Blunted Hypoxic Pulmonary Vasoconstriction in Experimental Neonatal Chronic Lung Disease

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Rationale: Neonatal chronic lung disease (CLD), caused by prolonged mechanical ventilation (MV) with O₂-rich gas, is the most common cause of long-term hospitalization and recurrent respiratory illness in extremely premature infants. Recurrent episodes of hypoxemia and associated ventilator adjustments often lead to worsening CLD. The mechanism that causes these hypoxemic episodes is unknown. Hypoxic pulmonary vasoconstriction (HPV), which is partially controlled by O₂-sensitive voltage-gated potassium (K_v) channels, is an important adaptive response to local hypoxia that helps to match perfusion and ventilation in the lung.

Objectives: To test the hypothesis that chronic lung injury (CLI) impairs HPV.

Methods: We studied preterm lambs that had MV with O₂-rich gas for 3 weeks and newborn rats that breathed 95%-O₂ for 2 weeks, both of which resulted in airspace enlargement and pulmonary vascular changes consistent with CLD.

Measurements and Main Results: HPV was attenuated in preterm lambs with CLI after 2 weeks of MV and in newborn rats with CLI after 2 weeks of hyperoxia. HPV and constriction to the K_v1.x-specific inhibitor, correolide, were preferentially blunted in excised distal pulmonary arteries (dPAs) from hyperoxic rats, whose dPAs exhibited decreased K_v1.5 and K_v2.1 mRNA and K⁺ current. Intrapulmonary gene transfer of K_v1.5, encoding the ion channel that is thought to trigger HPV, increased O₂-sensitive K⁺ current in cultured smooth muscle cells from rat dPAs, and restored HPV in hyperoxic rats.

Conclusions: Reduced expression/activity of O₂-sensitive K_v channels in dPAs contributes to blunted HPV observed in neonatal CLD.

Keywords: hyperoxia; lung injury; gene therapy; potassium channels; bronchopulmonary dysplasia

Premature infants account for more than 12% of all U.S. births (National Institute of Medicine report, July 13, 2006, <http://www.iom.edu/CMS/3740/25471/35813.aspx>). Recent advances in obstetrical management and neonatal intensive care have increased survival of extremely premature infants (< 1,000 g). These infants are prone to respiratory failure, for which they receive mechanical

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Recurrent episodes of hypoxemia and associated ventilator adjustments often lead to worsening of neonatal chronic lung disease (CLD). The mechanism that causes these hypoxemic episodes is unknown.

What This Study Adds to the Field

Blunted hypoxic pulmonary vasoconstriction (HPV) due to reduced expression/activity of O₂-sensitive K_v channels may explain hypoxemic episodes. Restoring K_v channel activity restores HPV. Modulation of K_v channels may improve management of neonatal CLD.

ventilation (MV) with O₂-rich gas. Prolonged exposure to cyclic stretch and high concentrations of inspired O₂ often leads to a chronic form of lung disease (CLD) that remains the leading cause of long-term hospitalization and recurrent respiratory illness in infants born at less than 28 weeks of gestation (1).

Prominent histologic features of CLD include arrested alveolar growth and decreased capillary density (2–4). Extremely premature infants with this condition frequently experience severe episodes of hypoxemia. The mechanism accounting for these hypoxemic events in neonates is poorly understood, but more likely related to either acute changes in airway resistance (5, 6) or increased pulmonary vasoconstriction. It is unknown whether impaired ventilation in CLD may lead to mismatching of ventilation and perfusion, which could contribute to episodic hypoxemia and cyanosis.

Hypoxic pulmonary vasoconstriction (HPV) is a unique and important physiological response that facilitates matching of ventilation and perfusion in the lung. In segmental hypoxia, as occurs in CLD, constriction of distal pulmonary arteries (dPAs), which regulate vascular resistance, diverts blood from poorly ventilated areas to well-ventilated areas of the lung, thereby optimizing oxygenation, usually without increasing pulmonary vascular resistance (PVR) (7).

Although the mechanisms that control HPV have not been completely elucidated (8–10), it appears that hypoxia initiates HPV through a redox mechanism (11, 12) that inhibits K⁺ channels in pulmonary artery smooth muscle cells (PASMC) (13). O₂-sensitive voltage-gated K⁺ channels (K_v) play an important role in regulating HPV through modulation of cell membrane potential (E_M) (14). During HPV, hypoxia closes K_v channels. This leads to membrane depolarization, opening of voltage-gated L-type calcium (Ca²⁺) channels, and increased influx of extracellular Ca²⁺, thereby causing pulmonary vasoconstriction. Inhibition of O₂-sensitive K_v channels K_v1.5 and K_v2.1 contribute to initiation of HPV (15, 16).

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Figure 1. Pulmonary vascular response to hypoxia induced by a reduction in FiO_2 sufficient to lower PaO_2 to less than 40 mm Hg at the end of Weeks 1, 2, and 3 of mechanical ventilation in preterm lambs. Pulmonary vascular resistance increased significantly during steady-state hypoxia at the end of Week 1, but this response was lost at the end of Weeks 2 and 3. Values are mean and SD. *Significant difference compared with baseline, $P < 0.05$.

Thus, we hypothesize that HPV is impaired in neonatal CLD and that the basis for impaired HPV is reduced expression and function of O_2 -sensitive K_v channels.

Some of the results of these studies have been previously reported in the form of an abstract (17).

METHODS

Detailed methods are provided in the online supplement.

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Utah in Salt Lake City (preterm lambs) and the University of Alberta (newborn rats).

Chronic Lung Injury in Preterm Lambs

Preterm lambs were mechanically ventilated for 3 weeks and chronically catheterized as previously described (18–21). PVR was measured during a 2-hour steady-state baseline period, followed by a 2-hour steady-state period of hypoxemia, induced by lowering the FiO_2 so that PaO_2 decreased to less than 40 mm Hg.

Chronic Lung Injury in Newborn Rats

Sprague-Dawley rat pups were exposed to normoxia (21% O_2 , control group) or hyperoxia (95% O_2 , O_2 -chronic lung injury [CLI] group) from birth to Postnatal Day (P)14 in sealed Plexiglas chambers with continuous O_2 monitoring (22–25). After P14, pups were killed and lungs processed for various analyses.

Exposure of newborn rats to hyperoxia from birth to P14 during the critical period of alveolar development impairs alveolar growth (larger but fewer alveoli) and is associated with right ventricular hypertrophy, an indirect marker of pulmonary hypertension. These results have been previously reported (26).

Echo-Doppler in Newborn Rats

Pulmonary artery acceleration time (PAAT) (Vevo 770B; VisualSonics, Inc., Toronto, ON, Canada) was measured from the onset of the pulmonary flow to its peak as previously described (26). For the HPV challenge, animals were briefly (~30 s) exposed to hypoxia (2.5% O_2) until a saturation of approximately 60% (MouseOx; Starr Life Sciences Corp., Oakmont, PA) was obtained.

Figure 2. (A) Echo-Doppler showing that the pulmonary arterial acceleration time (PAAT) is significantly decreased in O_2 -induced chronic lung injury (CLI) rats as compared with controls. During brief hypoxic exposure, PAAT decreased in controls but not in O_2 -induced CLI. (B) Representative trace and mean data showing that distal pulmonary arteries of O_2 -induced CLI do not constrict in response to hypoxia as compared with distal pulmonary arteries of room air-housed control rats.

Organ Bath Studies of Newborn Rat PAs

Third-generation PAs (diameter < 100 μ m, length = 2 mm, here referred to as dPAs) were mounted in a wire myograph (MyoDaq; Danish Myo Technology, Aarhus, Denmark) and bathed in Krebs-Henseleit buffer bubbled with 21% O₂–5% CO₂–balance N₂ (normoxia, PO₂ $\sim$ 120 mm Hg) or 5% O₂–5% CO₂–balance N₂ (hypoxia, PO₂ $\sim$ 50 mm Hg) maintained at 37°C, pH 7.35 to 7.45 (27, 28). Isometric changes in response to phenylephrine (10^{−5} mol/L), HPV, the nonspecific K_v channel inhibitor 4-aminopyridine (10 mM), a specific K_v1.x channel inhibitor correolide (100 μ M; gift from John Obenchain, Merck and Co., Inc., Rahway, NJ), the calcium-sensitive K⁺ channel (BK_{Ca}) inhibitor iberiotoxin (10^{−7} M), and the ATP-dependent K⁺ channel (K_{ATP}) inhibitor glyburide (5 $\times$ 10^{−6} M) and to 80 mmol/L KCl were compared between control and O₂-induced CLI PAs.

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on isolated dPAs from newborn rats as previously described (29, 30), using purchased primers for human K_v 1.5, rat K_v 1.5, and K_v 2.1, BK_{Ca} (Applied Biosystems, Foster City, CA). Levels of mRNA were normalized to a stable control gene (β 2 microglobulin [β 2MG]) and expressed as 2^{− $\Delta\Delta$ Ct} (Ct is cycle time) (29, 30).

Immunofluorescence

Immunofluorescence for anti-K_v1.5 antibody (P9357; Sigma-Aldrich, Oakville, ON, Canada) was performed on whole rat lung tissue as described (29, 30).

Adenovirus-Mediated Gene Transfer

A 2.1-kb cDNA fragment of K_v1.5 was obtained by reverse transcription of mRNA derived from the proximal PA of a cardiac transplant donor (29). The virus was given intratracheally to CLI rats at P10 (26).

Electrophysiology

PASMC were freshly isolated from dPAs of control, O₂-induced CLI, Ad5-K_v1.5-treated, and Ad5-green fluorescent protein (GFP)-treated rats. Their response to hypoxia was studied using a whole cell patch clamping technique. PASMC were voltage-clamped at −70 mV, and currents were evoked from −70 to +50 mV by steps of 200 milliseconds, as previously described (29).

Statistical Analysis

Data are expressed as mean $\pm$ SE, except where stated otherwise. Statistical analysis was performed using unpaired Student's *t* test or ANOVA, *post hoc* test (least significant difference) and Mann-Whitney as appropriate. Values were considered significant with *P* < 0.05.

RESULTS

HPV Is Blunted in Chronically Ventilated Newborn Lambs

Chronically mechanically ventilated preterm lambs had persistent baseline elevation of PVR for the entire 3 weeks. PVR increased significantly during hypoxia at the end of Week 1, but HPV was blunted during Weeks 2 and 3 (Figure 1).

HPV Is Blunted in Newborn Rats with O₂-induced CLI *In Vivo* and *Ex Vivo*

In vivo echo-Doppler studies showed that PAAT was significantly decreased in newborn rats with O₂-induced CLI as compared with normoxic controls (Figure 2A). During the hypoxic challenge, PAAT decreased in control pups, but not in pups with O₂-induced CLI (Figure 2A).

Figure 3. Contractile responses from distal pulmonary arteries (dPAs) of newborn rats. Representative trace and mean data $\pm$ SEM in response to (A) phenylephrine, (B) KCl, (C) the calcium-sensitive K⁺ channel inhibitor iberiotoxin and the ATP-sensitive K⁺ channel (K_{ATP}) inhibitor glyburide, (D) 4-AP, and (E) to the specific K_v1.x inhibitor correolide. (A) Phenylephrine-induced constriction is significantly decreased in dPAs from O₂-induced chronic lung injury (CLI) rats as compared with dPAs of room air-housed control rats. (E) The contractile response of O₂-induced CLI arteries to correolide is significantly decreased as compared with normoxic control arteries.

Figure 4. (A) K_v1.5 and K_v2.1 mRNA is decreased in O₂-induced chronic lung injury (CLI) distal pulmonary arteries compared with controls. (B) calcium-sensitive K⁺ channel (BK_{Ca}) mRNA expression in O₂-induced CLI distal pulmonary arteries compared with controls. Mean $\pm$ SEM of K_v1.5, 2.1, and calcium-sensitive K⁺ channel (BK_{Ca}) mRNA measured using quantitative real-time polymerase chain reaction.

In vitro, dPAs constricted in response to hypoxia while dPAs from newborn rats with O₂-induced CLI failed to constrict in response to hypoxia (Figure 2B).

Vascular Tone in Rat Distal Pulmonary Arteries

The contractile response to the α -agonist phenylephrine (10 mmol/L) was decreased significantly in O₂-induced CLI as compared with controls (Figure 3A). KCl constriction (Figure 3B) and the response to the BK_{Ca} and K_{ATP} channel blockers iberiotoxin and glyburide were similar between groups (Figure

3C). There was no difference in constriction in response to the K_v channel inhibitor 4-AP (10 mmol/L) (Figure 3D). In contrast, the vascular reactivity in O₂-induced CLI arteries to the specific K_v1.x inhibitor correolide was significantly decreased as compared with normoxic control arteries (Figure 3E).

K_v mRNA Expression Is Decreased in dPAs from Newborn Rats with CLI

Blunted HPV in newborn rats exposed to O₂ was associated with a decrease in mRNA expression of K_v1.5 and K_v2.1 in dPAs as

In vivo Echo-Doppler

Figure 5. (A) Echo-Doppler shows that the pulmonary arterial acceleration time (PAAT) is significantly decreased in O₂-induced chronic lung injury (CLI) rats, Ad5-K_v O₂-induced CLI, and Ad5-green fluorescent protein (GFP) O₂-induced CLI as compared with controls. During brief hypoxic exposure, PAAT is significantly decreased in controls and Ad5-K_v O₂-induced CLI but not in O₂-induced CLI and Ad5-GFP O₂-induced CLI groups. Representative trace and mean data $\pm$ SEM.

compared with controls (Figure 4A). There was no difference in BK_{Ca} mRNA expression between groups (Figure 4B).

Intratracheal Adenovirus-mediated K_v1.5 Gene Transfer Restores HPV in O₂-induced CLI in Newborn Rats

In vivo, echo-Doppler showed that PAAT was significantly decreased in newborn rats with O₂-induced CLI, Ad5-K_v O₂-induced CLI and Ad5-GFP O₂-induced CLI as compared with normoxic controls (Figure 5). During the hypoxic challenge, PAAT decreased only in Ad5-K_v O₂-induced CLI and control pups, but not in pups with O₂-induced CLI or Ad5-GFP O₂-induced CLI (Figure 5).

Intratracheal administration of Ad5-K_v1.5 increased K_v1.5 expression in PAs of O₂-induced CLI as shown by immunofluorescence imaging (Figure 6A) and qRT-PCR. qRT-PCR analysis using species-specific K_v primer revealed that human K_v1.5 was only expressed in Ad5-K_v1.5 transfected PAs (Figure 6B). In dPAs rings, Ad5-K_v1.5 gene transfer restored HPV as compared with Ad5-GFP dPAs and nontransfected O₂-induced CLI dPAs (Figure 6C).

Whole-cell K⁺ Current Is Restored by K_v1.5 Gene Transfer

Consistent with our *in vivo* and *in vitro* studies, whole-cell K⁺ current (I_K) was significantly decreased in PASMC from exper-

imental O₂-induced CLI compared with controls (Figures 7A and 7B). There was a loss of the outward rectifying portion of I_K (evident in a comparison of Figures 7A versus 7D as less total current and less upward deflection from the linear, ohmic current between voltage steps +10 to +70 mV). In control PASMC, I_K was inhibited by hypoxia (Figure 7A), whereas I_K in O₂-induced CLI remained unaffected (Figure 7B). CLI K_v1.5 gene therapy restored not only the net current, but specifically the outward rectifying portion of the current, so that Figures 7A and 7C look similar. This (together with the qRT data in Figure 4) indicates the loss of K⁺ current with CLI is largely due to loss of K_v current. Conversely, I_K was low in PASMC from Ad5-GFP-transfected animals (Figure 7D).

DISCUSSION

Many studies of CLI have focused on changes in lung mechanics and airway function (31–35) to explain hypoxic episodes in premature infants. However, little attention has been paid to the possible mismatch of ventilation and perfusion that may develop in CLD. In this study, we show for the first time *in vivo* and *in vitro* evidence that HPV is impaired in two experimental models, created in different species and by different approaches, that mimic human CLD. In addition, impaired HPV in newborn

Figure 6. Effective K_v1.5 gene transfer in distal pulmonary arteries (dPAs). (A) Confocal microscopy shows green autofluorescence of the elastic lamina in dPAs and red fluorescence for K_v1.5 protein; (B) quantitative real-time polymerase chain reaction (qRT-PCR) shows selective expression of Ad5-K_v1.5 mRNA for human K_v1.5 in infected arteries; (C) hypoxic pulmonary vasoconstriction (HPV) is restored in O₂-induced chronic lung injury (CLI) dPAs after intratracheal adenovirus-mediated K_v1.5 gene transfer. Representative trace and mean data ± SEM shows hypoxic-induced constriction in dPAs of Ad5-K_v1.5 infected rats. Ad5-green fluorescent protein (GFP) and O₂-induced CLI dPAs failed to constrict.

Figure 7. Representative patch-clamp recording and mean current density-voltage plots show hypoxic induced inhibition of whole-cell K^+ current (I_K) in control pulmonary artery smooth muscle cells (PASM) (A) but not in O_2 -induced chronic lung injury (CLI) PASM (B). Conversely, I_K is restored in PASM isolated from rats that had received intratracheal Ad5-Kv1.5 gene therapy (C), but not PASM of Ad5-green fluorescent protein (GFP) infected rats (D).

rats was associated with decreased expression of the O_2 -sensitive $K_v1.5$ channel. And finally, HPV was restored after adenovirus-associated gene transfer of $K_v1.5$. These findings support the notion that blunted HPV may contribute to the impaired gas exchange seen in CLD, and that therapeutic targeting of O_2 -sensitive K_v channels in the pulmonary circulation might restore HPV and thereby improve arterial oxygenation in lung vascular diseases associated with impaired O_2 responsiveness.

HPV is a unique and important physiologic response that facilitates matching of ventilation and perfusion in the lung. In segmental hypoxia the blood is diverted from poorly ventilated areas to well-ventilated segments of the lung, optimizing PO_2 without increasing systemic vascular resistance (14). Hypoxia depolarizes the PASM membrane and causes an increase in intracellular Ca^{2+} . This is related to inhibition of O_2 -sensitive K_v channels, particularly $K_v1.5$ (15, 16). Decreased HPV in chronic hypoxia results from loss of $K_v1.5$ and 2.1 (29, 36), and enhanced expression of $K_v1.5$ by adenovirus-mediated gene transfer restores HPV (29). Patients with pulmonary hypertension have decreased mRNA levels of the O_2 -sensitive $K_v1.5$ channel (37), suggesting an etiologic role for O_2 -sensitive K_v channels in the development of this disease.

During development, maturational changes in K^+ channel expression account for differences in O_2 constriction. In the fetus, the resting membrane potential in PASM is controlled by large-conductance BK_{Ca} , the predominant O_2 -sensitive channel (38). After birth, there is a shift to O_2 -sensitive K_v channels, presumably enhancing the capacity for HPV, without increasing pulmonary vascular resistance (38). Whether there is a delay in the maturational shift from BK_{Ca} to O_2 -sensitive K_v channels in experimentally induced BPD is unknown. O_2 toxicity and/or chronic ventilation, the two main causes contributing to lung injury in premature infants, may impede K_v channel expression/function in the pulmonary vasculature. More recently, the deleterious effect of even brief (30 min) hyperoxia (decreased vasodilation to nitric oxide and acetylcholine) on the pulmonary

circulation was reported (39, 40). Interestingly, the authors also show that lambs previously exposed for a brief period (30 min) to 100% O_2 did not have enhanced pulmonary vasoconstriction in response to subsequent hypoxic ventilation. Likewise, pulmonary vascular dysfunction, including pulmonary hypertension and altered response to inhaled nitric oxide in CLD, has been reported previously in preterm lambs (18–20, 41). Conversely, there is no information about the effects of hyperoxia on the regulation of K_v channels.

It is interesting that the PA and the ductus arteriosus (DA), although adjacent, behave in such opposite ways in response to O_2 and yet share a common pathway for O_2 sensing (10). dPAs exhibit HPV for optimizing gas exchange in the lung. In contrast, the DA is largely open in the hypoxic environment *in utero* and constricts when O_2 levels rise at birth, allowing the lung to take over its postnatal role of gas exchange. A patent DA is a common complication in premature infants. Decreased O_2 -induced constriction in the preterm rabbit DA is associated with decreased O_2 -sensitive I_K and O_2 -sensitive $K_v1.5$ expression, and overexpression of $K_v1.5$ in preterm rabbit DA restores O_2 -induced constriction (30). In this study we show similar results in dPAs, suggesting the importance for K_v channels in O_2 sensing.

Besides maturational changes in K_v channels, other factors may contribute to the altered response to hypoxia in experimental BPD. The mechanism by which hypoxia is sensed remains unknown. It has been proposed that HPV results from a redox sensor system present in dPAs, but also found in other O_2 -sensing organs (10). A sensor, the proximal portion of the mitochondria transport chain, produces a mediator (reactive oxygen species). This mediator would alter the effector (K^+ channels) through a process of reduction/oxidation. When the mitochondria detects a hypoxic environment, reactive oxygen species decrease thereby inhibiting redox-sensitive K^+ channels. Inhibitors of complex I and III of the electron transport chain in the mitochondria inhibit the formation of reactive oxygen species and mimic HPV in the isolated perfused lung (11, 42). In experimental BPD, a loss of O_2

sensing ability caused by chronic exposure to hyperoxia could alter mitochondrial function—reactive oxygen species production, making the pulmonary circulation unable to detect hypoxia and thus failing to constrict.

Abnormal vascular reactivity in older children with a history of severe BPD and pulmonary hypertension has been reported (43). Cardiac catheterization of these patients showed that the pulmonary vasculature remained responsive to O₂, but PVR was enhanced in response to hypoxia, suggesting increased HPV in this subpopulation of patients. The discrepancy with our data could be explained by differences in species, but is most likely due to a different timing in the assessment. HPV was assessed at a median of 5 years of age in the clinical study. Our study assessed animals during the neonatal period. As an adaptative and maturational response, the pulmonary vasculature with an initial blunted HPV could become hyperreactive to hypoxia later in life. Indications for such long-term consequences of injury during the perinatal period have been reported in the fawn-hooded rat (44) and more recently in rats exposed during fetal life to hypoxia showing that perinatal hypoxia alters the maturational shift in K⁺ channels and influences pulmonary vascular tone in adulthood (45). Remarkably, even ambient air may represent relative hyperoxia for infants born too early and impair lung development and function (46).

In conclusion, HPV is blunted in experimental CLD due, at least in part, to down-regulation of O₂-sensitive K_v channels. Hypoxic episodes in premature infants with CLD may result from impaired HPV. We speculate that restoration of O₂-sensitive K_v channels in the pulmonary circulation may decrease episodes of hypoxemia, facilitate management, and ultimately improve outcome of premature infants with CLD.

Conflict of Interest Statement: G.J.R.-P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.L.A. has a patent pending for the use of pyruvate dehydrogenase kinase inhibitors to treat human cancer. R.D.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.H.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.P.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.-C.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. F.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. X.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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