Restriction of placental function alters heart development in the sheep fetus

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1Center for the Early Origins of Adult Health, Discipline of Physiology, University of Adelaide, Adelaide, South Australia; 2Early Origins of Adult Health Research Group, Sanson Institute, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, South Australia, Australia; and 3Heart Research Center, Oregon Health and Science University, Portland, Oregon

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Morrison JL, Botting KJ, Dyer JL, Williams SJ, Thornburg KL, McMillen IC. Restriction of placental function alters heart development in the sheep fetus. Am J Physiol Regul Integr Comp Physiol 293: R306–R313, 2007. First published April 11, 2007; doi:10.1152/ajpregu.00798.2006.—Placental insufficiency, resulting in restriction of fetal substrate supply, is a major cause of intrauterine growth restriction (IUGR) and increased neonatal morbidity. Fetal adaptations to placental restriction maintain the growth of key organs, including the heart, but the impact of these adaptations on individual cardiomyocytes is unknown. Placental and hence fetal growth restriction was induced in fetal sheep by removing the majority of caruncles in the ewe before mating (placental restriction, PR). Vascular surgery was performed on 13 control and 11 PR fetuses at 110–125 days of gestation (term: 150 ± 3 days). PR fetuses with a mean gestational Po2 < 17 mmHg were defined as hypoxic. At postmortem (<135 or >135 days), fetal hearts were collected, and cardiomyocytes were isolated and fixed. Proliferating cardiomyocytes were counted by immunohistochemistry of Ki67 protein. Cardiomyocytes were stained with methylene blue to visualize the nuclei, and the proportion of mononucleated cells and length and width of cardiomyocytes were measured. PR resulted in chronic fetal hypoxia, IUGR, and elevated plasma cortisol concentrations. Although there was no difference in relative heart weights between control and PR fetuses, there was an increase in the proportion of mononucleated cardiomyocytes in PR fetuses. Whereas mononucleated and binucleated cardiomyocytes were smaller, the relative size of cardiomyocytes when expressed relative to heart weight was larger in PR compared with control fetuses. The increase in the relative proportion of mononucleated cardiomyocytes and the relative sparing of the growth of individual cardiomyocytes in the growth-restricted fetus are adaptations that may have long-term consequences for heart development in postnatal life.

hypoxygenation; cardiomyocyte; fetal growth restriction; hyperplasia; hypertrophy; placental restriction

LOW BIRTH WEIGHT is associated with an increased risk of heart disease in adult life (5); however, few studies have investigated the development of cardiomyocytes in the hearts of low birth weight fetuses. Fetal heart development involves proliferation and growth of cardiomyocytes. Heart growth early in development occurs via hyperplastic growth of mononucleated cardiomyocytes (45). There is a transition from hyperplastic to hypertrophic growth as increasing numbers of cardiomyocytes become binucleated and are terminally differentiated by cytokinesis in the absence of cytokinesis (9, 22). In the human heart, most cardiomyocytes undergo binucleation during fetal life, with 90% of cardiomyocytes being binucleated late in gestation and up to 97% being binucleated by 7 wk after birth (1). Thus, at birth, the human heart contains almost the full complement of cardiomyocytes that it will have for life (50), and this is important, because there is a limited capacity for cellular regeneration within the postnatal heart after injury (34, 44). The sheep is similar to the human in that binucleation of cardiomyocytes also occurs predominantly before birth, with hyperplastic growth of the cardiomyocytes occurring before 110 days of gestation (term: 150 ± 3 days), followed by a transition to hypertrophic growth by term (7, 43). Several studies have investigated the signals for cardiomyocyte development and found that IGF-1 (46), angiotensin (47), and cortisol (17) each can stimulate hyperplastic growth and that phenylephrine (46) and cortisol (28) can also cause hypertrophic growth in fetal sheep cardiomyocytes. Given the relative maturity and limited ability for regeneration of the heart at birth (34, 50), environmental factors that alter the timing of cardiomyocyte binucleation in late gestation are likely to have long-lasting consequences for heart growth and function (27). An example of an important change in the fetal environment that may have an impact on cardiomyocyte development is intrauterine growth restriction (IUGR). In humans, IUGR is defined as birth weight below the tenth centile (8) and is associated with chronic hypoxia (12) and increased plasma cortisol concentrations (13) as well as increased perinatal mortality (16) and neonatal morbidity (15, 18). Despite a degree of cardiac sparing, IUGR is also associated with altered structural development of the heart (19) and heart disease (5, 30) in postnatal life.

In one experimental model of IUGR, exposure to chronic hypoxia (10% inspired air) during late gestation in pregnant rats resulted in an increase in relative heart weight (3) and a decrease in DNA content in the heart at birth (33). Importantly, the IUGR offspring also had an increased vulnerability to ischemia-reperfusion injury (25) in adult life. There was also an increase in the proportion and size of binucleated cardiomyocytes at birth in this model (3). Given the significant differences among species in the developmental trajectory of cardiomyocyte development relative to birth, it is important to determine the impact of IUGR on fetal heart development in a species, such as the human, where cardiomyocyte binucleation occurs before birth. In the sheep, surgical ablation of the majority of uterine caruncles before conception results in a reduction in placental mass and fetal growth throughout gestation (2), offering an excellent model of fetal growth restriction. Placentally-restricted (PR) sheep fetuses have a similar arterial blood gas and hormonal profile [hypoxemia, hypercap-
HEART DEVELOPMENT IN THE GROWTH-RESTRICTED FETUS

Table 1. Number of animals in experimental groups

<table>
<thead>
<tr>
<th></th>
<th>&lt;135 Days</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PR</td>
</tr>
<tr>
<td>No. of fetuses</td>
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<td>4</td>
</tr>
<tr>
<td>No. of singles</td>
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<td>4</td>
</tr>
<tr>
<td>No. of twins</td>
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<tr>
<td>No. of females</td>
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<td>3</td>
</tr>
<tr>
<td>No. of males</td>
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</table>

Values are means ± SE. PR, placental restriction.

nia, hypoglycemia, increased plasma cortisol concentrations (29, 38) compared with growth-restricted human fetuses (13).

In the present study, we aimed to determine the impact of PR on the development of cardiomyocytes in the fetal sheep during late gestation. Cortisol is elevated in the PR fetus (37) and also stimulates hyperplastic and hypertrophic growth of cardiomyocytes (17, 28, 39). Because there is a prepartum rise in cortisol in both the human (49) and sheep (32, 37), we collected hearts from fetal sheep at two stages in late gestation, i.e., before (<135 days) and after (>135 days) the prepartum increase in fetal cortisol (35). We hypothesized that PR, possibly acting via an increase in circulating cortisol concentrations, would result in a change in the proportion and size of binucleated cardiomyocytes present in the heart of the growth-restricted fetal sheep in late gestation.

MATERIALS AND METHODS

All experiments were performed according to guidelines of both the University of Adelaide and the University of South Australia Animal Ethics Committees.

Animals and Surgery

The majority of uterine caruncules were removed in 11 Merino ewes before conception as previously described (2, 11, 14). The ewes’ recovery from surgery was observed for 4–7 days. After 10 wk, these 11 ewes plus 18 control ewes entered a mating program. At 110–125 days, surgery was performed in 13 control and 11 PR fetuses under aseptic conditions with general anesthesia induced by thiopentone sodium (1.25 g of Pentothal; Rhone Merieux, Pinkenba, Australia) and maintained by inhalation of halothane (2.5–4%) in oxygen. Briefly, vascular catheters (Critchley Electrical Products, Silverwater, Australia) were inserted as previously described in the maternal jugular vein, the fetal femoral and carotid arteries, the jugular vein, and the amniotic cavity (14, 31). Fetal catheters were exteriorized through a small incision in the ewe’s flank. At surgery, antibiotics were administered to the ewe (153.5 mg of Procaine penicillin, 393 mg of benzathine penicillin, 500 mg of dihydrostreptomycin; Lyppards, Adelaide, Australia) and fetus (150 mg of Procaine penicillin, 112.5 mg of benzathine penicillin, 250 mg of dihydrostreptomycin; Lyppards). Antibiotics were administered intramuscularly to each ewe for 3 days after surgery and to each fetus intramuscularly (500 mg of ampicillin; Lyppards) for 4 days after surgery. Animals were allowed to recover from surgery for at least 4 days before experimentation.

Arterial Blood Gas Measurements

Fetal carotid arterial blood gas samples (0.5 ml) were collected daily for the measurement of PO2, PCO2, pH, oxygen saturation (SO2), and hemoglobin (Hb) at 39°C with an ABL 520 analyzer (Radiometer, Copenhagen, Denmark) calibrated for sheep blood.

Postmortem

At the time of mating, ewes were randomly allocated for postmortem at <135 or >135 days. Therefore, postmortem examination was performed at 132–134 or 137–141 days of gestation. Ewes were killed with an overdose of pentobarbitone sodium (8 g; Vibrac Australia, Peakhurst, Australia). The uterus was removed by hysterectomy, and the fetus was removed. The umbilical cord was cut and the fetus was weighed. The heart was quickly dissected and weighed. Heparin (20,000 IU in 10 ml of saline) followed by saturated KCl (10 ml) was perfused through the coronary vessels via the aorta. The mean time from maternal death to perfusion of the fetal heart with KCl was 11.2 ± 0.6 min.

Cardiomyocyte Dissociation

Fetal hearts were suspended in a Langendorf apparatus and perfused through the aorta with warmed (37°C), oxygenated Tyrode’s buffer (4) with a heating pump (Paratherm II; Julabo, Schwarzwald, West Germany) for 11.4 ± 0.4 min at a rate of 10.6 ml/min by using a Minipulse 3 pump (Gilion, Villeirs, France) to clear blood from the coronary vessels. Collagenase (120 U/ml, lot no. X3M6745; Worthington Biochemical, Lakewood, NJ) and protease (10 mg/200 ml, lot no. 083K0799; Sigma, Castle Hill, Australia) solution was perfused for 14.4 ± 1.5 min at 25.8 ± 0.6°C to digest the extracellular matrix as previously described (17, 20, 21, 46, 47). Approximately 300 ml of Kraftbrühe (KB) buffer (21, 47) was then perfused through the heart to flush out these enzymes. The entire right and left ventricle free walls and the septum were dissected and placed in Falcon tubes with KB buffer and triturated, and cardiomyocytes were collected. Cardiomyocytes were fixed in a 1% paraformaldehyde solution for storage.

Cardiomyocyte Characterization

Ratio of mononucleated to binucleated cardiomyocytes. Nuclei of fixed cardiomyocytes were stained with methylene blue. The percentage of mononucleated cardiomyocytes was determined in each ventricle from each fetus by counting the number of mononucleated cardiomyocytes in a total of 200 cardiomyocytes (6, 7) using an Olympus VANOX-T microscope (Olympus Optical, Tokyo, Japan). The percentage of binucleated cardiomyocytes was then calculated using the formula: binucleated myocytes / (binucleated + mononucleated) × 100.

Table 2. Characteristics of fetuses postmortem

<table>
<thead>
<tr>
<th></th>
<th>&lt;135 Days</th>
<th>&gt;135 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PR</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Gestational age, days</td>
<td>132.8±0.3</td>
<td>133.2±0.2</td>
</tr>
<tr>
<td>Fetal body weight, kg</td>
<td>3.8±0.3</td>
<td>2.2±0.3*</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>32.4±3.1</td>
<td>17.6±2.0*</td>
</tr>
<tr>
<td>Relative heart weight, g/kg</td>
<td>8.4±0.5</td>
<td>7.9±0.4</td>
</tr>
<tr>
<td>No. of fetuses</td>
<td>1.5±0.2</td>
<td>1.0±0.0*</td>
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</table>

Values are means ± SE 2-way ANOVA: treatment (control, PR) and age (<135 days, >135 days). *P < 0.05, treatment effect. †P < 0.05, age effect.

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Japan). Counters were blinded, and inter- and intraobserver variabilities were <10%.

Hyperplastic cardiomyocyte growth. To determine the proportion of proliferating cells in each ventricle (17, 40), we performed immunohistochemistry on fixed cells using a Ki67 antibody (1:100, mouse anti-human monoclonal antibody; DAKO, Dianova, Germany). Ki67 is a cell cycle-related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2, and M phase) but is absent in resting cells (G0) (23, 24, 41). Cardiomyocytes were treated with 3% hydrogen peroxide for 20 min to remove endogenous peroxidase activity. A Histostain-Plus broad spectrum kit (85-9043, lot no. 30276167; Zymed Laboratories, San Francisco, CA) was utilized with diaminobenzidene for visualization of positively stained nuclei. Nuclei were counterstained with 1/2 l of 1% methylene blue, and 10/8 ml of cell suspension were placed on a slide for analysis. Random, nonrepeating fields were analyzed to determine the percentage of cardiomyocytes undergoing proliferation from the number of mononucleated cardiomyocytes that were positive for Ki67 out of 200 mononucleated cardiomyocytes, using an Olympus VANOX-T microscope (Olympus Optical). Counters were blinded, and inter- and intraobserver variabilities were <10%.

Hypertrophic cardiomyocyte growth. Ten microliters of fixed cardiomyocytes were stained with 1/2 l of 1% methylene blue on a slide and visualized with an Olympus VANOX-T microscope (Olympus Optical), and images were captured with an Olympus C-35AD-4 camera (Olympus Optical), and images were captured with an Olympus C-35AD-4 camera (Olympus Optical). From these images, length and width measurements (4, 17, 46, 47) were made of 50 mononucleated and 50 binucleated cardiomyocytes (6) with AnalySIS software (Soft Imaging System, Adelaide, Australia) calibrated using a gradicule to 2/5 m, and a mean was determined for each animal. The length of the cardiomyocyte was the longest distance from one tip of the cardiomyocyte to the other, and the width was the distance across the midline of the nucleus in mononucleated cardiomyocytes and midway between the two nuclei in binucleated cardiomyocytes. Inter- and intraobserver variabilities were <5%.

Values are means ± SE 2-way ANOVA: treatment (control, PR) and age (<135 days, >135 days). *P < 0.05, treatment effect. †P < 0.05, age effect.

Table 3. Mean gestational blood gas and pH values

<table>
<thead>
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<th>&lt;135 Days</th>
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<tr>
<td></td>
<td>Control</td>
<td>PR</td>
<td>Control</td>
<td>PR</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>4</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Po2, mmHg</td>
<td>20.8±0.5</td>
<td>14.5±1.6*</td>
<td>23.2±1.0</td>
<td>13.8±0.7*</td>
</tr>
<tr>
<td>Oxygen saturation, %</td>
<td>68.2±2.4</td>
<td>43.2±6.0*</td>
<td>69.6±2.9</td>
<td>35.7±3.1*</td>
</tr>
<tr>
<td>Oxygen content, ml/dl</td>
<td>8.6±0.4</td>
<td>6.3±0.5*</td>
<td>10.3±0.4†</td>
<td>5.8±0.5†</td>
</tr>
<tr>
<td>Po2, mmHg</td>
<td>44.3±0.9</td>
<td>48.5±0.9*</td>
<td>46.5±1.4†</td>
<td>53.5±1.1†</td>
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<tr>
<td>pH</td>
<td>7.376±0.015</td>
<td>7.377±0.016*</td>
<td>7.413±0.009</td>
<td>7.361±0.004*</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>9.1±0.5</td>
<td>11.0±1.5</td>
<td>10.6±0.2</td>
<td>12.0±1.1</td>
</tr>
</tbody>
</table>

Fig. 1. Top: there was a higher percentage of mononucleated cardiomyocytes in the ventricles of placentally-restricted [PR (intrauterine growth restriction, IUGR)] compared with control fetuses [3-way ANOVA: treatment (control, PR; *P = 0.001), age (<135 days, >135 days), and ventricle (right, left)]. Bottom: there was a significant relationship between mean gestational arterial Po2 and the percentage of mononucleated cardiomyocytes in the right (RV: P < 0.01, r² = 0.272, %mononucleated = −1.15Po2 + 68.91) and left ventricle (LV: P < 0.001, r² = 0.475, %mononucleated = −2.14Po2 + 88.03). Circles, <135 days; triangles, >135 days; filled symbols, PR; open symbols, control.
Cortisol radioimmunoassay. Total plasma cortisol concentration was measured in extracts by using an 125I radioimmunoassay kit (GE Healthcare, Sydney, Australia) as previously described (48). The average efficiency of recovery of 125I-labeled cortisol using dichloromethane extraction was 90%. The sensitivity of the assay was 0.39 nM. The rabbit anti-cortisol antibody cross-reacted <1% with cortisone and 17-hydroxyprogesterone and <0.01% with aldosterone, pregnenolone, estradiol, and progesterone. The inter- and intra-assay coefficients of variation were <10%.

Statistical Analyses

All data are means ± SE. A probability value of 5% (P < 0.05) was considered significant.

Experimental groups. Animals were divided into treatment (control vs. PR) and age groups (<135 vs. >135 days) (Table 1). All control fetuses had a mean gestational arterial PO2 of >17 mmHg, or, if PO2 measurements were not available (<135 days, n = 2; >135 days, n = 3), their body weight postmortem was within two standard deviations of the mean body weight of all control fetuses in the relevant age group (29). Statistical analysis was performed to validate this approach, and there was no significant difference (unpaired t-test) in fetal weight, heart weight, proportion of mononucleated cardiomyocytes, percentage of Ki67-positive mononucleated cardiomyocytes, or size of cardiomyocytes between the fetuses that were included on the basis of mean gestational PO2 or body weight at birth. All PR fetuses had a mean gestational PO2 <17 mmHg and were therefore defined as chronically hypoxic (11, 14), and, in addition, they were growth restricted. Table 1 shows fetal number and sex in each group. A recent study showed no difference in cardiomyocyte parameters between twins and singletons (21).

Blood gas and fetal plasma cortisol concentration data. Mean gestational arterial PO2, SO2, Hb, PCO2, and pH were calculated as the means of the values for all samples collected between surgery and postmortem. A two-way analysis of variance (ANOVA) was used to determine differences in the mean fetal blood gases, pH, arterial oxygen content [oxygen content = (PO2 × 0.003) + [Hb] × (SO2/100) × 1.39 ml/dl (14)], and plasma cortisol concentration between treatment (control vs. PR fetuses) and age groups (<135 vs. >135 days).

Cardiomyocyte measurements. A multifactorial ANOVA was used to determine differences in the percentage of mononucleated cardiomyocytes, percentage of Ki67-positive mononucleated cardiomyocytes, and length and width of both mononucleated and binucleated cardiomyocytes with treatment (control vs. PR), age (<135 vs. >135 days), and ventricle (left vs. right) as the specified factors (SPSS, Chicago, IL). Linear regression was performed on cardiomyocyte data to investigate relationships with mean gestational PO2 using Sigma-Plot (SPSS).

RESULTS

Effects of PR on Fetal Growth, Gender, and Number of Fetuses

The gestational age postmortem was not different between control and PR fetuses in the <135- or >135-day groups (Table 2). PR fetuses were smaller than control fetuses at both <135 and >135 days (P < 0.01). All PR fetuses weighed less than the tenth centile of control fetuses in each age group (<135 days, <3.15 kg; >135 days, <4.25 kg). Whereas control fetuses were heavier after 135 days than before 135 days, this was not the case for PR fetuses. Heart weight was lower in PR than control fetuses at both <135 and >135 days (P < 0.01); however, there was no difference in the relative heart weight between treatment groups at either gestational age range. There was a significant difference in the number of fetuses per pregnancy between the control and PR groups at both ages (P < 0.01; Table 1). There was no difference in the sex distribution between the control and PR groups or across gestational age.

Effects of PR on Fetal Arterial Blood Gases

Mean gestational PO2 (P < 0.01), SO2 (P < 0.01), and oxygen content (P < 0.01) were lower in PR than control fetuses at both <135 and >135 days (Table 3). Oxygen content increased with gestational age in the control fetuses and decreased with gestational age in the PR fetuses (P < 0.05).

Plasma Cortisol Concentrations

Fetal plasma cortisol concentration increased with gestational age in control fetuses (<135 days, 5.5 ± 1.6 nM; >135 days, 12.9 ± 3.1 nM; P < 0.05) and was higher in PR fetuses (<135 days, 18.5 ± 2.6 nM; >135 days, 17.3 ± 4.1 nM) compared with control fetuses (P < 0.01). There was no relationship between plasma cortisol concentration and mean gestational PO2, the percentage of mononucleated cardiomyocytes, or the number of binucleated cardiomyocytes.
cytes or Ki67-positive cardiomyocytes, or the size of either mononucleated or binucleated cardiomyocytes.

**Effects of PR on the Proportion and Proliferation of Mononucleated Cardiomyocytes**

The proportion of mononucleated cardiomyocytes was higher in PR than control fetuses ($P < 0.01$; Fig. 1A). There was no effect of gestational age on the percentage of mononucleated cardiomyocytes present in either ventricle, and there was no difference in the percentage of mononucleated cells between the right and left ventricle. There was an inverse direct correlation between mean gestational PO$_2$ and the percentage of mononucleated cardiomyocytes in the right ($P < 0.01$, $r^2 = 0.272$) and the left ventricle ($P < 0.01$, $r^2 = 0.475$; Fig. 1B).

The percentage of mononucleated cardiomyocytes that expressed Ki67 was similar in control and PR fetuses (Fig. 2B). There was no effect of age on the percentage of Ki67-positive mononucleated cardiomyocytes, and there was also no difference between the right and left ventricles in the proportion of mononucleated cardiomyocytes that expressed Ki67. There was no relationship between mean gestational PO$_2$ and the percentage of Ki67-positive mononucleated cardiomyocytes.

**Effects of PR on the Morphometry of Cardiomyocytes**

Mononucleated cardiomyocytes from PR fetuses were significantly shorter ($P < 0.05$) and narrower ($P < 0.05$) than control fetuses. The mononucleated cardiomyocytes were also shorter ($P < 0.01$) and narrower ($P < 0.01$) in the left than in the right ventricle in both age groups (Table 4).

Binucleated cardiomyocytes were significantly narrower in hearts from PR than control fetuses ($P < 0.05$; Table 4) and became more narrow with increasing gestational age ($P < 0.01$). In contrast, there was no difference in the length of the binucleated cardiomyocytes in the PR and control fetuses. In both groups, binucleated cardiomyocytes were longer ($P < 0.01$) and wider ($P < 0.05$) in the right compared with the left ventricle.

When the size of the cardiomyocytes was expressed relative to heart weight, both mononucleated and binucleated cardiomyocytes were wider (mononucleated, $P < 0.01$, Fig. 3A; binucleated, $P < 0.01$, Fig. 3C) and longer (mononucleated, $P < 0.01$, Fig. 3B; binucleated, $P < 0.01$, Fig. 3D) relative to heart weight in the PR group compared with control fetuses. In addition, with increasing gestational age, there was a decline in the relative width of both mononucleated ($P < 0.05$) and binucleated ($P < 0.05$) cardiomyocytes in both treatment groups. There was no relationship between fetal plasma cortisol concentration and the relative cardiomyocyte size. There was, however, a relationship between mean gestational PO$_2$ and the dimensions of the cardiomyocytes relative to heart weight in both the right and left ventricle ($P < 0.05$; Fig. 3, E and F).

**DISCUSSION**

This study investigated the impact of placental and fetal growth restriction on cardiomyocyte development. As expected, the PR fetuses were growth restricted, hypoxic, and relatively hypercortisolemic compared with age-matched control fetuses (11, 14, 37, 42). Importantly, we have found that although the relative heart weight was not different in the PR compared with the control fetuses, there was an increased proportion of mononucleated cardiomyocytes present in the hearts of PR fetuses both before and after 135 days of gestation. This increase occurred in the absence of a change in the relative proportion of proliferating mononucleated cardiomyocytes. Although mononucleated and binucleated cardiomyocytes were smaller in the PR fetuses, the relative size of these cardiomyocytes when expressed relative to heart weight was larger in the PR compared with the control fetuses. These novel findings emphasize that chronic fetal substrate restriction has a significant impact on the development of the heart at the cellular level in a large animal model in which the development of the cardiomyocyte profile is virtually complete by birth, as in the human.

### Table 4. Length and width of mononucleated cardiomyocytes isolated from hearts of fetuses

<table>
<thead>
<tr>
<th>Age</th>
<th>Treatment</th>
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<th>Length, μm</th>
<th>Width, μm</th>
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<tbody>
<tr>
<td>&lt;135 Days</td>
<td>Control</td>
<td>9</td>
<td>68.1±1.8</td>
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<td>PR</td>
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<td>64.8±1.0†</td>
<td>10.0±0.4‡</td>
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<tr>
<td>&gt;135 Days</td>
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<tr>
<td></td>
<td>PR</td>
<td>7</td>
<td>64.9±2.3‡</td>
<td>9.8±0.4‡</td>
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**Mononucleated cardiomyocytes**

<table>
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<th>Age</th>
<th>Treatment</th>
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<th>Length, μm</th>
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<td>10.8±0.2†</td>
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<td>93.5±2.5</td>
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<td>PR</td>
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<td>84.8±0.5‡</td>
<td>10.0±0.3‡</td>
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**Binucleated cardiomyocytes**

<table>
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<th>Age</th>
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<td>64.7±4.8‡</td>
<td>10.1±0.3*‡</td>
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<td>83.2±2.7‡</td>
<td>9.1±0.2*‡</td>
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</table>

Values are means ± SE 3-way ANOVA: treatment (control, PR), age (<135 days, >135 days), and ventricle (right, left). *$P < 0.05$, treatment effect. †$P < 0.05$, age effect. ‡$P < 0.05$, ventricle effect.
In the present study, we found that ~40% of cardiomyocytes were mononucleated at both gestational age ranges in the control fetuses. This is consistent with previous studies, which have reported that there are ~30–50% mononucleated cardiomyocytes present in the fetal sheep heart at this stage of gestation (4, 7, 17, 46). We found that there was no change in the proportion of mononucleated cardiomyocytes present in either the PR or control fetuses across the gestational age range of animals used in this study. This is not inconsistent with previous studies in which the range of values cited for the proportion of mononucleated cardiomyocytes present during late gestation spans 30–75% at 133 to 134 days of gestation and between 20 and 40% after 140 days of gestation (7). The major finding of the present study is the increased proportion of mononucleated cardiomyocytes present in both ventricles of hearts from the PR fetus compared with control fetuses. One interpretation of this finding is that there has been a delay in binucleation of the cardiomyocytes in the growth-restricted fetus. Interestingly, recent studies have reported that there also is a lower incidence of cardiomyocyte proliferation (26) and binucleation in the fetal heart after exposure to uteroplacental embolization between 110 and 130 days of gestation in the fetal sheep (6, 26). Placental embolization results in acute changes in fetal arterial PO2 from the onset of embolization and results in a 30% decrease in fetal growth by 130 days of gestation. In our study, we found that there was a direct relationship between the degree of fetal hypoxemia and the proportion of mononucleated cardiomyocytes present in the fetal heart. There was no difference, however, in the expression of a marker of cellular proliferation within the mononucleated cardiomyocytes between the PR and control fetuses. Thus it appears that fetal hypoxemia, experienced either as a result of acute placental failure in late gestation (as in placental embolization) or as a restriction of placental growth and function from conception (as in the PR fetuses) results in a delay in cardiomyocyte binucleation. Acute placental restriction, however, results in a decrease in cellular proliferation within the fetal heart, in contrast to chronic placental insufficiency, which does not result in changes in cardiomyocyte proliferation. Interestingly, both mononucleated and binucleated cardiomyocytes were smaller in PR compared with control fetuses. Although cardiomyocytes were smaller in the heart of the PR
fetuses, when cardiomyocyte size was expressed relative to heart weight, the relative size of cardiomyocytes was greater in PR fetuses compared with controls. Although this might suggest that there are fewer but relatively larger cardiomyocytes present in the heart of the growth restricted fetus, further studies are required to determine the impact of placental restriction on the number of cardiomyocytes in the heart of the PR fetus. Acute placental restriction induced by uteroplacental embolization did not alter cardiomyocyte size (6, 26). This is likely to be due to the difference in duration and degree of fetal hypoxemia and to fetal neuroendocrine responses in the embolization model compared with the chronic PR model of fetal growth restriction. There are a series of neuroendocrine responses to the decrease in fetal substrate supply in the PR fetus. Such responses include increased plasma cortisol concentrations (37), decreased plasma IGF-1 and IGF-2 concentrations (36), and increased circulating plasma noradrenaline concentrations (42). There is conflicting evidence for a role for cortisol in determining the normal profile of cardiomyocyte maturation or growth in late gestation. Intrafetal infusion of cortisol to levels that mimic the prepartum rise result in an increase in cardiomyocyte proliferation after 2 days of infusion (~7 nM) (17). In contrast, infusion of cortisol at levels that result in supraphysiological fetal plasma cortisol concentrations (~1,000 nM) and fetal hypertension has led to an increase in cardiomyocyte size (28). In the present study, there was no relationship between fetal plasma cortisol concentrations and any cardiomyocyte parameter measured, which may suggest that there are factors other than cortisol in the growth-restricted fetus that determine the growth profile of the cardiomyocytes within the heart. Further investigation of the mechanisms underlying the altered development of the cardiomyocyte population in the heart of the PR fetus may provide new insights into the mechanisms regulating the transition from hyperplastic to hypertrophic heart growth.

IUGR as a result of chronic hypoxia or maternal protein restriction in rats caused an increase in apoptosis of cardiomyocytes (3) and a reduction in cardiomyocyte number at birth (10), respectively. Although we did not directly measure the number of cardiomyocytes present in the fetal heart of the IUGR fetus, the possibility remains that there is an overall decrease in the number of cardiomyocytes in the heart of the PR fetal sheep. A decrease in cardiomyocyte number is supported by our finding that relative cardiomyocyte size is greater in PR fetuses and would result in an increase in postnatal hypertrophic growth (27) to maintain heart growth. This could be one mechanism that results in an increased susceptibility to cardiac injury during adult life (25).

In summary, we have shown that restriction of placental growth in the sheep results in chronic fetal hypoxia, fetal growth restriction, an increase in the proportion of mononucleated cardiomyocytes, and cardiomyocytes that are smaller in absolute terms but larger relative to heart size. These changes in the fetal heart may be an adaptation to chronic hypoxia that results in fetal growth restriction associated with more mononucleated cardiomyocytes, a delay in bincucleation, and relatively larger cardiomyocytes in postnatal life. These findings are consistent with a reduction in cardiomyocyte number in the heart of the growth-restricted fetus. There may be a neonatal period when interventions may normalize cardiomyocyte number in the heart and lead to a reduction in the adult vulnerability to heart disease.

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