Sequential growth of fetal sheep cardiac myocytes in response to simultaneous arterial and venous hypertension

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Am J Physiol Regul Integr Comp Physiol 292: R913–R919, 2007. First published October 5, 2006; doi:10.1152/ajpregu.00484.2006.—While the fetal heart grows by myocyte enlargement and proliferation, myocytes lose their capacity for proliferation in the perinatal period after terminal differentiation. The relationship between myocyte enlargement, proliferation, and terminal differentiation has not been studied under conditions of combined arterial and venous hypertension, as occurs in some clinical conditions. We hypothesize that fetal arterial and venous hypertension initially leads to cardiomyocyte proliferation, followed by myocyte enlargement. Two groups of fetal sheep received intravascular plasma infusions for 4 or 8 days (from 130 days gestation) to increase vascular pressures. Fetal hearts were arrested in diastole and dissociated. Myocyte size, terminal differentiation (%binucleation), and cell cycle activity (Ki-67+) cells as a % of mononucleated myocytes) were measured. We found that chronic plasma infusion greatly increased venous and arterial pressures. Heart (but not body) weights were ~30% greater in hypertensive fetuses than controls. The incidence of cell cycle activity doubled in hypertensive fetuses compared with controls. After 4 days of hypertension, myocytes were (~11%) longer, but only after 8 days were they wider (~12%). After 8 days, %binucleation was ~50% greater in hypertensive fetuses. We observed two phases of cardiomyocyte growth and maturation in response to fetal arterial and venous hypertension. In the early phase, the incidence of cell cycle activity increased and myocytes elongated. In the later phase, the incidence of cell cycle activity remained elevated, %binucleation increased, and cross sections were greater. This study highlights unique fetal adaptations of the myocardium and the importance of experimental duration when interpreting fetal cardiac growth data.

hypothesis: intrauterine hypertension; hyperplasia; terminal differentiation; cardiomyocyte

The fetal heart grows by proliferation and enlargement of cardiac myocytes (23). Among mammals, proliferative growth of cardiac myocytes ceases at or soon after birth through a process known as terminal differentiation (18). Thereafter, the postnatal heart grows almost exclusively by cellular enlargement. In the fetal sheep, as in many other species, terminal differentiation is marked by the appearance of two nuclei in cardiac myocytes that were formerly mononucleated. The rate of proliferation during intrauterine life and the occurrence of terminal differentiation in the perinatal period together determine the maximum number of myocytes in the heart for life.

Intrauterine conditions affect cardiac myocyte proliferation, enlargement, and terminal differentiation, but myocyte responses to abnormal conditions depend on the level of maturity of the heart. Increased systolic or diastolic hemodynamic loads in near-term fetal sheep have been shown to increase myocardial mass (6, 7, 10). It is unknown if the growth of these loaded hearts occurs by increased myocyte proliferation or by myocyte enlargement. Adult hearts typically respond to increases in hemodynamic load in a predictable manner by a combination of myocyte enlargement and ventricular remodeling to reduce myocardial systolic wall stress (15, 20). Growth patterns of fetal cardiac myocytes under the influence of combined increases in preload and afterload, as occurs in conditions such as arteriovenous malformations and twin-twin transfusion syndrome, have not been reported.

It has been suggested that myocardial growth resulting from long-term aortic coarctation in the fetal sheep may first occur by myocyte enlargement, followed by myocyte proliferation (21). Previously, we investigated cardiac growth that resulted from increased right ventricular systolic pressure load in the fetal sheep (4). We found that, after 10 days, right ventricular cardiac myocytes had both proliferated and enlarged. It was not clear from this study whether proliferation and enlargement occurred simultaneously or sequentially. We also found that the myofibrillar volume fraction was decreased in cardiac myocytes. This could result from increased hyperplasia with a lag in contractile element formation (a situation that would only occur in the immature myocardium). Because in utero cardiac growth results primarily from hyperplasia, we reasoned that myocyte proliferation would be the most immediate growth response to increased load. Therefore, we tested the hypothesis that, in response to arterial and venous hypertension, the fetal myocardium increases mass initially by myocyte proliferation and later by myocyte enlargement. To test this hypothesis, we slowly infused sterile sheep plasma in the vascular space of fetal sheep for either 4 or 8 days. The advantage of this technique is that it gradually imposes a substantial and quantifiable hemodynamic load by elevating both arterial and venous pressures (13). We then measured fetal heart and body weights and isolated the cardiac myocytes to determine the incidence of myocyte cell cycle activity, myocyte size, and the fraction of myocyte binucleation (as an index of terminal differentiation).

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MATERIALS AND METHODS

Animal Preparation

All surgical and experimental methods were approved by the Institutional Animal Care and Use Committee. Time-bred ewes of mixed Western breeds were obtained from a commercial supplier and acclimatized to the laboratory. Ewes were given an intramuscular injection of 7.5 mg atropine, and anesthesia was induced with intravenous injection of 10 mg diazepam and 400 mg ketamine. A surgical level of anesthesia was maintained with an oxygen and nitrous oxide mixture (2:1) with 1.5% isoflurane. Indwelling 0.86-mm internal diameter catheters were placed in the fetal pedal artery and saphenous vein (Bolab, Lake Havasu City, AZ), and an amniotic fluid catheter with multiple side openings (1.19-mm internal diameter) was anchored to the skin. In some fetuses, catheters (1.19-mm internal diameter) were instead placed in the jugular vein and carotid artery, and flow probes (Transonic Systems, Ithaca, NY) were placed on the brachiocephalic artery, the descending thoracic aorta, and/or the common umbilical artery; physiological parameters from some fetuses have been published (9, 12). Incisions were closed in layers, and catheters were tunneled subcutaneously to emerge on the ewe’s flank. After closure of the uterus, 1 million units of penicillin G were infused and on the final experimental day.

Experimental Protocol

During the experimental period, intravascular pressures were measured continuously with the ewe in a stanchion where she was afforded free access to food and water and freedom to stand or lie down. Pressures were recorded with Transpac pressure transducers (Abbott, Abbott Park, IL) on a calibrated computerized system (AD- Instruments, Colorado Springs, CO; Apple, Cupertino, CA). Fetal intravascular pressures were referred to amniotic fluid pressure and reported as arithmetic means calculated from computer tracings. Heart rates were calculated from arterial pressure tracings. Fetal arterial blood samples were taken for determination of plasma renin activity, for plasma protein concentration, and for blood gases, pH, and hematocrit. All experiments were begun at an average of 130 ± 2 days of gestation (term is 145 days). Adult sheep plasma protein was infused in the fetal circulation as previously described (13). In brief, after baseline pressure measurements and blood samples were taken, intravenous infusion of plasma was started using a Gilson Minipuls-3 roller pump (Gilson, Middleton, WI). The infusion rate was increased by 3.5%/day to correspond to the estimated percent increase in fetal size resulting from growth.

Two experimental groups were studied, a 4-day (early phase) plasma infusion group and an 8-day (late phase) plasma infusion group. Physiological data were obtained from these groups at baseline and on the final experimental day.

4-Day study group. The eight fetuses in the 4-day experimental group received a total of 1,169 ± 230 ml plasma containing 62 ± 18 g plasma protein. The 4-day group experiments were concluded when the fetuses were 134 ± 1 days gestational age. The hearts and cardiac myocytes of the 4-day experimental group were compared with eight age-matched control fetuses.

Eight-day study group. The eight fetuses in the 8-day experimental group received a total of 2,466 ± 685 ml plasma containing 135 ± 28 g plasma protein. Physiological data were also obtained from these fetuses at 4 days to compare the early and late phases of hypertension. These experiments were concluded when the fetuses were 138 ± 1 days of gestational age. The hearts and cardiac myocytes of the 8-day experimental group were compared with eight age-matched control fetuses. Occasional catheter failures prevented the measurement of some experimental variables in all fetuses (noted in the legends for Figs. 1–3).

Tissue Collection

Sheep plasma for fetal infusion was obtained from ewes that were to be killed, as previously described (13). Fetuses were collected following euthanasia of the ewe with a commercial pentobarbital sodium solution (~65 mg/kg) or anesthesia of the ewe (induced with an iv injection of 10 mg diazepam and 400 mg ketamine, then maintained at a deep level with additional doses of ketamine) for blood collection. Deeply anesthetized fetuses were given 10,000 units of heparin and an intravenous injection of saturated potassium chloride to arrest hearts in diastole. Fetuses were weighed at postmortem, their hearts were removed, great vessels were immediately trimmed from hearts in a standard manner, and hearts were weighed. The hearts were then enzymatically dissociated for cellular analysis.

Myocyte Analysis

Cardiac myocytes were dissociated using collagenase and protease, as described previously (4), with some modifications. The hearts were cannulated by the aorta, and the coronary arteries were perfused initially with Tyrode solution (in mM: 140 NaCl, 5 KCl, 1 MgCl2, 10 dextrose, and 10 mM HEPES, pH adjusted to 7.35 with NaOH) until the myocardium had become blanched. The hearts were then perfused with Tyrode solution containing 160 U/ml Worthington’s type II collagenase (Worthington, Lakewood, NJ) and 0.78 U/ml protease type XIV (Sigma, St. Louis, MO) for ~5 min. The perfusion solution was changed to 300 ml calcium-free Kraftbrühe (KB) buffer (in mM: 74 l-glutamic acid, 30 KCl, 30 KH2PO4, 20 taurine, 3 MgSO4, 0.5 EGTA, 10 HEPES, and 10 dextrose, pH adjusted to 7.37 with KOH) to rinse out the collagenase. All perfusion solutions were preheated to 39°C and bubbled with a 95% O2-5% CO2 gas mixture. The ventricular free walls were dissected from the heart, and each was shaken gently for a few seconds in KB buffer to separate myocytes. The myocyte slurry was rested in KB buffer for 30 min on the bench top before fixation with an equal volume of 2% formaldehyde freshly made in PBS.

Myocyte sizes. Cardiac myocytes were measured from photomicrographs of methylene blue-stained wet mounts at ×400 magnification on a Zeiss microscope (Zeiss Axioshot; Bartels and Stout, Bellevue, WA). Cardiac myocyte lengths and widths were measured using calibrated Optimas software (Optimas, Seattle, WA). At least 100 cells were measured per ventricle per fetus without selecting for nucleation. For all analyses of dissociated cardiac myocytes, cells were selected by nonrepeating, random sampling across the microscope slide. Methylene blue staining allowed the number of nuclei per cardiac myocyte to be determined.

Percent binucleation. At least 300 myocytes per ventricle per animal were counted to determine percent binucleation, separately of the cells measured for size.

Cell cycle activity. Cell cycle activity in cardiac myocytes was detected using the Ki-67 antibody MIB-1 (DAKO, Carpinteria, CA). The Ki-67 antigen is present during G1, S, G2, and M phases, but not in G0 (11). The Ki-67 antigen may play a role in nucleolar organization during the cell cycle (22) and is necessary for nuclear proliferation (8). Cardiac myocytes were dried on Superfrost Plus slides and postfixed in cold acetone for 30 min. Antigen retrieval was achieved by boiling the slides in 10 mM sodium citrate (pH 6.0) for 6 min. Endogenous peroxidase activity was blocked by incubating the slides for 5 min in 0.03% hydrogen peroxide. Slides were then incubated at 4°C overnight with a 1:200 Ki-67 antibody dilution. The secondary antibody (biotinylated anti-mouse IgG; Vector Laboratories, Burlingame, CA) was applied to the slides at a dilution of 1:200 and incubated at room temperature for 30 min. Incubation with avidin-biotin complex (Vector Laboratories) followed for another 30 min at room temperature. Positive cells developed dark brown nuclei when
Table 1. Vascular pressures, heart rates, and blood chemistry of hypertensive fetuses

<table>
<thead>
<tr>
<th></th>
<th>4-Day Hypertensive Group</th>
<th>8-Day Hypertensive Group</th>
<th>P Value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Day 4</td>
<td></td>
</tr>
<tr>
<td>Arterial pressure, mmHg</td>
<td>43 ± 2</td>
<td>63 ± 4a</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>Venous pressure, mmHg</td>
<td>3.2 ± 0.6</td>
<td>5.0 ± 1.2b</td>
<td>3.0 ± 0.9</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>166 ± 9</td>
<td>165 ± 11</td>
<td>174 ± 12</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>7.36 ± 0.02</td>
<td>7.32 ± 0.05</td>
<td>7.35 ± 0.04</td>
</tr>
<tr>
<td>Arterial PO2, mmHg</td>
<td>19.9 ± 1.7</td>
<td>14.3 ± 2.3c</td>
<td>20.7 ± 2.1</td>
</tr>
<tr>
<td>Arterial P02, mmHg</td>
<td>53.4 ± 3.7</td>
<td>60.1 ± 2.0c</td>
<td>53.9 ± 3.2</td>
</tr>
<tr>
<td>Arterial O2Ct, ml/100 ml</td>
<td>7.7 ± 0.8</td>
<td>5.3 ± 1.5c</td>
<td>7.7 ± 1.6</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>34.0 ± 5.4</td>
<td>32.8 ± 4.6</td>
<td>34.8 ± 5.2</td>
</tr>
<tr>
<td>Plasma protein, mg/ml</td>
<td>32.1 ± 4.2</td>
<td>67.7 ± 11.1a</td>
<td>33.8 ± 3.8</td>
</tr>
<tr>
<td>Plasma renin activity, ng/ml⁻¹·h⁻¹</td>
<td>7.8 ± 6.5</td>
<td>1.1 ± 1.8</td>
<td>7.6 ± 3.3</td>
</tr>
</tbody>
</table>

Values are shown as means ± SD. For all values n = 8, except n = 7 for all venous pressures, plasma renin activity in the 4-day group, and day 4 of the 8-day study group for arterial pressure, heart rate, arterial blood pH, PO2, Pco2, and O2 content (Ct) (due to catheter failures). aP < 0.001, bP < 0.01, and cP < 0.05, different from baseline with Dunnett’s test. NS, not significant.

The slides were incubated with diaminobenzidine. All incubation steps were followed by three washes in PBS. Cells were lightly counterstained with methylene blue, dehydrated, and mounted with cover slips. In samples from this analysis, all myocytes containing a cleavage furrow stained positively for the Ki-67 antigen; however, not all positive myocytes contained a cleavage furrow. Many Ki-67-positive myocytes were indistinguishable in size and general appearance from other myocytes. At least 500 cells were counted per ventricle per fetus for cell cycle analysis.

Plasma Assays

Plasma renin activity was measured by RIA, as previously described (12), using a kit from DiaSorin (Stillwater, MN). Protein concentrations were measured using the bicinchoninic acid-based assay from Pierce (Rockford, IL).

Statistics

This study was designed to measure longitudinal changes in intra-vascular pressures, arterial blood gas contents, pH, hematocrit, and plasma protein over the course of the plasma infusion. Unpaired t-tests were used to compare baseline with final day hemodynamic variables in the 4-day study group. Three time points are presented for the hemodynamic variables in the 8-day study group. These data are compared with one-way ANOVA, and the day 4 and day 8 time points are compared with baseline with Dunnett’s post hoc test. For comparisons of heart and body weight, myocyte size, percent binucleation, and cell cycle activity, each experimental group was compared with its age-matched control group using an unpaired t-test. All statistics were performed using Graphpad Prism version 4.0a for Mac OS X (Graphpad Software, San Diego, CA). P ≤ 0.05 was accepted as significant. All data are presented as means ± SD.

RESULTS

Four and eight days of chronic intravascular plasma infusion in the near-term fetal sheep progressively increased the concentration of protein in the fetal plasma (Table 1). Plasma-infused fetuses had profound arterial and venous hypertension. Arterial pressures were increased by ~43% over baseline values. Venous pressures were elevated by 60% over their baseline values after 4 days of plasma infusion and by 93% after 8 days. The heart rates of plasma-infused fetuses decreased somewhat between the baseline day, the 4th day, and the 8th day (by ~0.8%/day, not statistically significant); this trend is typical of near-term fetal sheep. Fetal hemodynamics and arterial blood chemistry tended to be similar on days 4 or 8 following initiation of plasma infusion. Arterial oxygen content decreased following initiation of intravascular plasma infusion. The fetal hematocrit did not change significantly with treatment, and so the decrease in oxygen content was mostly because of the lower fetal arterial oxygen pressure that occurred following onset of infusion. Plasma renin activity in plasma-infused fetuses was substantially depressed to ~28% of the baseline values.

The combined venous and arterial hypertension associated with intravascular infusion of plasma accelerated fetal cardiac growth. Heart weights in hypertensive fetuses (after 4 or 8 days) were increased by ~30% over those of age-matched controls (Table 2). Because fetal body weights were not altered by treatment, the heart-to-body weight ratios of the hypertensive fetuses were greater than those of controls. In hypertensive fetuses, the incidence of cell cycle activity, expressed as a percent of mononucleated cardiac myocytes (those believed to be capable of proliferation), was increased 108–131% over age-matched controls. After 4 or 8 days of hypertension, binucleated (terminally differentiated) myocytes of both ventricles were longer [left ventricle (LV): 10%; right ventricle (RV): 11%] than control binucleated myocytes (Table 3). This elongation persisted at 8 days of hypertension, when binucleated LV myocytes were 10% longer, and the RV myocytes were 12% longer than myocytes from control fetuses. Mononucleated myocytes from both ven-

Table 2. Body and heart weights of fetuses

<table>
<thead>
<tr>
<th></th>
<th>4-Day Study Group</th>
<th>8-Day Study Group</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hypertensive</td>
<td>Control</td>
</tr>
<tr>
<td>Heart wt, g</td>
<td>24.6 ± 5.4</td>
<td>32.0 ± 5.4d</td>
<td>30.5 ± 9.0</td>
</tr>
<tr>
<td>Body wt, kg</td>
<td>4.0 ± 0.8</td>
<td>4.1 ± 0.6</td>
<td>4.5 ± 0.8</td>
</tr>
<tr>
<td>Heart/body wt, g/kg</td>
<td>6.2 ± 0.5</td>
<td>7.8 ± 1.5d</td>
<td>6.7 ± 1.0</td>
</tr>
</tbody>
</table>

Values are shown as means ± SD. For all values n = 8. aP < 0.05 and bP < 0.01 different from age-matched control.
tricles also had a tendency to be longer in hypertensive fetuses at both time points, although this difference was only statistically significant after 4 days in the LV (10% longer than control myocytes) and 8 days in the RV (12% longer).

After 4 days of hypertension, cardiac myocytes were not significantly wider than controls. However, after 8 days, the diameters of binucleated myocytes of both ventricles were greater than controls (LV: 11%; RV: 13%). This trend was also true in mononucleated cells, although it was only statistically significant in the RV (LV: 10%; RV: 14%).

The percent binucleation of cardiac myocytes was not altered after 4 days of plasma infusion. However, after 8 days, the percent binucleation in the hypertensive fetuses was increased in both ventricles by 45% (RV) and 54% (LV) compared with control fetuses (Fig. 2).

**DISCUSSION**

To understand the effects of increased pressure load on fetal cardiac growth, researchers have developed models in fetal sheep, whereby either arterial pressure is increased by means of vascular occluders or volume load is imposed by means of arterial to venous fistulas. This model of plasma infusion was developed to increase both fetal preload and afterload. As is illustrated by daily measurements (13), there is a gradual increase in mean fetal arterial pressure and central venous pressure with plasma infusion when compared with twin fetuses receiving the same volume of lactated Ringer solution. This model offers the unique opportunity to study the effects of both increased diastolic and systolic pressure load on the growing fetal heart. Fetuses tolerate these very high intravascular pressures well, perhaps because the load is imposed gradually and steadily. Fetal shunts (the foramen ovale and the ductus arteriosus) cause the ventricles to operate in parallel (1); thus, an additional benefit of this model is that it loads both fetal ventricles equally.

We hypothesized that, in response to chronic arterial and venous hypertension, fetal cardiac myocytes would grow by proliferation during the early loading phase, followed by myocyte enlargement. We also hypothesized that myocyte enlargement would be associated with an increased proportion of cardiac myocytes that were terminally differentiated. Consistent with our hypotheses, we found that the hearts of hypertensive fetuses were heavier than those of controls. This growth may be considered adaptive in that it permits fetuses to maintain biventricular stroke volume in the face of greatly increased arterial pressures (13). During the first 4 days of plasma infusion (early phase), there was a large increase in hemodynamic load and cardiac mass, such that the heart-to-body weight ratio increased by 30% compared with controls (Table 2). During the next 4 days (late phase), the hemodynamic load increased only slightly, and growth of the heart was no longer accelerated, as evidenced by the fact that the heart-to-body weight ratio did not further increase.

**Table 3. Cardiac myocytes lengths and widths**

<table>
<thead>
<tr>
<th></th>
<th>4-Day Study Group</th>
<th>8-Day Study Group</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hypertensive</td>
</tr>
<tr>
<td><strong>Binucleated cardiac myocytes length, μm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left ventricle</td>
<td>81.2±4.0</td>
<td>89.3±6.5</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>89.9±5.7</td>
<td>99.5±6.3</td>
</tr>
<tr>
<td><strong>Binucleated cardiac myocytes width, μm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left ventricle</td>
<td>12.2±0.8</td>
<td>12.6±0.7</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>15.2±1.2</td>
<td>15.6±1.7</td>
</tr>
<tr>
<td><strong>Mononucleated cardiac myocytes length, μm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left ventricle</td>
<td>61.5±4.0</td>
<td>67.5±5.9</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>68.0±4.1</td>
<td>73.8±7.5</td>
</tr>
<tr>
<td><strong>Mononucleated cardiac myocytes width, μm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left ventricle</td>
<td>11.1±0.9</td>
<td>11.6±0.6</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>13.5±0.7</td>
<td>13.4±1.2</td>
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Values are shown as means ± SD. For all values, n = 8. *P < 0.05 and **P < 0.01 different from age-matched control group with t-test.
Cell Cycle Activity: Proliferation and Terminal Differentiation

The incidence of myocyte cycle activity was increased over control levels in the hearts of fetuses that had been hypertensive for 4 days (Fig. 1) and was sustained at high levels after 8 days. Cell cycle activity in fetal cardiac myocytes results in the generation of new myocytes or nuclear division without cellular division (18). When a mononucleated myocyte enters the cell cycle and divides, the result is a net addition of one mononucleated cell to the myocyte population. If a mononucleated myocyte enters the cell cycle and terminally differentiates, the net result is a loss of one mononucleated cell and the addition of one binucleated cell to the myocyte population. Because percent binucleation was not different between hypertensive and control fetuses on experimental day 4, we conclude that most of the cell cycle activity during this early period resulted in myocyte hyperplasia. In contrast, the elevated level of cell cycle activity during the later period of hypertension was more directed toward terminal differentiation than in the early period because percent binucleation in hypertensive fetuses on day 8 was greater than in controls.

A surge of myocyte proliferation may enable rapid cardiac growth in response to increased hemodynamic load, but, in the near-term fetal heart, it may soon be followed by terminal differentiation, as occurred in this experiment. This may increase myocyte number in the short term but prematurely reduces the proliferative potential of the heart. Cardiac myocyte proliferation normally generates a substantial number of new myocytes after 138 days of gestation in the sheep (5). Premature terminal differentiation, even following a surge of induced proliferation, may reduce the number of cardiac myocytes in the heart at birth; this question can be settled only by actually counting the total number of myocytes. It has been thought that the maximum number of cardiac myocytes for the life of an individual is set during the perinatal period when cardiac myocyte proliferation ceases. Myocyte number in the adult heart can be reduced by apoptosis and necrosis. Whether it may increase by proliferation is still a topic of debate (3, 17), although the proliferation and terminal differentiation of fetal cardiac myocytes are not (2).

Myocyte Enlargement

We observed that cardiac myocytes of hypertensive fetuses tended to become longer in the early phase of growth and wider during the later phase. Although independent longitudinal or cross-sectional enlargement of myocytes has been described in the adult heart, the regulatory mechanisms for these processes are not well understood (20). We also observed that binucleated myocytes were longer and wider than mononucleated myocytes in both normal and hypertensive fetuses, in both the 4- and 8-day groups.

In our primary analysis, we compared mean myocyte lengths and widths. However, we also sought to determine if, after 8 days of hypertension, those myocytes that had elongated were the same as those that had thickened. We found that in neither control nor hypertensive fetuses was there a relationship between myocyte length and width (Fig. 3). Nevertheless, regional variations in wall stress may have stimulated different types of myocyte enlargement (longitudinal or cross-sectional) depending on the location of the cells. It may be informative in future studies to collect myocytes from specific locations within the ventricular free walls to test this hypothesis. How mechanical stress regulates cardiomyocyte enlargement may be different in the fetal heart because the fetal myocardium undergoes hyperplasia as a part of the normal process of heart growth.

Fig. 2. Percent binucleation of the myocytes of the left and right ventricular free walls following either 4 or 8 days of hypertension compared with age-matched control fetuses. *P < 0.05 and **P < 0.01. For all values, n = 8. Values are shown as means ± SD.

Fig. 3. Myocyte lengths and widths from a pair of twin fetuses (138dGA) demonstrate the typical lack of a relationship between length and width of binucleated right ventricular myocytes. There is no correlation between myocyte length and width in the hypertensive fetus for 8 days or its control twin (Pearson r value: control = −0.236, hypertensive = 0.005).
We noted with interest that mononucleated myocytes from hypertensive fetuses were larger than those from controls (Table 3). Previously, in vitro studies had led us to conclude that mononucleated fetal sheep cardiac myocytes would respond to growth stimuli only by proliferation (24). However, Lumbers et al. (16) have reported increased mononucleated myocyte volumes following a period of hypertension induced by cortisol in fetal sheep, although this may have been because of hypertension rather than the direct effect of cortisol on the cardiac myocytes (14). It remains to be seen what other stimuli are capable of causing mononucleated cardiac myocytes to enlarge in the fetus in vivo.

**Growth Signals for Cardiac Myocytes**

Mechanical stress is a powerful modulator of cardiac growth. For pressure (p), circumferential radius of curvature (r), and ventricular wall thickness (h), the Law of Laplace describes wall stress as proportional to pr/2h. As arterial pressure rose to ~60 mmHg (from 43 mmHg) by the fourth day of plasma infusion (Table 1), systolic wall stress would have increased. Likewise, as venous pressure rose to ~5 mmHg (from ~3.1 mmHg), diastolic wall stress would have increased. The large difference in cardiac mass between hypertensive and control fetuses at 4 days probably represented growth that would have tended to normalize wall stress by increasing wall thickness, although these parameters were not measured. The magnitude of increases in arterial and venous pressures was less between 4 and 8 days, although the fetuses continued to grow at a normal rate (Table 2). The early and late differences in growth may have resulted from differences in ventricular wall stress between these periods of hypertension.

At any given arterial pressure, the fetal RV free wall experiences greater stress than the LV free wall because the fetal RV has a larger radius of curvature and a thinner free wall (19). As we increased intravascular pressures, we expect wall stress in the RV free wall to be greater than in the LV. Because mechanical stress is a powerful modulator of myocyte growth, we expected to observe an earlier and greater growth response in the RV than the LV. We were surprised to find that the ventricles responded similarly at both time points.

In summary, we showed that the fetal myocardial proliferative response to arterial and venous hypertension occurred in two phases. During the early phase, increased cardiomyocyte cell cycle activity predominantly reflects hyperplasia because terminal differentiation is not yet increased above control levels. In the later phase, increased cell cycle activity reflects increased terminal differentiation. Cardiac myocyte enlargement also occurred in two phases. Myocyte elongation in the early phase preceded later myocyte cross-sectional growth. Independent increases in myocyte length and diameter occur in the mature heart (20), but this is the first such demonstration in the fetal heart. Myocyte enlargement does not appear to occur only in binucleated myocytes but in mononucleated myocytes as well. Finally, the conversion from mononucleation to binucleation is associated with a increase in cell length and width.

In conclusion, these data show that the growth responses of the fetal myocardium to increased arterial and venous pressure are not constant with respect to time. This is significant for two reasons. First, it highlights the importance of the variable of time when interpreting studies investigating fetal cardiac growth. Both the period of gestation and the duration of the experiment are key factors that influence the outcome of studies and the conclusions that may be drawn from them. Second, the increased terminal differentiation and myocyte enlargement that occurred in the later phase suggests that cardiomyocyte hyperplasia may be only a limited, early response of the near-term fetal heart to sustained hypertension.

**ACKNOWLEDGMENTS**

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