Fetal Ductus Arteriosus Ligation

Pulmonary Vascular Smooth Muscle Biochemical and Mechanical Changes

Jaques Belik, A.J. Halayko, K. Rao, and Newman L. Stephens

To evaluate the smooth muscle mechanical and biochemical changes associated with persistent pulmonary hypertension syndrome of the newborn, we studied 31 fetal sheep in which the ductus arteriosus was ligated at 125 days of gestation. Sixty-one noninstrumented and six sham-operated fetuses served as controls. All animals were delivered by cesarean section at 137–140 days of gestation, and the experimental group had the ductus arteriosus ligated for 12±3 days. The ligated group demonstrated a higher mean ±SEM pulmonary artery pressure (72.3±3.8 versus 54.1±2 mm Hg, p<0.01) and right ventricular mean free wall weight (12.5±0.7 versus 6.8±0.3 g, p<0.01) as compared with the sham-operated group. Significant changes in the pulmonary vascular smooth muscle of the ligated group were observed. The myosin content of vessels from the second through fifth generation demonstrated a significant increase in actin and myosin content (p<0.01), but given their disproportional changes, the noninstrumented group demonstrated a lower actin/myosin ratio than the experimental group (p<0.01). Changes in the myosin heavy chain isoform stoichiometry, characterized by an increase in both the mean high/low myosin heavy chain isoform ratio (1.8±0.3 versus 1.0±0.1, p<0.05) and the nonmuscle isoform as a percentage of the total myosin heavy chain (12.4±0.7% versus 2.7±0.9%, p<0.01), were also observed in the ligated as compared with the noninstrumented animals. In addition, the muscle Mg-ATPase activity was significantly (p<0.05) reduced in the experimental group. The pulmonary vascular smooth muscle of ligated animals developed less force (p<0.01) and shared similar maximum shortening capacity and longer isotonic half-relaxation time (p<0.05) as compared with the noninstrumented group. To the extent that the present data can be extrapolated to persistent pulmonary hypertension syndrome of the newborn, it is unlikely that the postnatal maintenance of a high resistance in this syndrome is the result of greater vascular muscle contractility. (Circulation Research 1993;72:588–596)

KEY WORDS • ductus arteriosus • pulmonary hypertension • hypoxemia

Persistent pulmonary hypertension syndrome of the newborn (PPHN) is a clinical entity characterized by persistent hypoxemia secondary to the maintenance of a high pulmonary vascular resistance and right-to-left shunting across the fetal channels after birth. Its etiology and pathogenesis are unknown but believed to be secondary to intrauterine abnormal events.

It has recently been recognized that complete or partial occlusion of the fetal ductus arteriosus in prenatal sheep is associated with lung histological changes similar to those observed in infants dying with the PPHN syndrome. After birth, the ductus arteriosusligated animal has a high pulmonary vascular resistance, abnormal pulmonary vasoreactivity to increased inspired oxygen, and evidence of right-to-left shunting at the foramen ovale level. Thus, the ductus arteriosusligated fetal sheep is the first animal model of PPHN representative of the human disease and is perhaps suitable for determining the mechanism involved in the maintenance of a high pulmonary vascular resistance after birth in affected newborns.

Concerning the pathogenesis of PPHN syndrome, a commonly held view is that the failure of the pulmonary vascular resistance to decrease after birth is secondary to the increased muscularization of the pulmonary vasculature. Implicit in this hypothesis is that the persistence of a high pulmonary vascular resistance in the syndrome is secondary to the effect produced by smooth muscle cells that, either by their increased number or increased responsiveness to humoral factors, have a greater potential to induce and maintain vasoconstriction. Little is known, however, about the mechanical properties of the vascular smooth muscle in PPHN syndrome.

We recently demonstrated that perinatal pulmonary vascular smooth muscle is at a mechanical disadvantage as compared with adult muscle. In normal sheep, the fetal and newborn pulmonary vasculature generates less force and has a lower shortening capacity than the adult pulmonary vasculature, indicating that, under physiological conditions, the pulmonary vascular muscle of the fetus has less potential for shortening and thus less potential for increasing vascular resistance.

Furthermore, in at least one model of pulmonary hypertension—monocrotaline-induced pulmonary hy-
pertension in the rat—in which excessive pulmonary vascular muscularization is also seen, the pulmonary vascular muscle was shown to generate less force than in the control condition. Although morphological substrate differences between the fetal sheep and adult rat lung do exist, these data suggest that the medial hypertrophy of the pulmonary vasculature in the rat model of pulmonary hypertension consists of hypertrophied smooth muscle cells with reduced contractility.

Therefore, to ascertain the role of muscle contraction in the maintenance of a high resistance to blood flow after birth in the PPHN syndrome, we evaluated biochemical and mechanical property changes of pulmonary vascular smooth muscle after ductus arteriosus ligation in fetal sheep.

Materials and Methods

Animal Preparation

Pregnant ewes between 123 and 127 days of gestation (term, 145 days) were fasted for at least 24 hours before surgery. The ewes were anesthetized with intravenous pentobarbital sodium (loading dose, 20 mg/kg; maintenance, 1 mg/kg every 30 minutes). Under sterile conditions, the fetal head and upper chest were delivered through a uterine incision. A left fetal thoracotomy was performed, exposing the heart and great vessels. The ductus arteriosus was identified and ligated with 2-0 silk suture in 31 fetuses (ligated group). The chest was closed in layers. The fetus was carefully returned to the uterus, the wall of which was closed in layers. Particular care was taken to avoid any significant amniotic fluid loss. Catheters, when placed, were tunneled to the flank of each ewe, and ampicillin (1 g) was injected into the amniotic sac and abdominal cavity at the time of surgical closure. Thereafter, both ewe and fetus received antibiotics for 4 days. The ewe received $1 \times 10^4$ units penicillin and 50 mg gentamicin intramuscularly twice daily, and the fetus received one half of this dose intravenously once a day when a catheter was in place. All wounds were cleaned daily, and the catheters were filled with fresh heparin solution (1,000 units/ml) to prevent clotting.

Six ewes were subjected to the same surgical procedure, but the ductus arteriosus was only visualized and not ligated (sham-operated group). In twelve of the ligated and in all sham-operated animals, polyvinyl catheters (5F) were placed into the carotid artery and jugular vein for hemodynamic measurements. In these fetuses, blood samples (0.3 ml) were obtained daily from the carotid artery for blood gas and pH measurements carried out with a blood gas analyzer (model ABL-3, Radiometer, Copenhagen). In the ductus arteriosus–ligated fetuses in which no intravascular catheters were placed, daily two-dimensional real time ultrasound images were obtained to confirm that the fetuses were alive by documenting the presence of heart beats. Sixty-one noninstrumented sheep fetuses served as controls (noninstrumented group).

Experimental Design

Between 137 and 140 days of gestation the noninstrumented, sham-operated and ligated ewes were subjected to a cesarian section under pentobarbital sodium anesthesia. The fetal head was carefully exteriorized, and a 5F Swan-Ganz catheter was advanced from the jugular vein into the pulmonary artery. Sequential measurements of mean right atrial, ventricular, and pulmonary arterial pressures were obtained. Systemic arterial pressure was also recorded by the carotid artery catheter. Vascular and intracardiac pressures were measured with pressure transducers (Gould Inc., Oxnard, Calif.) and continuously recorded on paper. Measurements of heart rate were derived from the pressure-wave frequency. Subsequently, the fetus was subjected to a left thoracotomy, and a precalibrated electromagnetic flow probe (flowmeter and probe, Carolina Medical Electronics, Inc., King, N.C.) of adequate size (less than 20% constriction of the vessel) was placed around the main pulmonary artery immediately proximal to the ductus arteriosus; then a measurement of right ventricular output was obtained. Finally, the left atrial pressure was obtained by direct puncture of the left atrium. All fetuses were rendered apneic at the time of cesarean section by the pentobarbital sodium anesthesia administered to the ewe; thus, the lungs were never inflated or oxygenated.

After the hemodynamic measurements, the fetus was rapidly killed with an intravenous dose of potassium chloride, and after the body weight was obtained, the lungs and heart were removed en bloc.

Confirmation of the effective ligation of the ductus arteriosus was always sought by probing the ductus arteriosus from the pulmonary artery side during the postmortem examination.

Organ Weights

The hearts from all animals were dissected to obtain the free wall weights of the right and left ventricles, septum, and atria. In addition, the liver weights were also obtained from all animals. These measurements were obtained to evaluate the impact of chronic increased afterload on the fetal heart. Total fresh wet lung weight was obtained as a crude measurement of lung edema.

Vascular Smooth Muscle Mechanics

A segment of the second generation intralobar pulmonary artery of randomly selected ligated and noninstrumented animals was carefully dissected and placed in ice-cold Krebs-Henseleit solution. Using our lever system to obtain pulmonary vascular muscle mechanical measurements in pilot experiments in sheep, we observed that only second-generation intralobar pulmonary arterial strips yielded consistent results. Smaller arteries (third and fourth generations) showed poor force development and shortening (authors’ unpublished data), likely related to the deleterious effect of tissue dissection and handling on the contractility of the smooth muscle cells. Thus, in the present study the mechanical properties of only the second-generation intralobar pulmonary arterial strips were delineated.

The pulmonary vessel rings were transversely opened, yielding a 2-mm-wide rectangular strip. The strips were attached to a clamp fixed to the bottom of a 10-ml bath containing Krebs-Henseleit solution at 37°C, pH 7.4, Po2 of 600 mm Hg, and Pco2 of 40 mm Hg. The upper end was tied by 7-0 graded silk to the magnesium arm of an electromagnetic muscle lever system.
The following mechanical measurements were obtained: 1) Length–force curves were initially obtained to establish the optimal length and the maximum isometric tension. 2) Maximal shortening capacity was obtained by the quick-release method, which consisted of releasing the muscle to a quasi-zero load (0.5 mN) under passive and active contraction. For the passive contractions, the degree of muscle shortening after passive recoil (releasing the muscle to a quasi-zero load from its resting tension without stimulation) was measured. For the measurement of active contraction, 2 seconds after the onset of the electrical stimulation (18 V, 10 seconds), the muscle was released to a quasi-zero load and allowed to shorten maximally. The maximal active shortening capacity was represented by the difference between total and passive shortening and expressed as a percentage of the optimal length. 3) Isometric half-relaxation time was measured as the time for the force generated during an electrically stimulated isometric contraction to decrease by 50%. 4) Isotonic half-time relaxation was measured by a previously described technique to obtain a load and initial contractile element–length independent index of isotonic relaxation time, based on the fact that muscle relaxation depends on the load that it is subjected to, the state of muscle activation, and the length of the muscle contractile element at the onset of relaxation. Brieﬂy, at their ideal lengths, the muscle strips were supramaximally electrically stimulated to elicit maximal isotonic shortening. The stimulus was turned off at peak shortening, and the muscle strips were allowed to elongate. Immediately after the termination of the electrical stimulation, a series of load clamps were applied. The application of instantaneous load clamps at the same point in time for each contraction ensures that the muscle strips will begin to relax at the same length of the muscle contractile element even under different loads. For each of the loads, the time to elongate 50% of the initial length (half-time) was determined. The half-times for each curve with their respective loads were ﬁtted by linear regression, and the zero-load half-time was extrapolated. For all vessel strips, the correlation coefﬁcient for the load versus half-time was 0.95 or higher (p<0.01). The intercept of the linear regression equation yields the isotonic relaxation index, which is load and initial contractile element–length independent. 5) Vessel wall compliance was measured as the slope of the changes in length induced by loads of different magnitude after papaverine (10⁻⁴ M) was added to the muscle bath to prevent stretch-induced contraction.

Measurement of Myosin and Actin Content

Right and left pulmonary arterial strips were carefully dissected from lung parenchyma and connective tissue in Krebs-Henseleit solution (4°C) within 2 hours of procurement. The pulmonary vascular generations chosen for study were selected according to the classiﬁcation of Levin et al. The dissected vascular tissues were stored separately at −70°C until required for myosin and protein extraction. The method used has been previously described. Briefly, thawed tissue was homogenized for 1 minute in 4 vol extraction buffer (40 mM Na₂HPO₄, 1 mM MgCl₂, 1 mM EGTA, 20 μM leupeptin, 1 mM dithiothreitol, and 250 μM phenylmethylsulfonyl fluoride, pH 8.0) with a Potter-Elvehjem tissue grinder. After homogenization, the crude supernatant extracts obtained were assayed for protein content by the method of Bradford. This technique enabled us to measure the tissue smooth muscle cell protein content, as represented by the supernatant protein concentration. All collagen- and elastin-derived proteins have been previously shown to fully precipitate after centrifugation as presently carried out in a procedure used by us. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the procedure of Laemmli18 by using 4–20% linear polyacrylamide gradient separating gels with 3% stacking gels. Known amounts of crude original supernatant and pellet extracts were applied to the gels, and electrophoresis was carried out for 3–3.5 hours using a constant current of 20 mA at 15°C. Myosin heavy chain and actin contents of the gels were determined by quantitative densitometry using an LKB Ultrosca XL laser densitometer. To avoid any overestimation or underestimation of the contractile proteins, the relative dye-binding capacities of puriﬁed myosin heavy chain and actin were determined as described by Sutoh. The total amounts of myosin heavy chain and actin in the supernatant and pellet extracts were summed and then normalized to the original fresh wet weight of the tissue sample to obtain the myosin content of the whole tissue. The identity of the myosin heavy chain band detected by SDS-PAGE was conﬁrmed by Western immunoblot using antibodies speciﬁc for smooth muscle (rabbit anti–chicken gizzard myosin supplied by Dr. Ute Groschel-Stewart, University of Darmstadt [FRG]). The identity of bands corresponding speciﬁcally to actin was conﬁrmed by using commercially available monoclonal antibodies speciﬁc for smooth muscle α-actin (Boehringer Mannheim Corp., Indianapolis, Ind.).

Myosin Heavy Chain Isoform Distribution

Smooth muscle and nonmuscle myosin heavy chain isoforms were separated by 4% SDS-PAGE at 10°C on 8×10-cm minigels using the Laemmli18 buffer system. Crude pulmonary artery sample loads were kept below 3 μg and at the smallest possible volume (usually under 10 μl) to obtain maximal separation of heavy chains. Gels were run at a constant voltage of 200 V for 60–65 minutes and immediately used in Western blotting experiments or were ﬁxed in 40% (vol/vol) methanol–10% (vol/vol) acetic acid for silver staining. Protein bands in unblotted gels were visualized by use of a commercial silver-staining kit (Rapid-Ag-Stain, ICM Biomedicals, Cleveland, Ohio). Gels were scanned with an LKB Ultrosca XL laser densitometer (663 nm) to evaluate myosin heavy chain isoform stoichiometry. For Western blot studies, proteins were electrobotted to nitrocellulose paper at 4–8°C by a 25 mM Tris–192 mM glycine (pH 8.3) transfer buffer.20 Blots were blocked overnight and then were shaken in phosphate-buffered saline (0.05% Tween 20) containing either 10 μg/ml rabbit anti–chicken gizzard myosin immunoglobulin G21 or 10 μg/ml rabbit anti–chicken thrombocyte myosin immunoglobulin G22 for 2.5 hours to identify smooth muscle and nonmuscle myosin heavy chains. Goat anti-rabbit immunoglobulin G conjugated with hors eradish peroxidase, diluted 1:2,000 as per the...
manufacturer’s instructions (ICN ImmunoBiologicals, Cleveland, Ohio), was used as the secondary antibody; then blots were developed in Tris-buffered saline containing 0.05% chloronaphthol, 16.7% (vol/vol) methanol, and 0.015% H₂O₂.

**Measurement of Myosin ATPase Activity**

Myosin ATPase activity was measured in crude homogenates of ligated and noninstrumented pulmonary arteries prepared as described previously. Briefly, Mg-ATP (final concentration, 2 mM) was added to the crude homogenates to initiate the reaction, which was subsequently halted after 30 seconds with the addition of trichloroacetic acid to 10% (vol/vol). The concentration of inorganic phosphate liberated was quantified by the malachite green colorimetric assay. Rates of inorganic phosphate liberation (ATP hydrolysis) were normalized to crude homogenate myosin heavy chain content determined by the quantitative SDS-PAGE described earlier.

To exclude any error related to differences in vascular tissue water content, the wet/dry weight ratios of second generation strips from the noninstrumented and ligated groups were obtained.

**Statistical Analysis**

Data were processed by Students’ t test and two-way analysis of variance. All data are reported as mean±SEM and p<0.05 was considered significant.

**Results**

Of all ligated fetuses, 10 were alive at the time of the scheduled cesarean section, and another two fetuses were delivered vaginally after natural labor within 24 hours from the scheduled cesarean date (total of 12 ligated animals). Nineteen other ligated fetuses were stillborn. The mean gestational age of the animals studied at cesarean section was 136±2 days, and the ductus arteriosus of these animals was ligated for 12±3 days. All stillborn animals were alive at least 24 hours before abortion, based on daily fetal ultrasound assessment, and had a gestational age of 135±1 days and duration of ductus arteriosus ligation of 6±1 days. All six sham-operated animals were alive at the time of the cesarean section, had a gestational age of 136±2 days, and were surgically instrumented 8±3 days before delivery. The noninstrumented fetuses (n=61) had a gestational age of 137±3 days and were all alive at the time of cesarean section.

For the first 3 days after surgery, arterial blood gases and pH were consistently obtained in all animals in which a catheter had been placed but were not available on a daily basis from every fetus thereafter because of technical problems (obstruction or displacement of the arterial catheter). At 24 and 72 hours after surgery, the values were similar in both groups except for a small but significantly higher PaCO₂ at 24 and 72 hours and lower pH at 72 hours in the ligated group (Table 1).

**Hemodynamic Measurements**

The ligated fetuses had a significantly higher pulmonary arterial pressure (p<0.01) with no differences in systemic arterial pressure, atrial pressure, and heart rate when compared with the sham-operated group. The mean right ventricular output was reduced by almost 50% in the ligated animals (p<0.05) (Table 2).

**Organ Weights**

The measured organ weights for the noninstrumented, sham-operated, and ligated groups are summarized in Table 3. Ductus arteriosus ligation resulted in a 65% increase in total heart weight (p<0.01) and a small increase in liver weight (p<0.01 when compared with the noninstrumented group only). The lungs and total body weight did not significantly vary between groups. As a percentage of the total heart weight, the right ventricular free wall was greater and the left was lower when compared with the noninstrumented and sham-operated groups (p<0.01, Table 4). No significant differences between the noninstrumented and sham-operated animals were present for these variables.

**Table 1. Fetal Arterial Blood Gases and pH After Surgery**

<table>
<thead>
<tr>
<th>Time after surgery (hours)</th>
<th>Sham-operated (n=6)</th>
<th>Ligated (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>72</td>
</tr>
<tr>
<td>pH</td>
<td>7.30±0.01</td>
<td>7.38±0.00</td>
</tr>
<tr>
<td>PaCO₂ (mm Hg)</td>
<td>49.1±1.2</td>
<td>47.3±1.4</td>
</tr>
<tr>
<td>PaO₂ (mm Hg)</td>
<td>22.3±1.5</td>
<td>14.7±1.0</td>
</tr>
<tr>
<td>Standard bicarbonate (mM/l)</td>
<td>21.6±0.6</td>
<td>24.4±0.6</td>
</tr>
</tbody>
</table>

n, Number of sheep. Values are mean±SEM.

*p<0.01 and tp<0.05 compared with respective values in the sham-operated group.

**Table 2. Hemodynamic Measurements**

<table>
<thead>
<tr>
<th></th>
<th>Sham-operated (n=6)</th>
<th>Ligated (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic arterial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>62.4±1.2</td>
<td>59.8±2.5</td>
</tr>
<tr>
<td>Diastolic</td>
<td>52.4±1.3</td>
<td>50.7±2.1</td>
</tr>
<tr>
<td>Mean</td>
<td>57.2±1.3</td>
<td>55.3±2.1</td>
</tr>
<tr>
<td>Pulmonary arterial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>59.7±2.4</td>
<td>81.7±6.6*</td>
</tr>
<tr>
<td>Diastolic</td>
<td>50.2±2.1</td>
<td>65.7±2.9*</td>
</tr>
<tr>
<td>Mean</td>
<td>54.1±2.0</td>
<td>72.3±3.8*</td>
</tr>
<tr>
<td>Right atrial (mm Hg)</td>
<td>3.9±0.8</td>
<td>5.9±1.4</td>
</tr>
<tr>
<td>Left atrial (mm Hg)</td>
<td>3.2±0.4</td>
<td>2.5±0.7</td>
</tr>
<tr>
<td>Right ventricular output (mL·min⁻¹·kg⁻¹)</td>
<td>117.7±23.9</td>
<td>63.2±17.8*</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>221±8.0</td>
<td>191.7±13.9</td>
</tr>
</tbody>
</table>

n, Number of sheep; bpm, beats per minute. Values are mean±SEM.

*p<0.01 and tp<0.05 compared with the sham-operated group.
TABLE 3. Body and Organ Weights

<table>
<thead>
<tr>
<th>Weight</th>
<th>Noninstrumented (n=61)</th>
<th>Sham-operated (n=6)</th>
<th>Ligated (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body (kg)</td>
<td>3.1±0.1</td>
<td>3.3±0.2</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>Heart (g/kg body wt)</td>
<td>7.9±0.1</td>
<td>7.34±0.2</td>
<td>11.9±0.6*</td>
</tr>
<tr>
<td>Liver (g/kg body wt)</td>
<td>24.3±0.6</td>
<td>29.1±3.8</td>
<td>31.7±1.7*</td>
</tr>
<tr>
<td>Lungs (g/kg body wt)</td>
<td>25.6±1.4</td>
<td>31.7±1.3</td>
<td>27.2±2.0</td>
</tr>
</tbody>
</table>

n, Number of sheep. Values are mean±SEM. *p<0.01 compared with the two other groups; †p<0.01 compared with the noninstrumented group only.

Pulmonary Vascular Mechanics

The pulmonary vascular muscle stresses following supramaximal electrical and high potassium stimulation were significantly decreased in the experimental animals (Figure 1), whereas the shortening capacity and the isometric relaxation half-time were not significantly different between groups. The isotonic relaxation half-time was significantly prolonged in the ligated group (Table 5). The vascular wall compliance was 23±5 μm/mN (n=12) in the noninstrumented and 20±4 (n=12) in the ligated group (p=NS).

Biochemical Changes

The myosin heavy chain content of second through fifth generation vessels was at least twofold greater in the ligated group as compared with the noninstrumented group (Figure 2). The second through fifth generation combined actin content of the ligated group also increased (2.20±0.19 [n=11] versus 0.85±0.06 [n=25] μg/mg wet tissue in the noninstrumented group, p<0.01). However, given the disproportional changes between the two proteins, the actin/myosin ratio was significantly lower in the ligated group (p<0.01) (Figure 3).

The stoichiometry of the pulmonary vascular muscle myosin heavy chain isoforms was significantly altered by ductus arteriosus ligation (Figure 4). For the second generation vascular tissue, the high/low myosin heavy chain isoform ratio was significantly increased in the ligated group (p<0.01). A third myosin heavy chain isoform, the nonmuscle fraction, was greatly increased in the ductus arteriosus–ligated pulmonary vascular tissue, representing up to 12% of the total myosin content. This represents a greater than fourfold increase in this isoform as compared with the noninstrumented vascular tissue (Table 6).

TABLE 4. Heart Chamber Weight

<table>
<thead>
<tr>
<th>Site</th>
<th>Noninstrumented (n=61)</th>
<th>Sham-operated (n=6)</th>
<th>Ligated (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV free wall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>6.8±0.3</td>
<td>7.3±0.6</td>
<td>12.5±0.7*</td>
</tr>
<tr>
<td>Percent of total heart weight</td>
<td>27.8±0.4</td>
<td>29.9±1.1</td>
<td>34.8±0.6*</td>
</tr>
<tr>
<td>LV free wall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>7.9±0.4</td>
<td>8.1±0.6</td>
<td>9.2±0.5*</td>
</tr>
<tr>
<td>Percent of total heart weight</td>
<td>32.3±0.5</td>
<td>33.0±0.8</td>
<td>25.7±0.5*</td>
</tr>
<tr>
<td>Septum (g)</td>
<td>4.4±0.2</td>
<td>4.3±0.5</td>
<td>7.0±0.5*</td>
</tr>
<tr>
<td>Atria (g)</td>
<td>5.3±0.2</td>
<td>4.7±0.2</td>
<td>7.2±0.4*</td>
</tr>
</tbody>
</table>

n, Number of sheep; RV, right ventricular; LV, left ventricular. Values are mean±SEM. *p<0.01 compared with the noninstrumented and sham-operated groups.

Figure 1. Bar graph showing pulmonary vascular smooth muscle mechanical stress in response to electrical and high potassium stimulation in the noninstrumented (control, n=10) and ligated (n=12) groups. **p<0.01 compared with the corresponding noninstrumented group.

The second generation pulmonary vascular muscle myosin ATPase activity was significantly decreased in the experimental group (Figure 5), indicating a lower potential for myosin phosphorylation. No significant difference in the wet/dry weight ratio for the second generation vascular strips of the noninstrumented (5.78±0.73, n=10) and ligated (6.05±0.42, n=10) groups was observed.

Newborn Data

Hemodynamic, blood gas, and pH data were obtained in one of the two ductus arteriosus–ligated fetuses born alive. The hemodynamic measurements were obtained while the animal was awake and breathing room air. The results were as follows: systemic arterial pressures of 79, 56, and 64 mm Hg (systolic, diastolic, and mean pressures, respectively) and pulmonary arterial pressures of 79, 45, and 56 mm Hg. The arterial blood gas and pH values while the animal was breathing room air were as follows: pH 7.25; PaO₂, 39.6 mm Hg; PaCO₂, 46.2 mm Hg; and standard bicarbonate, 19.1 mM/L. After 15 minutes of 100% oxygen exposure, the results were as follows: pH 7.24; Pao₂, 64 mm Hg; PacO₂, 48 mm Hg; and standard bicarbonate, 19 mM/L.

Discussion

Ductus arteriosus ligation in fetal sheep for an average of 12 days was associated with a sustained increase...
TABLE 5. Smooth Muscle Mechanics

<table>
<thead>
<tr>
<th></th>
<th>Noninstrumented (n=10)</th>
<th>Ligated (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$l_0$ (mm)</td>
<td>4.7±0.5</td>
<td>3.8±0.8</td>
</tr>
<tr>
<td>Resting tension (mN)</td>
<td>8.4±0.8</td>
<td>6.7±1.2</td>
</tr>
<tr>
<td>Shortening capacity (percent of $l_0$)</td>
<td>5.9±1.0</td>
<td>5.9±2.0</td>
</tr>
<tr>
<td>Relaxation half-time (seconds)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isometric</td>
<td>32.2±4.8</td>
<td>39.1±5.5</td>
</tr>
<tr>
<td>Isotonic</td>
<td>166.5±48.5</td>
<td>516.0±157.6*</td>
</tr>
</tbody>
</table>

$n$, Number of sheep; $l_0$, optimal length. Values are mean±SEM. *p<0.05 compared with the noninstrumented group.

In pulmonary arterial pressure, decreased right ventricular output, and increased total heart and right ventricular free wall weight. The myosin heavy chain content of the smooth muscle tissue was characterized by a greater proportion of the non–vascular smooth muscle myosin isoform and an increased high/low MHC isoform ratio. In addition, both decreased actin/myosin ratio and myofibrillar ATPase activity were observed in the ductus arteriosus-ligated pulmonary vessels, suggesting a lower potential for crossbridge cycling and myosin light chain phosphorylation. In keeping with these changes, the pulmonary vascular muscle in the ligated group developed less response to electrical and high potassium stimulation and showed prolonged isotonic relaxation.

In sheep, we have previously reported that the fetal pulmonary vascular smooth muscle develops less force and has a lower shortening capacity than do the newborn and adult counterparts. On the basis of the previous suggestion that the maintenance of a high pulmonary vascular resistance in the PPHN syndrome is secondary to the increased muscularization of the pulmonary vasculature, and an enhanced pulmonary vascular muscle ability to vasoconstrict (greater stress and shortening capacity) was expected. The present data demonstrated that, after ligation of the ductus arteriosus, the pulmonary vascular tissue has an even lower stress in response to electrical and high potassium stimulation. The shortening capacity was comparable to the control fetal vascular tissue but still much reduced as compared with newborn and adult sheep vascular tissue. A decrease in smooth muscle force generation in response to pressure overload has also been observed in the rat portal vein and grafted saphenous vein. In contrast, the smooth muscle force generation of large and small resistance systemic arterial vessels is maintained in response to a sustained increase in arterial pressure in rats with either experimentally induced or spontaneous hypertension. The reasons for the discrepancy in smooth muscle contractility in response to pressure load between the veins and arteries is unclear, but the present data suggest that the fetal pulmonary artery behavior is more in keeping with that of the veins. Nevertheless, the lower pulmonary arterial muscle stress observed in the pulmonary hypertensive fetuses suggests that either structural reorganization of the contractile proteins or changes in the excitation–contraction coupling are responsible for the altered mechanical properties of the smooth muscle. Based on the present data, the former is the likely mechanism.

The smooth muscle cell changes that are presently described in pulmonary arterial vessels after ductus arteriosus ligation are not unique. Pressure overload “in vivo” or vascular stretch “in vitro” is known to induce smooth muscle hypertrophy, hyperplasia, and an increase in extracellular matrix components. These changes can also be accompanied by smooth muscle cell alterations, but the extent to which pulmonary hypertension influences smooth muscle cell differentiation at a given time in gestation in a certain species is presently unknown. After portal vein hypertension in rabbits, hypertrophy of the smooth muscle is associated with a disproportionate increase in thin, intermediate, and thick filaments. In this model of hypertension, the portal vein smooth muscle develops an increase in the thin/thick filament ratio and a reduction in force-generating ability. In rats, after portal vein hypertension the muscle force-generating ability is increased in proportion to the smooth muscle mass, but the maximal shortening velocity is decreased. Although the pulmo-

Figure 2. Graph showing pulmonary vascular wall myosin content for second through fifth generation vessels in the noninstrumented (control, n=25) and ligated (n=11) groups. **p<0.01 compared with the noninstrumented group.

Figure 3. Bar graph showing actin/myosin ratio of combined second through fifth generation values for pulmonary vascular smooth muscle in the noninstrumented (control, n=25) and ligated (n=11) groups. **p<0.01 compared with the noninstrumented group.
nary vascular muscle shortening velocity was not measured in our ligated animal group, the lower ATPase activity is indicative of abnormalities in this parameter after pulmonary hypertension.

Together, the decreased pulmonary vascular muscle force generation and biochemical changes observed in our fetal pulmonary hypertension animals, especially with respect to myosin heavy chain, suggest that prenatal ductus arteriosus ligation induces phenotypic modulation of the smooth muscle. This phenomenon is characterized by a change in cell phenotypic expression of the cell from a contractile to a synthetic form. Synthetic smooth muscle cells predominantly proliferate and produce extracellular matrix components. In smooth muscle cell culture, recent data indicate that a change from a contractile to a synthetic phenotype is associated with an increase in the nonmuscle myosin heavy chain isoform, suggesting that the latter is a marker for phenotypic modulation. Thus, although we have not actually measured smooth muscle synthetic function, the fourfold increase in content of pulmonary vascular myosin heavy chain nonmuscle isoform, the lower myosin heavy chain ATPase activity, and the decreased muscle-force generation presently reported in this model of pulmonary hypertension suggest phenotypic modulation of vascular smooth muscle.

Phenotypic changes in pulmonary vascular smooth muscle cells have also been previously reported in newborn animals after chronic hypoxia-induced pulmonary hypertension. The pulmonary vascular smooth muscle cells in these animals undergo functional changes, and a mixed population of phenotypically distinct cells is seen in the arterial wall. Synthesis of one or more low molecular weight elastogenic factors that stimulate the production of elastin in both fibroblasts and smooth muscle cells have been demonstrated after chronic hypoxia in the newborn calf. This evidence strongly suggests that phenotypically altered pulmonary vascular smooth muscle cells participate in the process of vascular remodeling characteristically seen in pulmonary hypertension. Also, in children with pulmonary hypertensive congenital heart disease, marked changes in immunostaining for smooth muscle contractile proteins and intermediate filaments precede intimal proliferation, suggesting that vascular remodeling is a late manifestation of increased pressure overload.

Among the mechanical properties of muscle studied, a prolonged isotonic relaxation half-time of the pulmonary vascular smooth muscle was observed in the ligated group. We have previously reported that the isotonic relaxation half-time is significantly increased in fetal and newborn pulmonary vascular muscle as compared with adult muscle. Abnormalities of the relaxation phase of smooth muscle contraction have already been implicated in the pathogenesis of systemic arterial hypertension. Since at birth the pulmonary vascular smooth muscle has to relax to allow for the decrease in lung resistance to blood flow to take place, abnormalities of the muscle relaxation phase may also account for

**TABLE 6. Myosin Heavy Chain Isoform Stoichiometry**

<table>
<thead>
<tr>
<th></th>
<th>Noninstrumented (n=10)</th>
<th>Ligated (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC1/MHC2</td>
<td>1.0±0.1</td>
<td>1.8±0.3*</td>
</tr>
<tr>
<td>NM-MHC</td>
<td>2.7±0.9</td>
<td>12.4±0.7†</td>
</tr>
</tbody>
</table>

n, Number of sheep; MHC1/MHC2, high/low molecular weight myosin heavy chain (MHC) ratio. Values are mean±SEM.

*p<0.05 and †p<0.01 compared with the noninstrumented group.

**FIGURE 4.** Representative 4% sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ligated fetal sheep second generation pulmonary arterial tissue is shown in lanes B and C. Lanes A and D correspond to Western blots that identify, respectively, nonmuscle myosin heavy chain (NM-MHC) and high and low molecular weight myosin heavy chain isoforms (MHC1 and MHC2, respectively).

**FIGURE 5.** Bar graph showing pulmonary vascular smooth muscle ATPase activity in the noninstrumented (control, n=6) and ligated (n=4) groups. MHC, myosin heavy chain. *p<0.05 compared with the noninstrumented group.
the maintenance of a high vascular resistance in this sheep model of PPHN syndrome. Whether any of the smooth muscle changes presently described are responsible for the prolonged relaxation is unclear. Since the myosin light chain phosphatase is the enzyme responsible for the vascular smooth muscle relaxation phase,46 we speculate that differences in quantity and/or distribution of this enzyme may account for the observed abnormalities in vascular smooth muscle relaxation presently reported. Further investigation of the role of abnormalities in pulmonary vascular muscle relaxation mechanics in this animal model of PPHN syndrome is warranted.

As judged by the increased myosin heavy chain content of large and medium-sized vessels (second through fifth generation), ductus arteriosus ligation in the fetal sheep induces increased muscleization not only of small vessels, as previously described,17 but also of larger vessels, such as the ones used for the mechanical and biochemical measurements in the present study. In the monocrotaline rat model of pulmonary hypertension, decreased smooth muscle force generation was observed in small resistance vessels as well as in large elastic arteries,12 suggesting that, whatever is the cause for the altered muscle mechanics, it affects the muscle mechanical properties of the entire pulmonary vasculature. Further studies addressing the mechanical properties of the smooth muscle mechanics of small resistance vessels in this model of PPHN are necessary before our results can be extrapolated to the human syndrome.

In summary, we have demonstrated that, in this fetal sheep model of persistent pulmonary hypertension syndrome, the pulmonary vascular smooth muscle undergoes significant changes in contractile protein content, stoichiometry of myosin heavy chain isoform distribution, and myosubtilin ATPase activity that may account for the decrease in muscle force generation and altered relaxation properties presently described. The present novel data indicate that the maintenance of a postnatal high pulmonary vascular resistance in this model of the PPHN syndrome is not due to pulmonary smooth muscle—enhanced potential for vasoconstriction, as previously proposed, but is possibly the result of abnormalities in the muscle relaxation properties and vessel wall geometric changes.

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