Right ventricular systolic pressure load alters myocyte maturation in fetal sheep

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Departments of 1Physiology and Pharmacology, 2Medicine (Cardiology), 4Pediatrics (Pediatric Cardiology), and 5Obstetrics and Gynecology and 3the Heart Research Center, Oregon Health Sciences University, Portland, Oregon 97201-3098

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Barbera, A., G. D. Giraud, M. D. Reller, J. Maylie, M. J. Morton, and K. L. Thornburg. Right ventricular systolic pressure load alters myocyte maturation in fetal sheep. Am J Physiol Regulatory Integrative Comp Physiol 279: R1157–R1164, 2000.—The effects of right ventricular (RV) systolic pressure (RVSP) load on fetal myocyte size and maturation were studied. Pulmonary artery (PA) pressure was increased by PA occlusion from mean 47.4 ± 5.0 (±SD) to 71 ± 13.6 mmHg (P < 0.0001) in eight RVSP-loaded near-term fetal sheep for 10 days. The maximal pressure generated by the RV with acute PA occlusion increased after RVSP load: 78 ± 7 to 101 ± 15 mmHg (P < 0.005). RVSP-load hearts were heavier (44.7 ± 8.4 g) than five nonloaded hearts (31.8 ± 0.2 g; P < 0.03); heart-to-body weight ratio (10.9 ± 1.1 and 6.5 ± 0.9 g/kg, respectively; P < 0.0001). RVSP-RV myocytes were longer (101.3 ± 10.2 μm) than nonloaded RV myocytes (88.2 ± 8.1 μm; P < 0.02) and were more often binucleated (82 ± 13%) than nonloaded myocytes (63 ± 7%; P < 0.02). RVSP-loaded myocytes had less myofibrillar volume than did nonloaded hearts (44.1 ± 4.4% and 56.1 ± 2.6%; P < 0.002). We conclude that RV systolic load 1) leads to RV myocyte enlargement, 2) has minor effects on left ventricular myocyte size, and 3) stimulates maturation (increased RV myocyte binucleation). Myocyte volume data suggest that RV systolic loading stimulates both hyperplastic and hypertrophic growth.

The cellular changes associated with successful adaptation to increased systolic pressure load on the immature sheep heart are the focus of the current investigation. To quantitate myocardial growth, contributions by hypertrophy and hyperplasia in response to loading, myocyte size, and numbers of myocytes per unit tissue volume must be made. We tested the hypothesis that the mass of the near-term fetal heart would increase in response to a selective right ventricular (RV) systolic pressure load by increased hyperplastic growth with cells increasing in size only to the extent predicted by normal myocyte growth in fetal sheep (19). To test this hypothesis, we applied a relatively severe pressure load to the RV of near-term fetal sheep for 10 days to stimulate RV growth. These quantitative data were used to estimate alterations in growth patterns of the fetal myocardium under pressure-load conditions. Arterial blood gas measurements were done to assess the condition of the instrumented fetal sheep. Hemodynamic parameters were monitored to assess adequacy of RV pressure load. The right atrial pressure (RAP)-RV stroke volume (SV; RVSV) relationship was assessed before and during RV systolic load to determine the effects of RV systolic pressure load on RV function. Similarly, RVSV as a function of pulmonary artery pressure was determined before and during RV systolic load to assess the adequacy of RV pressure load. We then measured myocyte

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size, degree of binucleation, and alterations in myocyte and organelar volume fraction in the loaded hearts and compared these measurements with unloaded hearts from age-matched controls. This study investigated the cellular response to RV obstruction severe enough to induce a change in RV mass but not associated with hemodynamic deterioration.

**METHODS**

Pregnant ewes (*Ovis aries*) of mixed Western breeds with dated gestational age were purchased from local farmers and were brought to the laboratory pens several days before surgery to become accustomed to new surroundings. Guidelines established by the Department of Animal Care, Oregon Health Sciences University for the care and use of sheep were followed.

**Experimental Groups**

Fetuses were assigned to two groups. *Group 1* consisted of 13 instrumented fetal sheep in which hemodynamic studies were performed before and after 10 days of RV systolic pressure load. *Group 1* fetuses were assigned to one of two subgroups for analysis: 1) for determination of myocyte size and nucleus number (*n* = 8) or 2) for determination of cytomorphometric features (*n* = 5). This subgrouping was necessary because the tissue-preparation methods were mutually exclusive. *Group 2* consisted of nine uninstrumented fetal sheep matched for gestational age with *group 1* fetuses, and subgroups served as their controls; five served as the control group for determination of myocyte size and myocyte maturational state, and four served as controls for the cytomorphometric group.

**Animals and Surgical Procedures**

Sterile surgery was performed as described previously (1, 18, 22, 23) on ewes of 121 ± 1 (mean ± SD) days gestation after a 24-h fasting period. Anesthesia was induced by administering an intravenous mixture of diazepam and ketamine. The ewe was intubated, and anesthesia was maintained using 1.0% halothane (Pittman-Moore, NJ) in a 70:30 mixture of oxygen and nitrous oxide.

The abdomen was opened in the midline, exposing the uterus. The uterus was incised, and the superior portion of the fetus was delivered through the uterine incision. The right jugular vein was cannulated with two 1.7-mm OD polyvinyl catheters (Bolab, V-8, Lake Havasu City, AZ), and the catheters were advanced to the right atrium. One jugular vein catheter was used to measure RAP, whereas the second jugular vein catheter was used to infuse drugs, withdraw blood, or infuse fluid. The right carotid artery was cannulated with 1.3- and 1.7-mm OD polyvinyl catheters (Bolab, V-5 and V-8), and the catheters were advanced to near the junction of the brachiocephalic artery and the aorta. The fetal heart was exposed through a left thoracotomy in the third intercostal space. The pericardium was opened over the lower margin of the main pulmonary artery exposing only the left atrial appendage, aorta, pulmonary artery, and ductus arteriosus. A 1.3-mm OD polyvinyl catheter (Bolab, V-5) was placed in the left atrium via the left atrial appendage. A 1.3-mm OD polyvinyl catheter (Bolab, V-5) was placed in the pulmonary artery proximal to the ductus arteriosus and secured in position using a purse-string suture. A 10-mm inflatable vascular occluder (In Vivo Metric, Healdsburg, CA) was placed around the pulmonary artery. An electromagnetic flow probe (In Vivo Metric or C&C Instruments (Culver City, CA)) was placed around the pulmonary artery between the proximal catheter and distal inflatable occluder. A 1.3-mm OD polyvinyl catheter (Bolab, V-5) with a 4-cm Silastic tip with three side holes was slipped into the anterior peri- cardial space via the pericardiotomy. The pericardium overlying the great vessels was left open, allowing passage of the catheters and flow probe connector cable. When prepared in this fashion, the pericardium reseals in a few days. The fetal chest was then closed in anatomic layers. A 1.7-mm OD polyvinyl catheter (Bolab, V-8) was attached to the fetal skin and used to measure amniotic fluid pressure. All catheters were anchored to the fetal skin. The fetus was returned to the uterus, and the uterus was closed. All catheters and the flow probe cable were passed through the ewe’s abdominal wall and tunneled to the ewe’s flank where they were stored in a nylon pouch sutured to the skin. The abdomen was closed, and 10 million units of penicillin G (Bristol-Myers Squibb, Princeton, NJ) were instilled into the amniotic space. Anesthesia was terminated, and the ewe was allowed to recover. After surgery, ewes were located in a clean pen, and 6 ± 1 recovery days were allowed before experiments were performed.

**Experimental Protocol**

**Laboratory procedure.** On the day of the experiment, the ewe was placed in a stanchion cart and allowed free access to water and food. Hydrostatic pressures from the amniotic fluid space, pericardial space, right atrium, pulmonary artery, left atrium, and carotid artery were measured using Statham Gould (Cleveland, OH) P23ID pressure transducers calibrated by mercury manometer. All vascular pressures were referenced to pericardial pressure.

The electromagnetic flow probes, previously calibrated using sheep blood and the appropriate size sheep vessel, were connected to a Gould (Oxnard, CA) SP2202 flowmeter. Flow zero was set on the diastolic plateau. The outputs from the flowmeter and the pressure transducers were connected to a Gould RS2000 eight-channel polygraph (Cleveland, OH). The analog signals from each channel of the polygraph were digitized (100 Hz) using a Hewlett-Packard 3497 data-acquisition unit (Fort Collins, CO), averaged every 5 s, and stored on disk using a Hewlett-Packard 9826S computer. Data were later checked against original polygraph records. RVSV was determined by dividing average pulmonary artery flow on 5-s intervals by heart rate for that interval. Arterial pH, PCO₂, and Po₂ values were determined using an Instrumentation Laboratories model 1306 blood-gas analyzer corrected to 39°C. Arterial oxygen content was determined using an Instrumentation Laboratories model 382 oximeter.

**Experimental protocol.** Baseline measurements of hydrostatic pressures, pulmonary artery flow, heart rate, and carotid artery blood gases were made. The fetuses were then given blocking doses of atropine (0.5 mg/kg; Elkins-Sinn, Cherry Hill, NJ) and propranolol (1 mg/kg; Smith Nephew, Franklin Park, IL) (22) to minimize the autonomic compensatory influences on the heart during assessment of RV function. Cholinergic and β-adrenergic blockade at these doses was demonstrated in pilot animals with increasing doses of agonist, acetylcholine, and isoproterenol. Postblock hemodynamic measurements were then made. RV performance was assessed from function curves relating RVSV to mean RAP. RV function curves were generated by rapidly withdrawing fetal blood into sterile, heparinized syringes until RVSV was approximately one-third the control value. The blood was then rapidly reinfused, and additional lactated...
Ringer solution was infused until a mean RAP of 8 mmHg was achieved.

RV systolic pressure load. In group 1 fetuses, systolic pressure load of the RV was then produced by partial inflation of the vascular occluder placed around the pulmonary artery. This pressure was increased by ~10–30 mmHg over the first 3 days of pressure loading. Pulmonary artery pressure and pulmonary artery flow profile were checked daily to ensure maintenance of adequacy of RV systolic pressure load. Elevated pulmonary artery pressure and thus increased RV systolic pressure load were maintained for 10 days. The total duration of occluder inflation and this RV systolic pressure load was 10 days.

Analysis of Function Curves

Each RAP-RVSV relationship consisted of a steep ascending limb, where SV increased rapidly with increasing mean RAP, and a plateau limb, where SV increased little with increasing RAP (22). The RAP-RVSV relationship was broken into an ascending limb and a plateau limb to allow statistical comparison of the position of the curves at day 0 and after 10 days of RV systolic pressure load. The intersection of the ascending and plateau limbs of the RAP-RVSV relationship (breakpoint) was mathematically determined for each curve by repeatedly fitting a line using the least-squares method through each of two changing sets of points along the curve until the sum of the residuals from the two least-squares fits was minimal (11–13, 18, 23).

Because of interanimal variability, breakpoint data were normalized to mean breakpoint values. SV data were normalized using the following equation (23): SVn = SVi – (BPSVi – BPSV), where SVn is normalized SV, BPSV is mean breakpoint SV, BPSVi is individual function curve breakpoint SV, and SVi is individual SV. RAP was normalized using the following equation: RAPn = RAPi – (BPRAPi – BPRAP), where RAPn is normalized RAP, RAPi is individual RAP, BPRAPi is individual function curve breakpoint RAP, and BPRAP is mean breakpoint RAP. The slopes and y-adjusted means of the two limbs of the function curves were compared using analysis of covariance (20).

After study, the ewe and fetus were killed with pentobarbital sodium. At autopsy, the fetuses were dissected to confirm catheter position and flow probe fit.

Myocyte Isolation Procedure

Myocytes from fetal hearts were dissociated using collagenase and protease as described by Klockner and Isenberg (9) and Mitra and Morad (10). The hearts were hung from a perfusion apparatus by cannulating the aorta and were perfused retrograde by a series of solutions at 39°C through the coronary arteries. The protocol was 5 min perfusion with oxygenated low-calcium buffer (Ca-Tyrode, no calcium added); 10 min with 50 μM Ca-Tyrode solution containing collagenase (Worthington type II, 300 units/ml), protease (type XIV 10 mg in 60 ml, Sigma), and albumin (0.1%); or 5 min with KB solution. Portions of the RV and left ventricular (LV) free walls and septum were separately removed with scissors, and the chunks were gently agitated in high-potassium solution (in mM: 74 glutamic acid, 30 KCl, 20 taurine, 0.5 EGTA, 10 HEPES, and 10 glucose with KH2PO4 and MgSO4 and adjusted to pH 7.37 using KCl) to release the cells. Isolated cells were filtered through a nylon mesh that removed tissue chunks, resulting in a cell slurry containing >95% isolated individual cells. In addition to myocytes, endothelial cells, fibroblasts, and red cells were also present. Isolated cells were divided into aliquots. One was fixed with 1.5% glutaraldehyde in monophosphate buffer, pH 7.4, and one was refrigerated. Freshly isolated myocytes (<2 h old) were measured for length and width using calibrated image analysis software (Optimas, Seattle, WA) at ×400 phase microscopy (Zeiss Axioshot, Bartels and Stout, Bellevue, WA).

Electron Microscope Morphometric Methods

Hearts were prepared for morphometric analyses as in our previous studies in fetal sheep and in pregnant guinea pigs (11, 16), with minor modifications as suggested by other researchers (6, 8, 19). The heart was removed from the chest, cooled, trimmed, measured, and weighed. An "outflow" catheter (3.0 mm OD) was tied in place in each ventricle through the atrium. The heart was immediately hung by the ascending aorta on a perfusion apparatus. Perfusate entered the coronary arteries via the aorta and flowed out of the heart through the atrial outflow catheters kept under physiological filling pressure determined by the height of the outflow tubes above the heart (5 cm). Hearts were initially perfused for 2 min at 30-mmHg pressure via the coronary arteries (which is associated with no apparent edema) with a heparinized Ringer solution containing 1% purified bovine serum albumin and 1% adenosine to dilate the coronary arteries. The heart was then perfused with a freshly made fixative solution containing 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M monophosphate buffer, pH 7.4, for 20 min and stored in the fixative overnight.

After fixation, the heart was dissected into its component parts: atria, interventricular septum, and RV and LV free walls, and each component was weighed. At a point one-third the distance from the base of the heart, a 3-mm-thick transverse section of the LV was removed for further processing. Five random tissue blocks (1-mm cubes) were removed from the midportion of the anterior and posterior aspects of the ventricular free walls and processed for a cascade sampling design at three magnifications (6, 8).

Tissue blocks were held at 4°C overnight in the glutaraldehyde-formaldehyde fixative. They were then rinsed four times in 0.1 M phosphate buffer, pH 7.4, postfixed for 120 min in buffered 2% OsO4, dehydrated in an ethanol series, and embedded in epoxy resin. “Thick” sections (2 μm) were cut from each block for analysis under the light microscope. Thin sections were also cut (Sorvall MT5000 ultramicrotome), stained with uranyl acetate and lead citrate, and photographed under a JEOL 100-S electron microscope.

Volume fraction of specific structures was calculated from an unbiased estimate based on random point counting techniques (24–26). Points from a randomly placed grid were placed over tissue photomicrographs, and each point was categorized as an item to be counted. All points were assigned a category or, if ambiguous, as one-half point in each of the appropriate categories. Cell and noncell volume fractions as well as intracellular organellar volume fractions were determined (2, 11, 14, 25, 26).

Estimation of Proliferative Growth during RV Systolic Pressure Load

The number of myocytes in the RV free wall can be estimated in the nonloaded and systolic pressure-loaded hearts if the free wall weight, the fraction of the volume occupied by myocytes, and the average volume of myocytes are each known. We had previously determined the proportions of total near-term fetal sheep heart mass contributed by the individual chambers (15). The RV free wall accounts for ~30% of the total heart weight in the normal fetus. On the
basis of our previous studies (15, 16), the RV free wall of the systolic pressure-loaded heart would have weighed ~10 g if not loaded. We also estimated from our previous RV systolic load studies that >80% of heart weight increases above normal are accounted for by increased mass of the RV free wall. Thus the estimated average RV free wall weight of the systolic pressure-loaded hearts can be determined. The fractional volumes occupied by myocytes in nonloaded fetal hearts and in hearts exposed to increased RV pressure were determined from cytomorphometric data. By dividing the total RV free wall myocyte volume (µm³) by the average volume of each myocyte (µm³) of nonloaded hearts, the average number of cardiomyocytes in the non-pressure-loaded RV free wall was determined. This process was repeated for the systolic pressure-loaded RV free wall. Inference regarding cell proliferation with loading was then possible.

Statistical Analysis

Experimental data were analyzed using one-way analysis of variance. When justified by significant F statistic, Tukey’s multiple-comparison test was used to test for significant differences (24). For the morphometric studies, nested analysis of variance was applied to the various levels (blocks, slides, and photographs) to estimate the variance at each level (27).

RESULTS

Hemodynamic Consequences of RV Systolic Pressure Load

Thirteen fetal sheep (group 1) were studied before and 10 days after RV systolic pressure load, at 127 ± 1.2 (mean ± SD) and at 137 ± 1.2 gestation days, respectively. There was no difference in arterial pH (7.41 ± 0.03 vs. 7.38 ± 0.04), Pco₂ (46.0 ± 5.0 vs. 48.2 ± 3.8 Torr), Po₂ (20.2 ± 1.3 vs. 19.9 ± 1.8 Torr), or O₂ content (8.0 ± 0.2 vs. 8.0 ± 0.6 ml/dl) after 10 days of RV systolic pressure load.

Hemodynamic data collected at day 0 (baseline) and after 10 days of RV systolic load are shown in Table 1. There was no difference in mean RAP or carotid arterial pressure between baseline and loaded conditions. Partial pulmonary artery occlusion elevated mean proximal pulmonary artery pressure by an average of ~30 mmHg above baseline and therefore the pressure against which the RV ejected during the experimental period. Mean heart rate was unchanged by RV systolic pressure load. RVSV was not statistically significantly different between baseline and loaded conditions. Similarly, RV output was not statistically significantly different between baseline and loaded conditions.

RV Function Curve

Composite RV function curves relating RVSV to mean RAP before and at the end of 10 days of RV systolic pressure load are shown in Fig. 1. Although the position of the RVSV-RAP relationship appeared to be shifted downward after 10 days of RV systolic load, this shift was not statistically significant. RAP at the breakpoint of the RAP-RVSV relationship was unchanged. The RAP at the breakpoint of the RAP-RVSV relationship was 3.1 ± 1.2 mmHg before and 2.9 ± 1.4 mmHg after 10 days of RV systolic pressure load. The RVSV at the breakpoint of the RAP-RVSV relationship was 1.30 ± 0.41 ml/kg before and 1.08 ± 0.32 ml/kg 10 days of RV systolic pressure load.

RVSV as a Function of Mean Pulmonary Artery Pressure

The maximal pressure that could be generated by the RV in response to acute pulmonary artery occlusion was assessed in hearts that had been conditioned by RV systolic pressure loading. This maximal pressure was compared with the maximal pressure that could be generated by the same RV in response to acute pulmonary artery occlusion before RV systolic pressure loading, the unloaded baseline state. The maximal systolic pressure that could be generated by the ventricle at unloaded baseline state was 78 ± 7 mmHg. This increased significantly to 101 ± 15 mmHg (P < 0.005) in hearts “conditioned” by RV systolic pressure loading.

Fetal Cardiac Mass, Cardiomyocyte Size, and Myocyte Maturation State

Fetal weights at autopsy were appropriate for the stage of gestation (4.8 ± 1.0 kg, nonloaded, and 4.5 ± 0.8 kg for fetuses in the RV systolic pressure-load group). Morphometric measurements and percent binucleation for the RV systolic pressure-loaded fetal sheep (group 1) and nonloaded controls (group 2) are
shown in Table 2. Gestational age was the same for both groups at 137 ± 1.2 days. The RV systolic pressure-loaded hearts were 40% heavier (44.7 ± 8.4 g) than nonloaded hearts (31.8 ± 10.2 g; P < 0.03). Similarly, the heart weight-to-fetal body weight ratio was greater for the RV-load fetuses (10.9 ± 1.1 g/kg compared with 6.5 ± 0.9 g/kg for nonloaded controls; P < 0.0001). RV myocytes were elongated under the effect of the systolic pressure load (101.3 ± 10.2 μm compared with 88.2 ± 8.1 μm; P < 0.02). The length of LV myocytes was not different between RV systolic pressure-loaded and nonloaded hearts. The width of RV myocytes from RV systolic pressure-loaded hearts (14.4 ± 2.3 μm) was not greater than the width of RV myocytes from the nonloaded hearts (13.2 ± 2.5 μm). The width of LV myocytes from hearts in which the RV was loaded was not different from nonloaded myocytes (Table 2). The proportion of RV myocytes that were binucleated was higher in the RV systolic pressure load group (82 ± 13%) compared with nonloaded hearts (63 ± 17%; P < 0.02). There was no significant difference in the portion of binucleated cells from the LV of loaded hearts compared with LV cells from nonloaded hearts (Table 2).

Organellar Volume Changes With Systolic Pressure Load

Table 3 shows the proportion of the total tissue volume occupied by myocytes, endothelium, fibroblast or pericyte, and noncellular matrix. Of these volume fractions, only the myocyte proportion of the systolic pressure-loaded hearts showed a statistically significant increase. The volume fraction occupied by myocytes was 75.5 ± 1.3% in the nonloaded group and was greater at 81.3 ± 4.6% in the systolic pressure-loaded group 2 (P < 0.05). Accompanying this increase in myocyte fraction, the mean volume fraction of endothelium and extracellular matrix was lower after systolic pressure loading, though neither decrease reached statistical significance.

The volume fraction of organelles within the myocytes for the RV systolic pressure load group showed an interesting change. RV systolic pressure load resulted in a decrease in the myofibrillar fraction (56.1 ± 2.6% compared with 44.1 ± 4.4%, P < 0.002). In addition, the fraction of intracellular cytosolic matrix was significantly greater (32.0 ± 7.6% compared with 16.2 ± 4.4%) in systolic pressure-loaded cells (P < 0.008). Nested analysis of variance indicated that the greatest source of variability was between groups as opposed to photomicrographs or tissue section blocks.

**DISCUSSION**

The hemodynamic signals that modulate growth of immature myocardium are not well studied. However, it is well known that embryonic and fetal hearts “grow” primarily by producing more myocytes via hyperplasia until after birth. The adult heart increases wall mass by hypertrophy, regardless of the nature of the growth stimulus. Soon after birth, the myocardium of mammals is thought to undergo a “switch” from hyperplastic myocyte growth to hypertrophic growth over several postnatal days (17). In the rat, cardiomyocytes become binucleate as they mature, an indication that they are unable to divide further (4). After switching, all myocardial “growth” is via cell enlargement and architectural rearrangement (remodeling). Although prenatal heart growth has been superficially studied in a number of animal species, it has been studied little in large mammals. Whereas it is known that canine and human hearts have varying degrees of binucleation and/or polyploidy as they become “terminally differentiated” and unable to divide (17), such indices have not been studied in sheep, a frequently used model for cardiovascular development. One excellent cross-sectional study by Smolich et al. (19) showed that RV myocytes have larger cross-sectional areas than LV myocytes in fetal sheep and that both RV and LV myocytes increase in size over the course of gestation. This group did not study cardiomyocyte nucleation. The growth mode (hyperplasia or hypertrophy) by

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**Table 2. Ventricular and myocyte size data**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>Loaded (n = 8)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation, days</td>
<td>137 ± 1.2</td>
<td>137 ± 1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Fetal weight, kg</td>
<td>4.8 ± 1.0</td>
<td>4.5 ± 0.8</td>
<td>NS</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>31.8 ± 10.2</td>
<td>44.7 ± 8.4</td>
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<tr>
<td>Heart weight/fetal weight</td>
<td>6.5 ± 0.9</td>
<td>10.9 ± 1.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Myocyte length, μm</td>
<td>88.2 ± 8.1</td>
<td>101.3 ± 10.2</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>82.5 ± 8.0</td>
<td>90.2 ± 5.5</td>
<td>NS</td>
</tr>
<tr>
<td>Myocyte width, μm</td>
<td>13.2 ± 2.5</td>
<td>14.4 ± 2.3</td>
<td>NS</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>11.4 ± 2.0</td>
<td>13.3 ± 1.3</td>
<td>NS</td>
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<tr>
<td>Binucleation, %</td>
<td>63 ± 17</td>
<td>82 ± 13</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>76 ± 15</td>
<td>86 ± 9</td>
<td>NS</td>
</tr>
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</table>

Values are means ± SD. NS, not significant.
which the fetal sheep heart can increase its mass in the face of a systolic pressure load has not heretofore been determined. We tested the hypothesis that the mass of the near-term fetal heart would increase in response to a selective systolic pressure load by increased hyperplastic growth accompanied by cell enlargement only to the extent predicted in normal myocyte growth in fetal sheep (19).

After 10 days of increased systolic pressure load to the fetal RV, the fetal blood gases did not change from their normal control values. Fetal weights at autopsy were appropriate for the stage of gestation (4.8 ± 1.0 kg, nonloaded; 4.5 ± 0.8 kg for fetuses in the RV systolic pressure load group). Baseline arterial pH and blood gas values were comparable with values from previous studies in our laboratory (1, 12, 22) and other laboratories (21). Hemodynamic measurements were also similar to those made during previous studies in our laboratory (12, 18). Pulmonary artery pressure was a little higher than carotid artery pressure at day 0, which is consistent with our previous study (1). There was no difference in mean RAP, carotid artery pressure, heart rate, stroke volume, or RV output between baseline and loaded conditions. The position of the RAP-RVSV relationship was not significantly different before and after 10 days of RV pressure load. The maximal systolic pressure that could be generated by the RV after 10 days of RV systolic pressure load increased significantly. These observations show that there was not a deterioration in hemodynamic state. Was the increase in the maximum pressure seen after RV systolic pressure load due to the effects of systolic load or merely a developmental change? We did not address this question in the present study. However, this issue was carefully addressed in our previous studies (15, 16). In those studies, the slope of the relationship between RVSV and increasing pulmonary artery pressure was unchanged over 10 days in the chronically instrumented fetal sheep of the same gestational period. The findings of the present study demonstrate that the systolic load on the RV was accompanied by augmented systolic function, as previously shown (15, 16), and not by functional deterioration.

Total heart weight was greater in systolic pressure-loaded animals compared with the nonloaded animals as expected. Accordingly, there was a marked increase in the fetal heart weight-to-body weight ratio. In our previous study of RV systolic pressure load, we found that most of the increase above normal growth was due to RV wall augmentation. On the basis of studies in the rat (4), prenatal exposure to maternal carbon monoxide caused “pure” RV enlargement through a hyperplastic response without enlargement of RV myocytes. We hypothesized a similar finding in these sheep studies. We were therefore surprised to find that after systolic pressure loading, RV myocytes were longer on average than myocytes from unloaded age-matched RVs. Thus we found that our hypothesis predicting pure hyperplasia was not entirely true.

There was a trend for the LV myocytes to be longer, wider, and more binucleated than the nonloaded age-matched LV myocytes. Because the RV and LV share the interventricular septum and, through the interventricular septum forces affecting the RV, can be translated to the LV, it is possible that RV systolic pressure load had an effect on the myocytes of the LV.

Because we unexpectedly found myocyte hypertrophy, we sought to determine the contributions of hyperplasia and hypertrophy to increases in RV free wall weight in these animals. On the basis of the application of length and width data, a cylindrical model for the myocyte (which overestimates myocyte volume by ~15% compared with confocal microscope volume estimates; K. Thornburg, J. Maylie, G. Giraud, and M. Morton, unpublished data), the average volume of the RV myocyte from fetal hearts after 10 days of systolic pressure loading was greater than in the nonloaded RV myocytes (1.21 × 10^4 and 1.65 × 10^4 μm^3, respectively, an increase of 36%). This is, by definition, myocyte hypertrophy.

We also sought to estimate the degree of proliferative growth that occurred with RV systolic pressure loading. Hypertrophy and hyperplasia are not mutually exclusive. According to the cytomorphic findings (Table 3), myocytes occupied 76% of the myocardial tissue volume in nonloaded and 81% in loaded animals. Dividing the total RV free wall myocyte volume (7.6 × 10^12 μm^3) by the average volume of each myocyte (1.2 × 10^4 μm^3) of nonloaded hearts gives 6.3 × 10^8 as the average number of cardiomyocytes in the non-pressure-loaded free wall. If one repeats the process for the loaded RV free wall based on an estimated 20 g of tissue (see METHODS), the total number of myocytes is calculated to be 9.8 × 10^8, an increase of 56% above the control number of cells accompanying myocyte hypertrophy. These rough estimates are compatible with our finding that cell enlargement does not account for all of the increased heart mass.

An important finding of this study is the greater percentage of RV myocytes containing two nuclei with systolic pressure loading (82% vs. 63%). If sheep myocytes behave like rat myocytes and binucleation is an indication of maturation, then the augmentation of binucleation appears to indicate that pressure loading caused a reprogramming of the normal maturation rate. If only mononucleate cells are able to divide and only binucleate cells are able to enlarge (as suggested by rat heart data), then a maturational shift toward a higher population of binucleate cells would favor use of the hypertrophic process of growth should the load continue. This could theoretically limit the total number of myocytes in the heart by the time of birth. The separate roles of mononucleate versus binucleate cells in myocyte maturation requires further study.

We were puzzled by the finding that the myofibrillar volume fraction was decreased in loaded cardiomyocytes (Table 3). We expected the opposite, that is, an increase in myofibrillar volume fraction, based on the well-known adult response. One explanation for this finding is that cell replication is so rapid during severe loading that there is a lag in the manufacture of contractile protein. If true, this situation could be expected...
only in immature myocardium but not in fully differentiated myocardium where hypertrophy accompanied by increased contractile protein is the usual growth response.

Our data are compatible with our previously published studies on the fetal RV (15, 16). The conditioned RV had a thickened wall and decreased meridional radius to wall thickness ratio. This was accompanied by improved mechanical performance in the face of acute increases in pulmonary arterial pressure. In both studies, wall thickening was at the expense of RV chamber volume. In the previous study (16), this caused a potent parallel leftward shift in the pressure volume curve. This growth pattern may underlie some pathophysiologic findings in humans in which outflow restriction defects are accompanied by notably small ventricular chambers.

In summary, we have shown that a severe systolic pressure load on the RV of the near-term fetal sheep heart caused an increased heart weight and an increased capability of the conditioned ventricle to eject against a systolic pressure load. This adaptation was accompanied by an increase in the average length of the RV myocytes and an increase in the portion of the myocytes bearing two nuclei. Estimates of cell numbers suggest a vigorous hyperplastic response in addition to the hypertrophic response. Finally, the rapidly growing cells had less myofibrillar material (contractile protein) as a fraction of the total myocyte volume than did control cells. These data indicate that the heart of a large precocial mammal may use different mechanisms to grow in response to increased systolic load conditions than those previously found for rodent hearts.

Perspectives

In the present study, we have addressed the question whether immature myocardium can use cell division, cell enlargement, or both to adapt to increased systolic load. This is a crucial question because it relates to the mechanisms used as the growing myocardium responds to congenital heart defects that cause increased systolic pressure load. Ventricular wall stress increases as systolic load increases, as predicted by the Laplace relationship. Both the fetal and the adult hearts remodel in response to pressure load, increasing wall thickness while normalizing wall stress. However, the adaptive processes in the fetal heart are more complex than in the adult heart. The immature hearts we studied were in the process of normal growth hyperplasia when a pressure load was applied. In our previous studies of fetal RV systolic pressure load, RV mass was accompanied by an increase in RV wall thickness and a decrease in RV chamber volume (15, 16). In the present study, we saw a similar increase in heart mass with RV systolic pressure loading, and we found modest RV myocyte hypertrophy. However, this hypertrophy could not account for the extent to which cardiac heart mass increased. Therefore, we estimated the number of myocytes in RV systolic loaded and nonloaded hearts. Our estimates suggest an approximate 50% increase in myocyte number that accompanied cell enlargement. These findings suggest that the adaptive mechanisms underlying RV response to RV systolic pressure load involve both myocardial hypertrophy and hyperplasia. Understanding the regulation of these adaptive mechanisms would advance our understanding of cardiac growth from the fetus to the adult and could lead to new therapeutic modalities for the fetus and throughout life.

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