylephrine-induced vasoconstriction (Figure 1, inset), indicating that the $K^+$ channel maturational change 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 $O_2$ sensing is immature in the preterm DA (Figure 7). Modulation of expression and activity of $O_2$-sensitive $Kv$ channels may have therapeutic potential for persistent DA and other diseases associated with disordered $O_2$ sensing.

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