Chronic intrauterine pulmonary hypertension decreases calcium-sensitive potassium channel mRNA expression

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The mechanisms responsible for the maintenance of the high-tone, low-flow fetal pulmonary vasculature and the rapid increase in pulmonary blood flow that occurs at birth remain incompletely understood. At birth, pulmonary blood flow increases 8- to 10-fold, and pulmonary arterial pressure declines steadily over the first several hours of life (17). Although physical factors and vasoactive products elaborated by the pulmonary vascular endothelium are involved in the regulation of perinatal pulmonary vascular tone (1, 8), sustained and progressive perinatal pulmonary vasodilation requires pulmonary arterial smooth muscle cell (PASMC) K+ channel activation (22). PASMC K+ activation causes membrane hyperpolarization, closure of voltage-operated calcium channels, and a decrease in cytosolic calcium (18).

Recent studies have demonstrated that activation of calcium-sensitive K+ (KCa) channels in the pulmonary vasculature plays a critical role in perinatal pulmonary vasodilation. Oxygen causes perinatal pulmonary vasodilation through kinase-dependent activation of the KCa channel (9). K+ channel inhibition with tetraethylammonium (TEA), a preferential KCa channel antagonist, blocks the perinatal pulmonary vasodilation caused by ventilation (22). This observation implies that ventilation causes sustained and progressive perinatal pulmonary vasodilation through activation of TEA-sensitive K+ channels. Nitric oxide (NO), a vasoactive agent that is essential for the normal transition of the pulmonary circulation, causes perinatal pulmonary vasodilation, in part, through activation of the KCa channel (19). Even shear stress-induced fetal pulmonary vasodilation induced by compression of the ductus arteriosus (DA) during fetal life requires KCa and voltage-gated K+ (KV) channel activation (21).

In some newborn infants, pulmonary vascular resistance (PVR) remains elevated after birth, resulting in a clinical syndrome termed persistent pulmonary hypertension of the newborn (PPHN), which is characterized by extrapulmonary right-to-left shunting of blood across the DA or patent foramen ovale causing severe hypoxemia (10). Although the mechanisms responsible for abnormal perinatal pulmonary vasoactivity remain incompletely understood, evidence suggests that adverse intrauterine stimuli such as chronic hypoxia or hypertension (11) can decrease NO synthase (NOS) activity and endothelial NOS (eNOS) gene and protein expression and limit perinatal NO production, thereby contributing to the pathophysiology of PPHN (2, 20, 24).

Because several critical birth-related stimuli such as an increase in oxygenation, ventilation, shear stress, and NO production cause vasodilation through KCa
channel activation, we hypothesized that 1) the fetal lung expresses K⁺ channels and 2) decreased Kᵥ channel activity contributes to failure of the pulmonary circulation to achieve and sustain low PVR after birth. To test this hypothesis, we measured Kᵥ and K⁺ channel mRNA levels from the lungs of normal fetal lambs and from the lungs of animals with experimental chronic intrauterine pulmonary hypertension caused by ligation of the DA. This experimental model of perinatal pulmonary hypertension has previously been shown (2, 15) to closely resemble the pathophysiology of PPHN in humans. We report that the ovine fetal lung expresses both Kᵥ and K⁺ channels and that chronic in utero pulmonary hypertension decreases lung mRNA of the Kᵥ channel but not of the K⁺ channel.

MATERIALS AND METHODS

RT-PCR. Lung tissue was suspended in liquid nitrogen and ground to powder with a prechilled mortar and pestle. Total RNA was extracted with the guanidinium thiocyanate-phenol-chloroform method (TRI Reagent, Sigma, St. Louis, MO). 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. One microgram of RNA 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. Oligonucleotide primers used to amplify Kᵥ2.1 cDNA were based on the human sequence (3) and were (forward) 5'-ACAGAGCAAACCAAAGGAAGAAC-3' and (reverse) 5'-CACCTCTCATGAACTGTATTTA-3', which yielded a product consistent with that expected for human Kᵥ2.1. The fragment size was 385 bp. Identity of the product was confirmed with sequence analysis.

Oligonucleotide primers used to amplify Kᵥ channel cDNA were based on the human sequence (23) and were (forward) 5'-TCGTCATCAAAACTGCATA-3' and (reverse) 5'-ACAGAGCAAACCAAAGGAAGAAC-3', which yielded a product consistent with that expected for human Kᵥ channel. Identity of the product was confirmed with sequence analysis.

Oligonucleotide primers used to amplify K⁺ channel-associated protein (KChAP) cDNA were based on the human sequence (25) and were (forward) 5'-GGATCTGTTGTCATTGGTCAATGG-3' and (reverse) 5'-GGCGGAAGGACAGTTGTTG-3', which yielded a product consistent with that expected for human KChAP. The fragment size was 801 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 semiquantification of the ethidium bromide bands. This control band appears as 488 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 modified to block extension by DNA polymerase. When combined with the functional primers for 18S cDNA, the amplification efficiency is reduced. Pilot experiments determined the correct ratio of primers to competimers, the cycle number, and the RT input required to yield multiplex PCR products, which are all in the linear range of amplification. The PCR cocktail consisted of 1× PCR buffer (Perkin-Elmer) with 1.5 mM Mg²⁺, 10 pM each Kᵥ2.1 primer, 10 nM deoxynucleotide triphosphate mixture, 20 pM 18S primer mixture (ratio of 1:9) of 18S primers/competimers, 1 U of AmpliTaq 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 was for 2 min at 90°C, followed by 28–32 cycles of 1 min at 94°C, 1 min at 54°C, and 2 min at 72°C, and then an extension of 2 min at 65°C Samples without reverse transcriptase were evaluated by PCR; the products were absent. Identity of the band was confirmed by sequencing the product (>91% homology with known sequences). Densitometry was used in relative semiquantitative assessment of the RT-PCR product (NIH Image, Scion, Frederick, MD). Each gel contained PCR product from both hypertensive and control animals. The relative density of the 18S ribosomal and K⁺ channel PCR products was compared in each individual gel.

Animals. Fetal and adult sheep were used. The procedures used in these studies were previously reviewed and approved by the Animal Care and Use Committee at the University of California, School of Medicine, Riverside, CO. Procedures ranged from 135 to 140 days gestation (term is 147 days). Ewes were sedated with intravenous pentobarbitol sodium (total dose: 2–4 g) and anesthetized with 1% tetracaine hydrochloride (3 mg) via lumbar puncture. Ewes were kept sedated but breathed spontaneously throughout the surgery and study periods. Under sterile conditions, the left forelimbs of fetal lambs were delivered through a uterine incision. Skin incisions were made under the left forelimb after local infiltration with lidocaine (2–3 ml, 1% solution). Polyvinyl catheters were advanced into the ascending aorta and the superior vena cava after insertion into the axillary artery and vein. A left thoracotomy exposed the heart and great vessels. In some animals, catheters were inserted into the main pulmonary artery between the DA and the pulmonic valve through purse-string sutures as previously described (2, 8). Catheters were guided into position with a 14- or 16-gauge intravenous placement unit (Angiocath, Travenol, Deerfield, IL). The catheters were secured by tightening the purse string as the introducer was withdrawn. A catheter was placed in the amniotic cavity to measure pressure. The hystero- tomy was closed, the uterus was returned to the maternal abdominal cavity, and the catheters were exteriorized via subcutaneous tunnels to an external flank pouch. The ewes recovered rapidly from surgery and were generally standing in their pens within 6 h. Food and water were provided ad libitum. After 8–12 days, the animals were killed rapidly after high-dose maternal and fetal infusions of pentobarbitol sodium, and lung tissue was harvested as described above.

Statistical analysis. In each individual experiment, the ratio of K⁺ channel to 18S mRNA content in hypertensive animals was compared with that in control animals. For each individual gel, data from hypertensive animals are expressed as a percentage of those from the control animals. In addition, the ratio of Kᵥ channel to 18S mRNA levels was
compared between control and hypertensive animals. Student’s t-test was used to assess differences between experimental groups. P values < 0.05 were considered significant.

RESULTS

Chronic ligation of the DA increased mean pulmonary arterial pressure and total pulmonary resistance without affecting mean aortic pressure. Perinatal pulmonary tissue expressed both KCa and KV channels as well as KChAP. RT-PCR yielded a 446-bp fragment of the ovine KCa channel molecule. A 385-bp fragment of the ovine Kv2.1 channel was obtained with RT-PCR analysis. Both KCa and KV channel mRNAs were highly homologous to the human sequences of the same molecules (Fig. 1). Fetal ovine KChAP mRNA was >92% homologous to human sequences of the same molecule. Intrauterine DA ligation decreased lung KCa channel mRNA levels by 26 ± 6% compared with those in control animals as determined by semiquantitative PCR (P < 0.02; Fig. 2). The ratio of KCa channel to 18S mRNA levels was 0.41 ± 0.16 in the hypertensive animals compared with 0.60 ± 0.14 in the control animals.
animals \( (P = 0.039) \). Intrauterine ligation of the DA had no effect on \( K_V \) channel mRNA levels (Fig. 3) or KChAP mRNA levels (Fig. 4).

**DISCUSSION**

Because sustained and progressive perinatal pulmonary vasodilation requires activation of pulmonary vascular \( K^+ \) channels (22), we hypothesized that 1) the perinatal lung expresses \( K_Ca \) channels and 2) decreased \( K_Ca \) channel production may contribute to a sustained elevation in PVR in neonatal pulmonary hypertension. To test these hypotheses, we measured steady-state mRNA levels of the \( K_Ca \) and \( K_V \) channels in lungs from fetal lambs after normal gestation and in an experimental model of perinatal pulmonary hypertension caused by intrauterine ligation of the DA. We report that fetal lung tissue expresses both \( K_Ca \) and \( K_V \) channels as well as KChAP (25). Partial sequence analysis of the fetal ovine pulmonary \( K^+ \) channels indicates high homology to the previously described molecular sequences of human \( K^+ \) channels (3, 23, 25). Compared with age-matched fetal lambs, chronic in utero pulmonary hypertension decreased lung \( K_Ca \) channel mRNA levels but not lung \( K_V \) channel mRNA levels. We concluded that intrauterine pulmonary hypertension attenuates \( K_Ca \) channel mRNA expression, which may lead to sustained pulmonary hypertension and decrease the normal perinatal pulmonary vasodilator response to stimuli such as NO, ventilation, oxygen, and shear stress.

Whereas previous studies (4, 5, 9, 16, 19, 21, 27) have demonstrated the physiological significance of \( K^+ \) channel activity in the pulmonary vasculature, the present study provides the first molecular evidence that the fetal lung expresses \( K^+ \) channels. The present study provides evidence that the fetal lung expresses mRNA for the \( K_Ca \) and \( K_V \) channels as well as for KChAP, a protein that modulates \( K^+ \) conductance through the \( K_V \) channel (25). The high degree of homology between known \( K^+ \) channel sequences and those demonstrated in these experiments provides support on a molecular basis for the physiological studies that have characterized the role of \( K^+ \) channels in the perinatal pulmonary circulation with the use of pharmacological probes (9, 19, 21, 22).

The observation that \( K_Ca \) channel mRNA levels, but not \( K_V \) channel or KChAP mRNA levels, were decreased by intrauterine DA closure provides support for physiological studies that have demonstrated a critically important role for the \( K_Ca \) channel in perinatal pulmonary vasodilation. This model of perinatal pulmonary hypertension has been used by many investigators (2, 6, 26) to examine the mechanisms responsible for the failure of postnatal adaptation of the pulmonary circulation. Investigators have shown that chronic in utero pulmonary hypertension caused by partial or complete closure of the DA increases fetal pulmonary arterial pressure and alters fetal pulmonary vasoreactivity while having no affect on the histology of airway epithelial or smooth muscle cells (26). After birth, these animals manifest a persistently elevated PVR that is relatively unresponsive to mechanical ventilation and/or increased concentrations of inspired oxygen (15). Previous studies with this model have demonstrated attenuated pulmonary vasodilation in response to ventilation, oxygen, and shear.

**Fig. 3.** Pulmonary vascular \( K_V \) channel mRNA levels (385-bp fragment) were not affected by chronic intrauterine pulmonary hypertension. mRNA levels, which were measured as RT-PCR products, were normalized with an internal standard (18S; 488-bp fragment) and compared in normal (N1–N4) and hypertensive (H1–H3) specimens taken from different animals.

**Fig. 4.** Pulmonary vascular mRNA levels of the \( K_Ca \) channel, but not of the \( K_V \) channels or KChAP, are decreased in an ovine model of chronic intrauterine pulmonary hypertension. \( n \), No. of animals. The ratio of \( K^+ \) channel to 18S mRNA content from hypertensive animals is expressed as a percentage of the ratio of \( K^+ \) channel to 18S mRNA content in control animals.
stress (14), physiological stimuli that mediate vasodilation, at least in part, through NO release (8).

Identical physiological stimuli, increased oxygen tension, ventilation, and NO, have been shown (9, 21, 22) to cause perinatal pulmonary vasodilation through activation of the KCa channel. KCa channel activation causes PASMC hyperpolarization, closure of voltage-operated calcium channels, a decrease in cytosolic calcium, and vasodilation (18). Although the attenuated perinatal pulmonary vasodilation in this model has been shown to be caused, in part, by a decrease in NOS expression (24), the present findings argue that the persistent elevation of PVR after birth may be caused, at least in part, by a decrease in KCa channel gene expression. Because NO causes pulmonary vasodilation through KCa channel activation (19), the attenuated response to NO-dependent vasodilators such as oxygen, shear stress, and ventilation in this model may be caused by a decrease in KCa channel expression as well as by the decrease in eNOS expression. Although the change in KCa channel mRNA levels might derive from changes in cells other than vascular smooth muscle cells, use of the specific model employed in these studies mitigates this concern to some degree. The histology of this model has been well described previously (2, 6, 26). The most prominent change in histology is in the pulmonary vasculature. No changes in airway smooth muscle cells or pulmonary epithelial cells have been reported in this model. Because activation of pulmonary vascular endothelial cell KCa channel plays a role in mediating the response to shear stress in the perinatal pulmonary circulation (21), a decrease in pulmonary vascular endothelial KCa channel mRNA expression would be consistent with the hypothesis outlined in the present study.

This study is not the first to demonstrate that blood pressure changes modulate KCa channel expression. Liu et al. (12) demonstrated that elevations in systemic blood pressure caused KCa channel protein expression to increase in cerebrovascular smooth muscle cell membranes. This observation suggests that KCa channel expression may be a homeostatic mechanism that limits cerebral vascular tone despite chronic hypertension. In a separate report (13), the same group of investigators reported that although KCa channel protein increased during hypertension, mRNA levels of the KCa channel were unaffected, suggesting that the upregulation of KCa channels in arterial muscle membranes during hypertension may rely on a posttranscriptional mechanism. Our finding that pulmonary parenchymal KCa channel mRNA levels decrease during chronic in utero pulmonary hypertension differs from these reports.

Given that KCa channel, but not Kv channel or KChAP, mRNA levels are decreased in this model, it is unlikely that chronic intrauterine pulmonary hypertension simply causes a global decrease in fetal lung KCa channel expression. A selective decrease in KCa channel expression fits well with previous studies showing that an acute increase in oxygen tension causes fetal pulmonary vasodilation through cyclic nucleotide-dependent kinase activation of the KCa channel (9). Similarly, ventilation and NO have been shown (19, 22) to cause perinatal pulmonary vasodilation through KCa but not through Kv channel activation. Shear stress causes perinatal pulmonary vasodilation through both NO release (8) and activation of both KCa and Kv channels (21). Thus a decrease in KCa channel expression may underlie the attenuated pulmonary vasodilator response to these birth-related stimuli in this model.

Based on data demonstrating that hypoxic pulmonary vasoconstriction results from pulmonary vascular smooth muscle cell Kv channel inactivation (4, 27), an increase in Kv channel gene expression might have been expected in this model, but neither Kv channel nor KChAP gene expression was altered. Because KChAP has been shown to cause a fourfold increase in K+ conductance through the Kv channel (25), an increased gene expression of KChAP may have pointed to an increased role for the Kv channel in the pathophysiology of PPHN. The absence of an increase in gene expression of either Kv channel or KChAP argues that the decrease in KCa channel gene expression seen in this model may be of central importance in PPHN. Thus the incomplete response to perinatal pulmonary vasodilator stimuli that characterizes these animals may derive, to a significant degree, from a decrease in KCa channel expression and compromised pulmonary vascular KCa channel activation. It is important to note that the present study only addressed alterations in Kv2.1 expression. There is evidence that alternative Kv channels such as Kv1.2 (7), Kv1.5 (5), and Kv9.3 (16) may play an essential role in mediating the response of the adult pulmonary vasculature to hypoxia.

In summary, we report that KCa channel, Kv channel, and KChAP genes are expressed in the ovine fetal lung. Chronic intrauterine pulmonary hypertension caused by DA closure decreases KCa channel but not Kv channel or KChAP mRNA levels. These findings support the notion that adverse intrauterine events such as premature closure of the DA can lead to an altered gene expression of an ion channel that plays a critically important role in mediating the response of the perinatal pulmonary circulation to vasodilator stimuli. A decrease in KCa channel gene expression may contribute to the pathophysiology that characterizes PPHN.

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