Pulmonary vascular $K^+$ channel expression and vasoreactivity in a model of congenital heart disease

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Received 31 October 2001; accepted in final form 29 May 2002

Cornfield, David N., Ernesto R. Resnik, Jean M. Herron, Olaf Reinhardt, and Jeffrey R. Fineman. Pulmonary vascular K+ channel expression and vasoreactivity in a model of congenital heart disease. Am J Physiol Lung Cell Mol Physiol 283: L1210–L1219, 2002. First published August 23, 2002; 10.1152/ajplung.00428.2001.—K+ channels play an important role in mediating pulmonary vasodilation caused by increased oxygen tension, nitric oxide, alkalosis, and shear stress. To test the hypothesis that lung K+ channel gene expression may be altered by chronic increases in pulmonary blood flow, we measured gene and protein expression of calcium-sensitive ($K_{Ca}$) and voltage-gated (Kv2.1) K+ channels, and a pH-sensitive K+ channel (TASK), in distal lung from fetal lambs in which an aortopulmonary shunt was placed at 139 days gestation. Under baseline conditions, animals with an aortopulmonary shunt showed elevated pulmonary artery pressure and pulmonary blood flow compared with twin controls. Hypoxia caused a greater increase in pulmonary vascular tone in shunt animals compared with controls. Alkalosis caused pulmonary vasodilation in control but not shunt animals. To determine lung K+ channel mRNA levels, we performed quantitative RT-PCR. In comparison with control animals, lung $K_{Ca}$ channel mRNA content was increased in shunt animals, whereas TASK mRNA levels were decreased. There was no difference in Kv2.1 mRNA levels. Channel protein expression was consistent with these findings. We conclude that, in the presence of elevated pulmonary blood flow, $K_{Ca}$ channel expression is increased and TASK is decreased.

pH-sensitive potassium channel; pulmonary hypertension; calcium-sensitive potassium channel; vascular reactivity

CONGENITAL HEART DISEASE remains a major source of morbidity and mortality in children. Although congenital heart disease is often associated with immunological, renal, or gastrointestinal abnormalities, pulmonary vascular disease is among the most frequent, and least well understood, pathological condition that complicates the management of children with congenital heart disease (19). As perinatal pulmonary vascular resistance decreases after birth, pulmonary blood flow increases (26). If pulmonary blood flow remains elevated, as is the case in many forms of congenital heart disease, pulmonary vascular smooth muscle extends into the periphery of the pulmonary circulation, extracellular matrix deposition increases (21), and endothelial-dependent pulmonary vasodilation is attenuated (9).

Recent evidence suggests that chronic elevations in pulmonary blood flow lead to an alteration in the production of vasoactive mediators from the pulmonary endothelium, with the production of vasodilator agents such as nitric oxide (NO) and prostacyclin diminished (17) and the production of the vasoconstrictor molecules thromboxane and endothelin increased (2, 3). Despite the lack of mechanistic understanding of abnormal pulmonary vasoreactivity associated with congenital heart disease, the physiology and histology have been well characterized. Initially, the ratio of pulmonary to systemic blood flow ($Q_p/Q_s$) is elevated. Over time, as intimal proliferation and smooth muscle cell hypertrophy occur (21), pulmonary vascular resistance increases and $Q_p/Q_s$ approaches one. Concomitant with the decrease in pulmonary blood flow, the response of the pulmonary circulation to vasodilator stimuli such as oxygen, adenosine, NO, and alkalosis is attenuated (4). The absence of the pulmonary vasodilator response may be so pronounced that surgical repair of the congenital heart disease is no longer feasible. Even when surgical repair of the heart lesion can be undertaken, postoperative management of children with congenital heart disease is frequently complicated by sudden increases in pulmonary vascular resistance (16).

Recent studies have demonstrated that pulmonary vascular K+ channels modulate the response of the pulmonary circulation to several critically important physiological stimuli. For example, in the perinatal pulmonary circulation, oxygen causes pulmonary vasodilation through kinase-dependent activation of Ca$^{2+}$-sensitive K+ channels ($K_{Ca}$; see Ref. 13). K+ channel inhibition with tetraethylammonium (TEA), but not...
glibenclamide, an ATP-sensitive K⁺ channel antagonist, blocks the pulmonary vasodilation caused by ventilation (32), suggesting that ventilation causes sustained and progressive pulmonary vasodilation through activation of TEA-sensitive K⁺ channels. NO, a vasoactive mediator that is essential for the normal transition of the pulmonary vasculature (1), causes perinatal pulmonary vasodilation, at least in part, through \( K_\text{Ca} \) channel activation (6, 29). Even the shear stress response, induced by compression of the ductus arteriosus during fetal life, involves \( K_\text{Ca} \) and voltage-dependent K⁺ (\( Kv \)) channel activation (31). In the adult pulmonary circulation, vasoconstriction in response to hypoxia is mediated by inactivation of a voltage-sensitive K⁺ channel (36).

Given these observations, we hypothesized that chronically elevated pulmonary blood flow 1) attenuates pulmonary vasodilation in response to oxygen and alkalosis; 2) modulates K⁺ channel expression; and 3) potentiates hypoxic pulmonary vasoconstriction. To test these hypotheses, an ovine model of congenital heart disease was utilized, physiological studies were performed, and pulmonary K⁺ channel mRNA levels were determined.

METHODS

Surgical Preparations and Care

Ewes. Twelve late twin-gestation mixed-breed Western pregnant ewes were operated under sterile conditions, as previously described (22). Ten animals, five shunt and five twin controls, were studied for hemodynamic measurements. Pulmonary tissue for molecular studies was obtained from 10 different animals, 5 shunted and their twin controls. Pulmonary vascular tissue for protein studies was obtained from two shunt and two twin control animals. Through a left lateral fetal thoracotomy, an 8.0-mm Goretx vascular graft (~2-mm length; W. L. Gore and Associates, Milpitas, CA) was anastomosed between the ascending aorta and main pulmonary artery of the fetus with 7.0 proline (Ethicon, Somerville, NJ) using a continuous suture technique, as previously described (22). After recovery from anesthesia, the ewe was returned to the cage with free access to food and water. Antibiotics (2 million units of penicillin G \( K_\text{H11001} \) and 100 mg gentamicin sulfate) were administered to the ewe during surgery and daily thereafter until 2 days after spontaneous delivery of the lamb (shunted lamb).

Lambs. After spontaneous delivery, antibiotics (1 million units of penicillin G \( K_\text{H11002} \) and 25 mg im gentamicin sulfate) were administered for 2 days. The lambs were weighed daily, and the respiratory rate and heart rate were obtained. Furosemide (1 mg/kg im) was administered daily to all lambs. Elemental iron (50 mg im) was given weekly. At 8 wk of age, five shunted lambs and their twin controls were killed by an intravenous injection of pentobarbital sodium (Euthanasia CII; Central City Medical, Union City, CA) followed by bilateral thoracotomy. An autopsy was performed to confirm patency of the vascular graft. The lungs were removed, and sections from each lobe were frozen in liquid nitrogen to allow extraction of RNA. All procedures and protocols were approved by the Committee on Animal Research of the University of California, San Francisco.

Tissue Preparation

The heart and lungs were removed en bloc. The lungs were dissected with care to preserve the integrity of the vascular endothelium. Sections (2–3 mm) from each lobe of the lung were removed. These tissues were snap-frozen in liquid nitrogen and stored at ~70°C until used. For protein studies, lung tissue was extracted from two shunt animals and two control animals and placed on ice. The main intralobar pulmonary arteries were exposed by gentle dissection, and third- and fourth-generation branches were carefully isolated and removed. After adventitial tissue was removed, intralobar pulmonary arteries were removed and frozen in liquid nitrogen.

Physiological Studies

At 2 mo of age, 10 additional lambs (5 shunted and 5 age-matched controls) had polyvinyl catheters placed in an artery and vein of one hindleg under local anesthesia with 1% lidocaine hydrochloride. These catheters were advanced to the descending aorta and the inferior vena cava, respectively. The lambs were then anesthetized with ketamine hydrochloride (~0.3 mg·kg⁻¹·min⁻¹) and diazepam (0.002 mg·kg⁻¹·min⁻¹), intubated with a 5.5-mm outer diameter endotracheal tube, and mechanically ventilated with a Healthdyne pediatric time-cycled, pressure-limited ventilator (Healthdyne, Marietta, GA). Succinylcholine chloride (2 mg·kg⁻¹·dose⁻¹) was given intermittently for muscle relaxation. Heart rate and systemic blood pressure were monitored continuously to ensure adequate anesthesia. Ventilation with 21% oxygen was adjusted to maintain an arterial PCO₂ between 35 and 45 torr. A midsternotomy incision was performed, and the pericardium was incised. Two single-lumen polyurethane catheters were inserted in the left and right atrium. A double-lumen polyurethane catheter was placed in the main pulmonary artery distal to the vascular graft. An ultrasonic flow probe (Transonsics Systems, Ithaca, NY) was placed around the left pulmonary artery (LPA) to measure left pulmonary blood flow. The chest was closed with towel clamps. The responses to vasodilating agents may be dependent on the resting tone of the vascular bed studied (27). Therefore, vasodilator responses in control lambs were studied during a similar degree of pulmonary hypertension induced by the intravenous infusion of U-46619 (a thromboxane A₂ mimetic).

Control Lambs

After a 45-min recovery period from surgery, baseline measurements of the hemodynamic variables (pulmonary and systemic arterial pressure, heart rate, left pulmonary blood flow, left and right atrial pressures) and systemic arterial blood gases and pH were measured. An infusion of U-46619 (a thromboxane A₂ mimetic) was then begun in the inferior vena cava. The dose (~1–2 \( \mu \)g·kg⁻¹·min⁻¹) was titrated to increase mean pulmonary arterial pressure to a similar value as shunted lambs. After 15 min of steady-state pulmonary hypertension, all variables were measured. Then, as the U-46619 infusion continued, hyperoxia or alkalosis (selected from a table of random numbers) was induced for 15 min. Normocarbic hyperoxia was induced by increasing the inspired oxygen concentration to 100%. After 15 min of ventilation with 100% oxygen, the hemodynamic variables and systemic arterial blood gases and pH were measured; normocarbic ventilation with 21% oxygen was then resumed. Alkalosis was induced by increasing the ventilatory rate by ~50% and administering sodium bicarbonate (1–2 meq/kg). After 15 min of alkalosis, the hemodynamic variables and systemic...
arterial blood gases and pH were measured and then normocarbic ventilation with 21% oxygen was resumed. A 15-min recovery period of normocarbic ventilation with 21% oxygen was allowed between conditions to allow the hemodynamic variables and systemic arterial blood gases and pH to return to precondition values before initiating the next condition. After recovery from the last agent, the infusion of U-46619 was stopped. After a 60-min recovery period, baseline measurements were obtained again, acute alveolar hypoxia (10% oxygen) was induced by adding nitrogen to the ventilation gas mixture, and the hemodynamic variables were measured as described above.

**Shunted Lambs**

After a 45-min recovery period from surgery, baseline measurements of the hemodynamic variables and systemic arterial blood gases and pH were measured. Hyperoxia, alkalosis, and alveolar hypoxia were administered in random order, as described above. All protocols and procedures were approved by the Committee on Animal Research of the University of California, San Francisco. All animals were killed using appropriate methods as described in the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*.

**Drug Preparation**

U-46619 (9,11-diepoxy-9-epoxymethano-PGP<sub>2,4</sub>; Sigma Chemical, St. Louis, MO) was suspended in 95% ethanol and stored at -20°C. Immediately before the study, 100 μg were dissolved in 20 ml of 0.9% saline.

**Physiological Measurements**

Pulmonary and systemic arterial and right and left atrial pressures were measured using Statham P23Db pressure transducers (Statham Instruments, Hato Rey, PR). Mean pressures were obtained by electrical integration. Heart rate was measured by a cardiotachometer triggered from the phasic systemic arterial pressure pulse wave. Left pulmonary blood flow was measured on an ultrasonic flowmeter (Transonic Systems). All hemodynamic variables were recorded continuously on a Gould multichannel electrostatic recorder (Gould, Cleveland, OH). Systemic arterial blood gases and pH were measured on a Corning 158 pH/blood gas analyzer (Corning Medical and Scientific, Medfield, MA). Hb concentration and oxygen saturation were measured by a hemoximeter (model OSM 2; Radiometer, Copenhagen, Denmark). Q<sub>R</sub>/Q<sub>S</sub> was calculated using the Fick equation. Pulmonary vascular resistance was calculated using standard formulas.

**RT-PCR**

Lung tissue was extracted and immediately frozen in liquid nitrogen and ground to powder with a prechilled mortar and pestle. Total RNA was extracted using the guanidium thiocyanate-phenol-chloroform method (Trireagent; Sigma). After homogenization, the samples were processed according to the reagent instructions, and the RNA was dissolved in diethyl pyrocarbonate-treated water and stored at -70°C. Optical density was measured to determine the RNA concentration. RNA (1 μg) was added to 11 μl of first-strand cDNA synthesis reagent (Pharmacia) with random hexamers as primers in a final volume of 33 μl. Two microliters of this RT reaction were added to each PCR reaction.

Oligonucleotide primers used to amplify the α-subunit of the maxi-K<sub>Ca</sub> channel were based on the human sequence and were 5'-CTACTGGGATGTTTCAGTGTG-3' (forward) and 5'-TGCTGTCAAAATGCTATA-3' (reverse), which yielded a product consistent with the expected 446-bp fragment for the human maxi-K<sub>Ca</sub>. Identity of the product was confirmed with sequence analysis.

Oligonucleotide primers used to amplify Kv2.1 cDNA were based on the human sequence and were 5'-ACAGAGCAACCCGAGGAGAAC-3' (forward) and 5'-CACCTTCATGGATTGACTT-3' (reverse), which yielded a product consistent with that expected for human Kv2.1 of 385 bp. Identity of the product was confirmed with sequence analysis.

Oligonucleotide primers used to amplify Twik-related acid-sensitive K<sup>+</sup> channel (TASK) cDNA were based on the human sequence and were 5'-TACGTGTTGACCATCTCAT-3' (forward) and 5'-GATACCTCACAAAGTGTGATT-3' (reverse), which yielded a product consistent with that expected for human TASK, 386 bp. Identity of the product was confirmed with sequence analysis.

18S rRNA was analyzed in RT-PCR as an internal control. 18S cDNA was amplified with a QuantumRNA primer/competimer set (Ambion) to allow relative quantitation of the cDNA. Primers for amplification were 48S bp. Because 18S rRNA is far more abundant than the mRNA under study, the 18S amplification reaction was modulated by the addition of "competimers." These primers are designed to regulate amplification by DNA polymerase. When combined with the functional primers for 18S cDNA amplification, efficiency is reduced in a linear fashion. Pilot experiments determined the correct ratio of primers to competimers, cycle number, and RT input to yield multiplex PCR products that are all in the linear range of amplification. PCR cocktail consisted of 1× PCR buffer (Perkin-Elmer) with 1.5 mmol/l Mg<sup>2+</sup>, 10 pmol/l each primer, 200 μmol/l dNTP mixture, 20 pmol/l 18S primer mixture (ratio of 1:9) of 18S primers, competitors, and 1 unit AmpliTag polymerase and water to make 50 μl. PCR was performed in an MJ Research thermocycler with a heated lid and 0.2 ml thin-walled tubes. The PCR reaction was 2 min at 90°C followed by 30–40 cycles of 1 min at 94°C, 1 min at 53°C, 2 min at 72°C, and then an extension of 2 min at 65°C. Samples without RT were evaluated in PCR; the products were absent. Identity of the bands was confirmed by sequencing the product. Densitometry was used in quantitative, internally controlled assessment of the RT-PCR product (NIH Image; Scion, Frederick, MD). The relative density of the 18S ribosomal and K<sup>+</sup> channel PCR products were compared in each individual gel. RT-PCR was performed three times for each mRNA sample.

**Western Blot Analysis**

Frozen pulmonary artery was ground to powder in liquid N<sub>2</sub> with a mortar and pestle. This was homogenized with a Dounce tissue grinder in RIPA buffer with protease inhibitor cocktail and 0.1% Triton X-100. The homogenate was sonicated briefly and then centrifuged at 1,000 g for 5 min. The supernatant protein concentration was determined with the bicinchoninic protein assay (Pierce). Protein (75 μg) was combined with SDS-PAGE reducing sample buffer and electrophoresed in a 4–20% gradient gel. The proteins were electrophorbed on a polyvinylidene difluoride membrane (Bio-Rad). Skim milk (6%) in 20 mM Tris-buffered saline was used for blocking and washing the membranes. Antibodies against maxi-K<sub>Ca</sub> channel and TASK channel (Alomone) were diluted 1:200 in milk-TBS, and membranes rotated in the solutions overnight at 4°C. In blocking experiments, the antibody was incubated 1 h, as recommended with specific antigen provided with the antibodies, and then diluted 1:200. Second
antibody was anti-rabbit IgG horseradish peroxidase conjugate (Jackson), 1:3,000 in milk-TBS with 0.01% Tween 20, rocked for 2 h at room temperature. After being washed, the membranes were incubated for 10 min at room temperature with Super Signal West Pico chemiluminescent reagent (Pierce). Kodak X-Omat fs-1 film was exposed for 10 min. Bands for TASK and K_{Ca} channels were seen at 55 and 125 kDa, respectively, and antibody binding to these bands was blocked by antigen preincubation. Separate gels were prepared and loaded with 75 μg protein and probed with an antibody against α-smooth muscle cell actin (Sigma). Kodak X-Omat fs-1 film was exposed for 1 min. For each antibody tested, three separate blots were prepared.

**Statistical Analysis**

The means ± SD were calculated for the baseline hemodynamic variables and systemic arterial blood gases and pH. The effects of each vasoactive agent were compared with their previous steady-state condition by the paired t-test using the Bonferroni correction when necessary. The percent change in pulmonary vascular resistance induced by these agents was compared between study groups by the unpaired t-test or ANOVA for repeated measures with multiple comparison testing. *P < 0.05* was considered statistically significant.

For the molecular data presented, values are expressed as means ± SE. Statistical significance was tested with the Student’s t-test (paired or unpaired, as appropriate). *P values < 0.05* were considered significant.

**RESULTS**

Under baseline conditions, animals with an aorto-pulmonary shunt showed elevated pulmonary artery pressure (PAP) and pulmonary blood flow compared with twin controls. In the control group, LPA blood flow was 0.8 l/min, PAP was 25% that of systemic pressure, and the Q_{p}/Q_{s} was 1.1. In shunted animals, LPA blood flow was 2.1 l/min, PAP was 45% that of systemic pressure, and Q_{p}/Q_{s} was 3.1:1 (Table 1). Mean PAP was 18 ± 3 mmHg in control animals compared with 28.3 ± 13.5 mmHg in shunt animals (Table 1; *P < 0.05 vs. control*).

In response to acute hypoxia, pulmonary blood flow was unchanged in both control and shunt animals. PAP increased in both shunt and control animals. Acute hypoxia caused pulmonary vascular resistance to increase to a greater degree in shunt compared with control animals (Fig. 1 and Tables 2 and 3; *P < 0.05* shunt vs. control). Aortic pressure was unaffected. In control lambs, neither systemic arterial pH (from 7.41 ± 0.02 to 7.45 ± 0.02) nor PCO_{2} (from 35.7 ± 3.3 to 33.6 ± 4.1 torr) changed. Systemic arterial PO_{2} decreased from 75.7 ± 10.8 to 28.4 ± 3.5 torr (*P < 0.05*). Similarly, in shunt lambs, neither arterial pH (from 7.41 ± 0.02 to 7.41 ± 0.03) nor PCO_{2} (from 35.9 ± 3.3 to 37.6 ± 4.3 torr) changed. Systemic arterial PO_{2} decreased from 70.0 ± 5.6 to 28.0 ± 2.7 torr (*P < 0.05; Tables 1 and 2*).

In response to an acute increase in oxygen tension, both shunt and control animals demonstrated a decrease in PAP. Pulmonary blood flow did not change. Pulmonary vascular resistance decreased by 12% in control animals and 22% in shunt animals (Tables 1 and 2; Fig. 2; *P ≠* significant vs. baseline for both shunt and control animals). In control lambs, neither systemic arterial pH (from 7.40 ± 0.03 to 7.39 ± 0.01) nor PCO_{2} (from 39.2 ± 1.0 to 41.2 ± 3.7 torr) changed. Systemic arterial PO_{2} increased from 64.8 ± 8.7 to 349.2 ± 104.2 torr (*P < 0.05*).

Acute alkalosis caused pulmonary vasodilation in control animals but not in shunt animals. In control animals, acute alkalosis caused a decrease in both PAP and pulmonary vascular resistance. Pulmonary blood flow did not change. In shunt animals, acute alkalosis had no effect on PAP, pulmonary artery blood flow, or pulmonary vascular resistance (Fig. 3 and Tables 2 and 3). Pulmonary vascular resistance decreased by 39% in control animals (*P < 0.05*), whereas there was no change in shunt animals (Tables 2 and 3). In control lambs, systemic arterial PO_{2} (from 61.2 ± 17.6 to 63.8 ± 12.6 torr) was unchanged. Systemic arterial pH increased from 7.36 ± 0.05 to 7.53 ± 0.06 (*P < 0.05*),

<table>
<thead>
<tr>
<th>Table 1. General hemodynamics of control and shunt lambs</th>
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<tbody>
<tr>
<td>Control Lambs</td>
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<tr>
<td>Age, days</td>
</tr>
<tr>
<td>Weight, kg</td>
</tr>
<tr>
<td>PAP, mmHg</td>
</tr>
<tr>
<td>SAP, mmHg</td>
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<tr>
<td>SAP/SAP</td>
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<tr>
<td>Left pulmonary blood flow, ml·kg^{-1}·min^{-1}</td>
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<tr>
<td>Q_{p}/Q_{s}</td>
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</table>

Values are means ± SD; n = 5 shunt lambs and their twin controls. PAP, pulmonary arterial pressure; SAP, systemic arterial pressure; Q_{p}/Q_{s}, ratio of pulmonary to systemic blood flow. *P < 0.05 vs. control lambs.
and $P_{CO_2}$ decreased from 39.6 ± 5.3 to 30.0 ± 3.7 torr ($P < 0.05$). Similarly, in shunt lambs, systemic arterial $P_O_2$ (from 74.3 ± 9.4 to 75.0 ± 8.3 torr) was unchanged, systemic arterial pH increased from 7.41 ± 0.02 to 7.55 ± 0.02 ($P < 0.05$), and $P_{CO_2}$ decreased from 38.3 ± 4.7 to 30.1 ± 3.0 torr ($P < 0.05$).

Pulmonary parenchymal tissue expressed the KCa and Kv2.1 channels as well as the TASK channel. RT-PCR yielded a 446-bp fragment of the ovine KCa channel transcript. Similarly, a 385-bp fragment was obtained for the ovine Kv2.1 channel. Both KCa and Kv channel mRNA were highly homologous to the human sequences of the same molecules. Neonatal lamb TASK mRNA was >92% homologous to its human counterpart.

KCa channel mRNA expression was increased in shunt compared with control animals, as determined by relative quantitative, internally controlled RT-PCR. A representative gel of KCa channel expression and 18S ribosomal mRNA expression in both control and shunt animals is provided in Fig. 4A. The ratio of KCa channel to 18S mRNA levels was 0.81 ± 0.06 in the shunt animals compared with 0.60 ± 0.14 in controls ($P = 0.039$; Fig. 4B). Lung KCa mRNA expression was increased by 34% (134 ± 6% that of the control) in shunt compared with control animals ($P < 0.05$; Fig. 5).

Lung TASK mRNA expression was decreased in shunt animals. The ratio of TASK channel to 18S mRNA levels was 0.57 ± 0.06 in the shunt animals compared with 0.76 ± 0.04 in the control animals ($P < 0.03$; Fig. 6B). Relative to control animals, lung TASK mRNA expression was decreased by 28% (72 ± 6% of control; $P < 0.05$). A representative gel of TASK channel expression and 18S ribosomal mRNA expression in both control and shunt animals is provided in Fig. 6A. The presence of an aortopulmonary shunt had no effect on Kv2.1 channel mRNA levels ($P = 0.16$, Fig. 7, A and B).

KCa channel and TASK protein expression were analyzed in vascular tissue derived from two control and two shunt animals. KCa bands of ~125 kDa were observed in both control and shunt samples. The 125-kDa band is consistent with previously published reports (20, 25, 28). Expression of the KCa channel protein was increased in shunt animals compared with controls. TASK protein was also expressed in pulmonary vascular tissue for both control and shunt animals. The 55-kDa band is consistent with previously published reports. TASK expression was decreased in shunt animals compared with controls (Fig. 8). Bands were not evident when samples were coincubated with both antigen and antibody, demonstrating antibody specificity. Separate gels incubated with α-smooth muscle cell actin antibody indicated similar amounts of protein in shunt and control samples. The results were entirely consistent with the mRNA expression experiments reported above.

**DISCUSSION**

Pulmonary vascular disease frequently complicates both surgical and medical management of congenital heart disease (18). Although pulmonary hypertension and abnormal pulmonary vascular reactivity are frequently associated with congenital heart disease (21), our understanding of both the physiology and the molecular mechanisms that underlie the pathophysiology remains incompletely understood. We report that chronic elevation (for 8 wk) in pulmonary blood flow in

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**Table 2. Hemodynamic changes in control lambs in response to hypoxia, an acute increase in oxygen tension, and alkalosis**

<table>
<thead>
<tr>
<th></th>
<th>Prehypoxia</th>
<th>Hypoxia</th>
<th>Preoxygen</th>
<th>Oxygen</th>
<th>Prealkalosis</th>
<th>Alkalosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPAP, mmHg</td>
<td>17.1 ± 4.3</td>
<td>24.3 ± 7.3*</td>
<td>33.6 ± 2.3</td>
<td>29.8 ± 2.8*</td>
<td>35.0 ± 4.1</td>
<td>27.6 ± 4.2*</td>
</tr>
<tr>
<td>Left pulmonary blood flow, ml·kg⁻¹·min⁻¹</td>
<td>38.3 ± 3.9</td>
<td>42.7 ± 8.1</td>
<td>31.5 ± 5.4</td>
<td>30.9 ± 7.9</td>
<td>28.9 ± 9.1</td>
<td>31.1 ± 5.2*</td>
</tr>
<tr>
<td>Left PVR, mmHg</td>
<td>0.230 ± 0.09</td>
<td>0.349 ± 0.19*</td>
<td>0.802 ± 0.19</td>
<td>0.718 ± 0.26</td>
<td>1.03 ± 0.57</td>
<td>0.58 ± 0.20*</td>
</tr>
<tr>
<td>Mean arterial BP, mmHg</td>
<td>86.5 ± 12.3</td>
<td>80.3 ± 12.3</td>
<td>113.2 ± 7.8</td>
<td>113.0 ± 9.5</td>
<td>107.0 ± 12.4</td>
<td>107.8 ± 20.9</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>140.7 ± 22.1</td>
<td>157.3 ± 34.2</td>
<td>146.8 ± 22.2</td>
<td>138.4 ± 25.0</td>
<td>146.0 ± 29.5</td>
<td>147.4 ± 31.2</td>
</tr>
</tbody>
</table>

Values are means ± SD; $n = 5$ animals in each group. *$P < 0.05$ vs. previous column. MPAP, mean PAP.

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**Table 3. Changes in hemodynamic variables in shunt lambs**

<table>
<thead>
<tr>
<th></th>
<th>Prehypoxia</th>
<th>Hypoxia</th>
<th>Preoxygen</th>
<th>Oxygen</th>
<th>Prealkalosis</th>
<th>Alkalosis</th>
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<tbody>
<tr>
<td>MPAP, mmHg</td>
<td>25.3 ± 8.5</td>
<td>34.6 ± 7.8*</td>
<td>26.2 ± 10.7</td>
<td>24.0 ± 10.0*</td>
<td>24.0 ± 26.2</td>
<td>23.0 ± 25.6</td>
</tr>
<tr>
<td>Left pulmonary blood flow, ml·kg⁻¹·min⁻¹</td>
<td>137.3 ± 43.8</td>
<td>135.5 ± 41.2</td>
<td>111.9 ± 31.8</td>
<td>114.9 ± 27.2</td>
<td>129.6 ± 52.3</td>
<td>125.2 ± 50.8</td>
</tr>
<tr>
<td>Left PVR, mmHg</td>
<td>0.124 ± 0.06</td>
<td>0.211 ± 0.10*</td>
<td>0.146 ± 0.06</td>
<td>0.114 ± 0.06</td>
<td>0.133 ± 0.08</td>
<td>0.124 ± 0.07</td>
</tr>
<tr>
<td>Mean systemic arterial pressure, mmHg</td>
<td>70.9 ± 17.8</td>
<td>65.4 ± 21.9</td>
<td>64.4 ± 4.6</td>
<td>65.4 ± 6.2</td>
<td>66.5 ± 6.8</td>
<td>66.2 ± 10.0</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>150.9 ± 27.6</td>
<td>164.9 ± 35.3</td>
<td>137.6 ± 11.1</td>
<td>131.6 ± 22.5</td>
<td>141.3 ± 9.4</td>
<td>140.5 ± 12.2</td>
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</table>

Values are mean ± SD; $n = 5$ animals in each group. *$P < 0.05$ vs. previous column.
a neonatal ovine model of congenital heart disease results in attenuated pulmonary vasodilation in response to alkalosis, enhanced hypoxic pulmonary vasoconstriction, and preservation of oxygen-induced pulmonary vasodilation. In association with these changes in pulmonary vasoreactivity, pulmonary K$^+$/H11001 channel expression changes as KCa channel mRNA expression increases and TASK mRNA expression decreases. Parallel changes were noted in KCa and TASK channel protein expression. These observations support the notion that alterations in blood flow and oxygen tension, given the relatively high oxygen content in the pulmonary arterial blood of shunt animals, may modulate K$^+$/H11001 channel expression in the pulmonary vasculature.

The observation that pulmonary blood flow is increased in the present model is consistent with previous reports that an aortopulmonary shunt placed in fetal lambs leads to an increase in pulmonary blood flow during postnatal life (22). $Q_p/Q_s$ is increased, and pulmonary artery blood pressure is at one-half the systemic levels. Previous studies using this model have identified histological alterations that are similar to those observed in children with pulmonary hypertension associated with congenital heart disease (22). At 2 mo of age, the oxygen-induced pulmonary vasodilation was similar in both control animals and in animals with an aortopulmonary shunt. In contrast, the response to alkalosis was absent in shunt animals, whereas the response to hypoxia was enhanced.

**Fig. 2.** Percent change in left PVR in response to an acute increase in alveolar oxygen tension (100%). Pulmonary vasodilation in response to an acute increase in oxygen tension is similar in both shunted and control lambs. Values are means ± SE; n = 5 shunt and 5 control lambs. $P = \text{not significant vs. baseline for both shunt and control animals.}$

**Fig. 3.** Percent change in left PVR in response to an acute increase in arterial pH. Pulmonary vasodilation in response to alkalosis is absent in shunted animals. Compared with control lambs, the pulmonary vasodilator response to alkalosis is attenuated. Values are means ± SE; $n = 5$ shunt and 5 control lambs. $*P < 0.05$ vs. controls. $**P < 0.05$ vs. baseline.

**Fig. 4.** A: representative gel of RT-PCR analysis of calcium-sensitive K$^+$/H11001 (KCa) channel mRNA expression in shunt and control lambs. The KCa band (446 bp) was compared with the control 18S band (488 bp) in shunt and control samples. B: KCa channel band intensity was determined by densitometry and normalized to that of the 18S band in several experiments. KCa channel mRNA levels are increased in shunt animals compared with control animals. $*P < 0.05$ vs. control.

**Fig. 5.** Pulmonary mRNA levels of the KCa channel are increased, voltage-gated K$^+$/H11001 channel (Kv) 2.1 mRNA levels are unchanged, and Twik-related acid-sensitive K$^+$/H11001 (TASK) mRNA levels are decreased in an ovine model of neonatal congenital heart disease. The ratio of KCa, Kv2.1, and TASK channels to 18S mRNA content from shunt animals is expressed as the percentage of the ratio of K$^+$/H11001 channel to 18S mRNA content in control animals. $*P < 0.05$ vs. control.
pattern of responses observed argues that pulmonary vasoreactivity in animals with a shunt was not globally diminished, since the vasodilator response to oxygen was retained. This is consistent with a previous report in this model demonstrating that the response to inhaled NO is preserved (23).

Because $K_{C_{a}}$ channels have been shown to mediate the response of the pulmonary vasculature to several physiological stimuli (6, 13, 31, 37), we determined the mRNA levels of specific $K_{C_{a}}$ channels. The results of the physiological studies were consistent with the mRNA measurements. First, the preservation of the response to an acute increase in oxygen tension might be explained, in part, through the increase in $K_{C_{a}}$ channel mRNA expression. Activation of the $K_{C_{a}}$ channel in pulmonary vascular smooth muscle cells has been shown to cause an increased $K^{+}$ efflux, membrane hyperpolarization, and a decrease in cytosolic calcium, resulting in relaxation. Because $K_{C_{a}}$ channels have been shown to mediate oxygen-induced perinatal pulmonary vasodilation (13), the increase in $K_{C_{a}}$ channel expression in shunt animals may enable the pulmonary vasculature to dilate in response to oxygen despite pulmonary vascular smooth muscle cell hypertrophy. The fact that the ratio of $K_{C_{a}}$ to 18S mRNA increases against the possibility that increases in mRNA expression derive from an overall increase in pulmonary vascular smooth muscle. Because $K_{C_{a}}$, but not $K_{v2.1}$, channel mRNA expression is affected in shunt animals, it seems unlikely that the present results derive from nonspecific changes in $K^{+}$ channel mRNA expression.

Recently, a $K^{+}$ channel that is sensitive to changes in extracellular pH has been identified. The TASK channel is a background channel that is constitutively open. The channel is thought to be voltage insensitive. However, with an increase in extracellular pH to 7.7, $K^{+}$ current through the channel increases to 90% of its

Fig. 6. A: representative gel of RT-PCR analysis of TASK mRNA expression in shunt and control lambs. The TASK band (386 bp) was compared with the control 18S band (488 bp) in shunt and control animals. B: TASK channel band intensity was determined by densitometry and normalized to that of the 18S band. TASK channel mRNA levels are decreased in shunt animals compared with control animals.

Fig. 7. A: representative gel of RT-PCR analysis of $K_{v2.1}$ mRNA expression in shunt and control lambs. The $K_{v2.1}$ band (385 bp) was compared with the control 18S band (488 bp) in shunt and control animals. B: $K_{v2.1}$ channel band intensity was determined by densitometry and normalized to that of the 18S band. $K_{v2.1}$ channel mRNA levels are similar in both shunt and control animals.

Fig. 8. Western blot analysis of $K^{+}$ channel expression in distal pulmonary arteries of shunt and control animals. A: representative gel of $K_{C_{a}}$ protein expression in shunt and control lambs. The 125-kDa band was increased in shunt animals compared with controls. B: representative gel of TASK protein expression. The 55-kDa TASK band was reduced in shunt animals compared with controls. C: Smooth muscle cell (SMC) actin was used as a control to indicate equal lane loading. In all cases, brain tissue was used as a positive control for the anti-$K_{C_{a}}$, and anti-TASK antibodies, and specificity was assessed by specific antigen-blocking experiments.
maximum. With a decrease in pH to 6.7, the K+ current through the channel decreases to ~10% of its maximum (24). Hence, the changes in acid-base status might affect pulmonary vascular tone through K+ efflux via the TASK channel. Presumably, alkalosis increases pulmonary artery smooth muscle cell K+ efflux through the TASK channel, leading to membrane hyperpolarization, closure of the voltage-operated calcium channels, and a decrease in cytosolic calcium. The decrease in pulmonary artery smooth muscle cell cytosolic calcium concentration results in pulmonary vasodilation (34).

Although the TASK channel has been described in vitro (24), the present report suggests that the TASK channel may have physiological relevance. The absence of a pulmonary vasodilator response to alkalosis, in combination with a decrease in TASK channel mRNA expression, lends credibility to the concept that the channel senses changes in extracellular pH. The absence of pulmonary vasodilation in response to alkalosis cannot be attributed simply to a decreased capacity for pulmonary vasodilation, since the response of shunt animals to an increase in oxygen tension is preserved.

Previous investigators have demonstrated that alkalosis-induced pulmonary vasodilation is mediated by endothelium-dependent NO release, prostacyclin, and KCa channel activation (35). The present study suggests that TASK activation also plays a role in alkalosis-induced pulmonary vasodilation. Because alkalosis induces pulmonary vasodilation through NO, prostacyclin release, and KCa channel activation (35), the role of the TASK channel may lie in sensing the change in extracellular pH. With the TASK channel conferring sensitivity to changes in extracellular pH, the pulmonary vasculature can respond with elaboration of vasoactive mediators from the pulmonary endothelium and effect KCa channel activation and pulmonary vasodilation (13, 29).

Among the limitations of this study is the inability to distinguish between alterations in endothelial compared with vascular smooth muscle K+ channel expression. Multiple lines of evidence suggest that vascular endothelium express both KCa and Kv channels (11, 30, 31, 33). Because RNA was extracted from whole lung homogenate, whether the changes in K+ channel mRNA levels result from alterations in the endothelium, vascular smooth muscle cells, or a combination is unknown. However, given that this model leads to pulmonary vascular remodeling and not remodeling of the airways, it is reasonable to conclude that the changes in mRNA expression between the shunt and control animals result from changes in K+ channel expression in the vasculature as opposed to the airway smooth muscle or airway epithelial cells. The protein studies were performed on pulmonary vascular tissue, as opposed to whole lung homogenate, providing further support for specific alterations in pulmonary vascular K+ channel expression in the present model. To ensure fidelity between alterations in K+ channel mRNA levels and K+ channel protein expression, Western analysis was performed. The present data support the notion that increases in mRNA expression of the α-subunit of the maxi-KCa channel lead to increases in protein expression of the α-subunit of the KCa channel. Similarly, the decrease in TASK mRNA expression correlated with a decrease in TASK protein expression. We speculate that a slight change in ion channel expression can have a significant effect on vascular endothelial and smooth muscle cell physiology (20, 28, 33, 34).

Given the accentuation of hypoxic pulmonary vasoconstriction in the shunt compared with the control animals, Kv2.1 channel mRNA levels might be expected to be greater in shunt animals. Several investigators have demonstrated that hypoxia causes pulmonary artery smooth muscle cell depolarization through inactivation of Kv channels (7, 37). The observation that Kv2.1 channel mRNA expression is similar in both shunt and control animals suggests that an alternative mechanism, such as proliferation of pulmonary vascular smooth muscle cell and extracellular matrix, might augment the contractile strength of the pulmonary vasculature. Alternatively, production of vasodilator compounds by the pulmonary endothelium, such as prostacyclin or NO, may be compromised, rendering the pulmonary vasculature more responsive to hypoxia. An additional explanation may be that increases in pulmonary blood flow enhance the sensitivity of the pulmonary artery smooth muscle contractile apparatus to calcium.

There is precedent for the notion that expression of vascular K+ channels is dynamic, changing with both maturation (15) and in response to hypertension (20, 28). Recent reports from our laboratory indicate that pulmonary vascular KCa channel expression decreases with maturation (25), whereas Kv2.1 channel expression increases with maturation (15). Systemic hypertension increases KCa channel expression in the systemic vasculature (20). In a model of chronic intrauterine pulmonary hypertension, KCa channel expression is decreased (14). Although the present findings may seem to diverge from those reported previously, it is essential to consider several important differences between the two models. With ligation of the ductus arteriosus, the pulmonary blood flow in the fetal lamb remains low, <10% of the biventricular output, whereas PAP is higher than systemic blood pressure, and oxygen tension is quite low. In contrast, the pulmonary blood flow in the neonatal lamb with an aortopulmonary shunt is significantly greater than systemic blood flow, PAP is less than one-half systemic pressure, and oxygen tension in the blood entering the pulmonary arteries is relatively high. These important physiological differences, superimposed on alterations in the pulmonary circulation during fetal as opposed to neonatal life, likely influence vascular K+ channel expression. Indeed, consideration of the changes in K+ channel expression in the present model compared with the model of chronic intrauterine hypertension may provide insight into the application of specific treatment modalities based on the etiology of the pul-
monary hypertension. It is also important to recognize that the study animals were only 8 wk old at the time of study and subsequent death. With more pronounced pulmonary hypertension, the changes in K⁺ channel mRNA expression may have been greater.

Despite the apparent changes in vascular K⁺ channel expression associated with an increase in pulmonary blood flow, the mechanisms responsible for alterations in K⁺ channel expression remain completely speculative. Perhaps, the relatively oxygen-rich blood that is present in pulmonary arteries of shunt animals provides a signal that modulates K⁺ channel expression. Alternatively, the increase in pressure in the pulmonary arteries of shunt animals may be the critical stimulus for modulating K⁺ channel expression. Because endothelial dysfunction occurs in neonatal pulmonary hypertension (5, 10), and in this model (8, 23), alterations in the production of vasoactive mediators such as endothelin or NO may modulate K⁺ channel expression.

In conclusion, we report that pulmonary vascular reactivity is altered in neonatal lambs with an aorto-pulmonary shunt. After 2 mo, shunt animals demonstrate no pulmonary vasodilator response to alkalosis, an accentuated response to hypoxia, and an intact vasodilator response to oxygen. In association with the changes in pulmonary vasoreactivity, pulmonary K⁺ channel expression is altered. In particular, TASK channel mRNA expression is decreased, Kv2.1 channel expression is unchanged, and KCa channel mRNA expression is increased. The specific alterations in K⁺ channel expression argue that pulmonary K⁺ channels mediate the response of the pulmonary circulation to alkalosis, hypoxia, and an acute increase in oxygen tension. In children with congenital heart disease, pharmacotherapy targeted to pulmonary vascular K⁺ channels may represent a novel therapeutic strategy.

This work was supported by National Heart, Lung, and Blood Institute Grants RO1 HL-60784 (D. N. Cornfield) and RO1 HL-61284 (J. R. Fineman), the Viking Children’s Fund (E. R. Resnik), and an American Heart Association Established Investigator Award (D. N. Cornfield).

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AJP-Lung Cell Mol Physiol • VOL 283 • DECEMBER 2002 • www.ajplung.org

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