Extracellular signal-regulated kinase and phosphoinositol-3 kinase mediate IGF-1 induced proliferation of fetal sheep cardiomyocytes

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Departments of 1Physiology and Pharmacology, 2Medicine (Cardiology), and 3Surgery, 3Portland Veterans Affairs Medical Center, 4Center for Research on Occupational and Environmental Toxicology, 5The Vollum Institute, and 4Heart Research Center, Oregon Health and Science University, Portland, Oregon 97239

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Sundgren, Nathan C., George D. Giraud, Jess M. Schultz, Michael R. Lasarev, Philip J. S. Stork, and Kent L. Thornburg. Extracellular signal-regulated kinase and phosphoinositol-3 kinase mediate IGF-1 induced proliferation of fetal sheep cardiomyocytes. Am J Physiol Regul Integr Comp Physiol 285: R1481–R1489, 2003. First published August 28, 2003; 10.1152/ajpregu.00232.2003.—Growth of the fetal heart involves cardiomyocyte enlargement, division, and maturation. Insulin-like growth factor-1 (IGF-1) is implicated in many aspects of growth and is likely to be important in developmental heart growth. IGF-1 stimulates the IGF-1 receptor (IGF1R) and downstream signaling pathways, including extracellular signal-regulated kinase (ERK) and phosphoinositol-3 kinase (PI3K). We hypothesized that IGF-1 stimulates cardiomyocyte proliferation and enlargement through stimulation of the ERK cascade and stimulates cardiomyocyte differentiation through the PI3K cascade. In vivo administration of Long R3 IGF-1 (LR3 IGF-1) did not stimulate cardiomyocyte hypertrophy but led to a decreased percentage of cells that were binucleated in vivo. In culture, LR3 IGF-1 increased myocyte bromodeoxyuridine (BrdU) uptake by three- to five-fold. The blockade of either ERK or PI3K signaling (by U0-126 or LY-294002, respectively) completely abolished BrdU uptake stimulated by LR3 IGF-1. LR3 IGF-1 did not increase footprint area, but as expected, phenylephrine stimulated an increase in binucleated cardiomyocyte size. We conclude that 1) IGF-1 through IGF1R stimulates cardiomyocyte division in vivo; hyperplastic growth is the most likely explanation of IGF-1 stimulated heart growth in vivo; 2) IGF-1 through IGF1R does not stimulate binucleation in vitro or in vivo; 3) IGF-1 through IGF1R does not stimulate hypertrophy either in vivo or in vitro; and 4) IGF-1 through IGF1R requires both ERK and PI3K signaling for proliferation of near-term fetal sheep cardiomyocytes in vitro.

hyperplasia; hypertrophy; UO-126; LY-294002

Prenatal malnutrition leads to “programming” of the fetus and increased susceptibility to adult-onset hypertension, insulin resistance syndrome, and ischemic heart disease (3, 10). Insulin-like growth factor-1 (IGF-1) is an important regulator of growth during fetal life and is regulated by fetal nutrient supply (29, 30). Maternal undernutrition lowers fetal IGF-1 levels and decreases fetal heart-to-body weight ratio while infusion of IGF-1 to the fetus increases heart-to-body weight ratio in sheep (23, 31). However, the mechanisms by which IGF-1 regulates heart size are unknown. IGF-1 is known to stimulate hypertrophy, hyperplasia, and/or cell differentiation, depending on the cell type and maturation state of the cell (9), but its role in cardiomyocyte growth and maturation is unclear.

Transgenic and knockout mice have proven IGF-1’s critical role in development and growth. Mice null for IGF-1 are born 60% of normal birth weight, and mice null for the IGF-1 receptor (IGF1R) are 45% of normal birth weight. The IGF-1 nulls that survive birth are stunted in growth compared with littermate controls (1). These data show that IGF-1 remains an important growth factor into the neonatal period in mice, a period comparable to fetal development of precocious mammals, such as sheep.

IGF-1 is an important regulator in the cell cycle progression from the G1 to S phase (4, 32). In primary cardiomyocyte cultures, IGF-1 causes a large increase in proliferating cell nuclear antigen expression and in several cyclins involved in cell cycle progression as well as increased bromodeoxyuridine (BrdU) labeling (16, 21). When IGF-1 is overexpressed in the heart using a transgenic approach, heart weight is increased, as is heart weight-to-body weight ratio, apparently due to an increase in cardiomyocyte number rather than cell size (36).

Several in vivo animal models have linked pressure load and hypertrophy with IGF-1 levels. In the adult pig, volume or pressure overload causes increases in IGF-1 protein and mRNA that last for the duration of loading (26). IGF-1 stimulates neonatal rat cardiomyocytes to enlarge in vitro, and the increase in size is accompanied by increased expression of myosin light chain 2 and skeletal α-actin (15, 18). In the mouse,...
IGF-1 is highly expressed during adult cardiac hypertrophy caused by transverse aortic banding compared with normal controls (39). However, interpreting the role of IGF-1 in promoting cardiomyocyte hypertrophy in vivo is difficult because studies of IGF-1 are usually performed in adult animals where most myocytes are terminally differentiated.

IGF-1 and -2, acting through the IGF1R, can lead to either the proliferation or differentiation of skeletal muscle myoblasts (35, 43, 44) through different signaling pathways (6). However, the binucleation of cardiac myocytes during fetal or early postnatal growth, while called “terminal differentiation,” is quite different from developmental tissue differentiation. Cardiomyocyte binucleation occurs by DNA replication that is followed by karyokinesis without the cytokinesis that would normally follow in the mitotic cycle (19, 20). In transgenic mice overexpressing IGF-1, the proportions of mononucleated and binucleated cells in the heart were unchanged (36). However, the cardiac myocytes in that study appear to have been beyond the age that binucleation normally occurs. The ratio of mononucleated to binucleated cardiomyocytes is likely to be a very important index of heart growth potential. We have recently shown in prenatal sheep that only binucleated cardiomyocytes are capable of hypertrophy and only mononucleated cardiomyocytes are capable of proliferation (45). The role of IGF-1 in heart maturation in the large mammal has not been examined.

IGF-1 through IGF1R stimulates a multitude of signaling pathways; the two best studied are the extracellular signal-regulated kinase (ERK) and phosphoinositol-3 kinase (PI3K) cascades. Distinctive and complementary roles for these IGF-1-stimulated signaling cascades have been found. In skeletal myoblasts, IGF-1 induced proliferation through ERK signaling and differentiation through PI3K signaling (6). However, in a neuroblastoma cell line (SH-SY5Y cells), IGF-1-induced growth and differentiation required both ERK and PI3K (17). ERK signaling is necessary in IGF-1-induced protein synthesis and hypertrophy in neonatal rat cardiomyocytes consistent with the ERK cascade’s obligatory role in many forms of agonist-induced hypertrophy (18, 47, 49). Given the importance of these cascades in various forms of growth and differentiation, it is important to dissect their roles in IGF-1-induced growth in the fetal myocardium of the large mammal.

Thus there are many forms of evidence that IGF-1 plays a major role in heart development. We are interested in the intermediate phase of late fetal growth when working cardiac myocytes transition from being entirely mononucleate to predominantly binucleate. This phase of heart growth includes the transition from the early fetal hyperplastic to adult-type hypertrophic mode of growth. We set out to determine the role of IGF-1 and its signaling cascades in heart growth and maturation of fetal cardiomyocytes of sheep. Because we discovered recently that binucleate but not mononucleate cardiomyocytes are capable of enlargement under the stimulus of phenylephrine (45), we tested the hypothesis that IGF-1 would lead to increased binucleation followed by hypertrophy simultaneously with proliferation in the mononucleate population of fetal sheep cardiomyocytes. We hypothesized that proliferation and hypertrophy would be controlled by ERK stimulation while binucleation would require PI3K stimulation.

**MATERIALS AND METHODS**

**Animals.** The Oregon Health and Science University Institutional Animal Care and Use Committee approved all procedures, protocol A050. On day 124.4 ± 1.4 days of gestation (mean ± SD, term = 145 days), surgery was performed on fetal sheep of mixed Western breed as described previously (2, 46). Polyvinyl catheters of 1.3-mm OD or 1.7-mm OD are placed in the right atrium (via jugular vein) and the aorta (via carotid artery). Fetuses are studied, unanesthetized, after >5 days postsurgery.

Whole animal experiments were performed on five animals infused with Long R3 (LR3) IGF-1 (experimental group), five uninstrumented twins of the experimental group (twin control group), and five vehicle-infused animals (vehicle control group) of which three were twins and two were singletons.

IGF-1 circulates largely bound to a family of at least six binding proteins in serum with a small fraction existing in the “free” form. The roles of the binding proteins are not entirely clear. The binding proteins may both sequester free IGF-1 and prevent binding to IGF1R, or they may potentiate uptake into tissue and/or receptor binding (8). The binding proteins themselves may also have actions independent of IGF-1 (8). To isolate the actions of IGF-1 unassociated with binding protein but bound to its receptor, we infused sheep fetuses with an IGF-1 analog, LR3 IGF-1 (Groep, Adelaide, Australia), which binds binding protein with much lower affinity (200- to 1,000-fold) than endogenous IGF-1 and which circulates mostly in a free form in plasma.

**Day 0 of experiments began on 130.5 ± 0.9 days of gestation. Baseline hemodynamics were measured, and blood was drawn from the aorta for blood gases and hematocrit. Plasma was collected and frozen for later determination of plasma IGF-1 levels. An ambulatory mechanical pump was attached to a catheter placed in the right atrium via the jugular vein and secured in a cloth pouch attached to the mother. Experimental fetuses were given 5 mg of LR3 IGF-1 over 7 days (29.8 μg/h, or ~6.6 ± 1.2 μg·h⁻¹·kg⁻¹ based on autopsy weight). This dose was calculated, based on published pharmacological data, to be equivalent to ~81 μg/h recombinant IGF-1 the form that has been shown in a previous study to increase fetal sheep heart weight (23). Control animals received an equal volume of vehicle (normal saline + 1% BSA) infused at the same rate and duration as for experimental animals (10.4 ml/day, 7 days). On days 4 and 7, hemodynamic measurements and blood samples were taken as on day 0. After 7 days of infusion, a lethal dose of commercial euthanasia solution containing pentobarbital sodium was given to the ewe, and the fetus was heparinized with 10,000 U of heparin sulfate and given 3 ml of saturated KCl solution to arrest the heart in diastole. Fetal body weight, total liver weight (including gall bladder), and combined kidney weights were measured, and the heart was taken for cell isolation.

**Plasma IGF-1 and LR3 IGF-1.** Plasma IGF-1 levels were measured using an ELISA kit that releases IGF-1 from its binding proteins and gives a value for the total IGF-1 (ALPCO Diagnostics, Windham, NH). Measurement of plasma LR3 IGF-1 used an ELISA kit from the makers of LR3 IGF-1

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(Gropec). Coating and capture antibodies were supplied in the kit and were used at the recommended concentrations. Anti-rabbit Ig horseradish peroxidase (HRP) antibody was used at 1:8,000 dilution (Jackson ImmunoResearch).

Cell isolation, measurement, and culture. Heart cells were isolated for both immediate fixation and for cell culture. Cardiomyocytes used for cell culture include the hearts from vehicle-infused instrumented fetuses, noninfused instrumented fetuses, and noninstrumented fetuses ranging from 127 to 139 days gestation. Our cell isolation procedure has been described previously (2, 45). A portion of the isolated cell slurry was fixed and measured for length and width under ×400 light microscopy (Zeiss Axiophot) as described previously (2). One hundred cells were measured for size, and 200 were counted for binucleation. The rest of the cell slurry from the cell isolation was used for culture.

Cardiomyocyte culture. Our cardiomyocyte culture protocols have been described elsewhere (45). Briefly, the isolated cell suspension was placed in an untreated tissue culture flask containing serum-enriched medium and incubated for 2 h (39°C, 5% CO2); the medium was changed twice to preplate fibroblasts and endothelial cells. After the second preplating, unattached cells were plated into 75-cm2 tissue culture dishes holding sterile 22 × 22-mm coverslips precoated with laminin (1–5 μg/ml) or plated into six-well plates that were laminin treated; 500,000 cardiomyocytes per

Cultured cardiomyocyte data. Protocols for staining and data collection of cultured cardiomyocytes have been described elsewhere (45).

Cell stimulation and Western blot analysis. At experiment, four wells were preincubated 20 min with 10 μM UO-126, four were preincubated 20 min with 10 μM LY-294002, and four were left in serum-free media alone. All wells were stimulated with 1 μg/ml LR3 IGF-1 for 0, 5, 10, or 20 min. Protein was collected and analyzed by Western blot analysis as described previously (45). All lanes were loaded with an equal amount of protein (40 μg) and probed for signaling cascade activity. Primary antibodies were phosho-44/42 MAP kinase (Thr202/Tyr204), phospho-AKT (p-AKT; Thr308), AKT (Cell Signaling, Beverly, MA), and ERK2 (Santa Cruz Biotechnology). Primary antibodies were used at 1:1,000, and secondary antibodies (anti-rabbit HRP or antimouse HRP) were used at 1:10,000. In the analysis, raw densitometry data were calculated as a ratio of active protein to total protein (p-ERK/ERK2 or p-AKT/AKT) and normalized to the 0-min time point of stimulation in the absence of either inhibitor for each experiment.

Table 1. Hemodynamic measures, arterial blood gases, hematocrit, pH, and IGF-1 plasma levels

<table>
<thead>
<tr>
<th></th>
<th>Vehicle Control</th>
<th>Experimental</th>
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<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 4</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>41.4 ± 2.2</td>
<td>45.6 ± 7.0</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>53.4 ± 5.6</td>
<td>56.0 ± 6.3</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>36.0 ± 2.4</td>
<td>38.0 ± 4.4</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>154 ± 6</td>
<td>156 ± 9</td>
</tr>
<tr>
<td>PaO2, mmHg</td>
<td>20.2 ± 1.1</td>
<td>19.8 ± 1.1</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>32.4 ± 2.7</td>
<td>34.0 ± 1.0</td>
</tr>
<tr>
<td>O2 content, ml/dl</td>
<td>7.2 ± 0.6</td>
<td>7.2 ± 0.9</td>
</tr>
<tr>
<td>PaCO2, mmHg</td>
<td>37.5 ± 6.6</td>
<td>46.9 ± 2.0</td>
</tr>
<tr>
<td>pH</td>
<td>7.35 ± 0.02</td>
<td>7.34 ± 0.02</td>
</tr>
<tr>
<td>IGF-1, ng/ml</td>
<td>58.1 ± 22.0</td>
<td>51.7 ± 19.9</td>
</tr>
<tr>
<td>LR3 IGF-1, ng/ml</td>
<td>0.4 ± 0.1‡</td>
<td>0.4 ± 0.1‡</td>
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Values are means ± SD. MAP, mean arterial pressure; BP, blood pressure; HR, heart rate; PaO2, and PaCO2, arterial O2 and CO2, respectively; IGF-1, insulin-like growth factor-1; LR3 IGF-1, Long R3 IGF-1. *P < 0.05 compared with day 0; †P < 0.05 compared to same-day control value; n = 5 for all values except ‡n = 4.

Two-way ANOVA was used to compare 2 groups. Comparisons were considered significant at P < 0.05.

RESULTS

Table 1 shows the arterial blood gas, pH, hematocrit, and hemodynamic data of experimental and vehicle control animals before infusion (day 0), after 4 days (day 4), and on the final day of infusion (day 7). Of note is the gradual decline in arterial Po2 (PaO2) and O2 content over the 7 days of infusion with LR3 IGF-1.
Other groups have reported this $P_{\text{aO}_2}$ decline with infusion of recombinant IGF-1 over several days, but the cause for this has not been found (23, 24). Experimental animals also showed an increase in heart rate on day 7 compared with vehicle controls (Table 1). Otherwise the animals were healthy and in the expected ranges for all parameters.

Plasma levels of endogenous IGF-1 are shown in Table 1. At day 0, experimental plasma IGF-1 levels were not different from vehicle control plasma IGF-1 levels. Animals infused with vehicle showed no change in plasma IGF-1 levels over 7 days. Experimental animals showed a dramatic decrease in endogenous IGF-1 levels in response to LR3 IGF-1 compared with vehicle at both days 4 and 7 (Table 1). LR3 IGF-1 plasma levels were increased on day 7 in experimental animals over vehicle-infused controls (Table 1).

Whole animal experiments were performed on five animals infused with LR3 IGF-1 (experimental group), five uninstrumented twins of the experimental group (twin control group), and five vehicle-infused animals (vehicle control group) of which three were twins and two were singletons. A 7-day infusion of LR3 IGF-1 did not alter body weight of experimental animals (4.69 ± 0.44 kg, mean ± SE) compared with vehicle-infused controls (4.61 ± 0.24 kg) or twin controls (4.28 ± 0.28 kg, Fig. 1A). Heart weight increased by 35% in experimental animals (41.4 ± 2.6 g) compared with vehicle control animals (30.6 ± 2.3 g). Previous work showed that IGF-1 infusion increased kidney and liver weight as well as heart weight (23). We measured these organ weights to assess the effectiveness of our dosing protocol. Combined kidney weight appeared to increase in experimental animals (31.0 ± 1.4 g) compared with vehicle control animals (25.1 ± 2.3 g) but was not significant until normalized to body weight (Fig. 1B). Liver weight was not different in experimental animals (83.7 ± 11.3 g) compared with vehicle control animals (88.4 ± 7.6 g). Vehicle control animals were not statistically different from twin control animals in any measurement (heart weight = 26.6 ± 2.3 g, kidney weight = 22.2 ± 2.1 g, liver weight = 78.9 ± 13.3 g).

Morphometric measurement of dissociated cells is summarized in Table 2. LR3 IGF-1 infusion did not change cell sizes. Experimental animals did, however, show a significant decrease in percentage of cells that were binucleated or, alternately stated, an increase in percentage of cells that were mononucleated.

We surmised that the increase in percentage of mononucleated cells resulting from LR3 IGF-1 infusion in vivo indicated a proliferation of mitotically competent mononucleated cells. Therefore, we used an in vitro cell culture system of ovine cardiomyocytes to measure BrdU uptake as an assay of proliferation. In culture, cardiomyocytes in serum-free conditions have a low rate of BrdU uptake over 48 h (1.11 ± 0.23%, n = 9) that is increased in serum-enriched media conditions (8.26 ± 2.32%, n = 9, P < 0.05). Figure 2A is an image of BrdU labeling (green) in a cardiomyocyte labeled for myosin (red). Two doses of LR3 IGF-1 were used in serum-free conditions, and BrdU uptake was measured. LR3 IGF-1 (0.1 μg/ml) increased BrdU uptake (3.59 ± 0.50%, n = 9, P < 0.05), which was further increased by 1 μg/ml LR3 IGF-1 (5.54 ± 0.77%, n = 8, P < 0.05, Fig. 2B). The BrdU uptake, stimulated by LR3 IGF-1, was dependent on both ERK and PI3K signaling cascades as demonstrated by the inhibition of BrdU uptake in the presence of either UO-126 or LY-294002, respectively (Fig. 2B). Neither of the inhibitors alone or in combination with high dose LR3 IGF-1 stimulated BrdU uptake above the serum-free levels (P > 0.05, ANOVA).

Although LR3 IGF-1 did not increase ovine cardiac myocyte cell size in vivo, IGF-1 has been shown to increase rat cardiomyocyte size in culture (15). We have previously shown that PE causes an increase in ventricular binucleated cell size while not changing cell size in mononucleated cells (45). In cardiomyocytes from either ventricle, there was no change in mononucleated cardiomyocyte cell sizes with any treatment [serum-free left ventricle (LV) = 908.51 ± 64.02 μm², right ventricle (RV) = 1,056.90 ± 76.06 μm², n = 7, Fig. 3]. In LV cardiomyocytes, LR3 IGF-1 did not stimulate an increase in cell size (1,612.04 ± 125.21 μm²) compared with cells grown in serum-free conditions (1,599.10 ± 96.48 μm²), while PE did increase cell size (2,125.03 ± 124.36 μm², P < 0.05, Fig. 3A). In RV cardiomyocytes, known to be larger than LV cardiomyocytes (42), the same pattern was true; LR3 IGF-1 did not cause hypertrophy (2,162.05 ± 138.08 μm²) com-
increase proliferation. To rule out a nonspecific and PI3K signaling are required for LR3 IGF-1 to stimulation. A representative blot of LR3 IGF-1 stimulation of p-ERK and p-AKT levels in response to LR3 IGF-1 tested the hypothesis that ERK signaling in the absence of PI3K signaling from IGF1R activation could induce hypertrophy. However, LR3 IGF-1 given in combination with LY-294002 did not increase binucleated cell size over baseline serum-free conditions (2,012.86 ± 288.95 μm², n = 4, Fig. 4). We have previously shown that PE induces cell enlargement in an ERK-dependent manner (45). We hypothesized that IGF1R stimulation would simulate the changes seen in a systolic pressure load by increasing proliferation, maturation, and cell size (2). Previous work established a dose of IGF-1 that increased fetal sheep heart weight by 26% and heart-to-body weight ratio by ~15% (23). This dose was sufficient to increase kidney and liver weight while not increasing overall body weight (23). We here report a dose of LR3 IGF-1 that similarly affected heart weight, increasing heart weight by 35% and heart-to-body weight ratio by 37% (Fig. 1). We also saw an increase in combined kidney weight but did not see any effect on liver weight (Fig. 1). Whereas infusion of IGF-1 increases plasma levels of IGF-1 (23, 24), our infusion of LR3 IGF-1 decreased endogenous levels of IGF-1 while increasing levels of LR3 IGF-1 (Table 1), p-ERK is shown in Fig. 5A. Differences across treatment were highly significant (F2,33 = 164.13; P < 0.01). Addition of UO-126 produced uniform decreases in median fold changes compared with LR3 IGF-1 stimulation at all time points (P < 0.01), and addition of LY-294002 produced a significant increase in baseline p-ERK (P < 0.05, Fig. 5B). Changes were also observed across the four time points (F3,33 = 5.21; P < 0.01). LR3 IGF-1 alone significantly increased stimulation over time relative to baseline (Fig. 5B). A representative blot of LR3 IGF-1 stimulation of p-AKT is shown in Fig. 5C. Median fold changes are significantly affected by treatment (F2,33 = 30.86; P < 0.01) but not by time (F3,33 = 1.33; P > 0.05, Fig. 5D). Addition of UO-126 resulted in median fold changes that were not significantly different from LR3 IGF-1 alone (P > 0.05 for all time points).

**DISCUSSION**

We have described experiments designed to elucidate the effects of IGF1R on fetal sheep heart growth and to determine the importance of ERK and PI3K signaling cascades as mediators of those effects. We hypothesized that IGF1R stimulation would simulate the changes seen in a systolic pressure load by increasing proliferation, maturation, and cell size (2).

**Table 2. In vivo myocyte size data**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle Control (n = 5)</th>
<th>Experimental (n = 5)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binucleation, %</td>
<td>Left ventricle 61.3 ± 8.1</td>
<td>45.8 ± 5.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Right ventricle 61.9 ± 8.7</td>
<td>50.5 ± 4.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Binucleated length, μm</td>
<td>Left ventricle 87.3 ± 4.3</td>
<td>90.5 ± 5.0</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Right ventricle 95.0 ± 7.4</td>
<td>98.9 ± 7.9</td>
<td>NS</td>
</tr>
<tr>
<td>Binucleated width, μm</td>
<td>Left ventricle 13.7 ± 1.1</td>
<td>15.1 ± 0.6</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Right ventricle 16.5 ± 1.7</td>
<td>17.3 ± 1.9</td>
<td>NS</td>
</tr>
<tr>
<td>Mononucleated width, μm</td>
<td>Left ventricle 66.8 ± 3.2</td>
<td>70.4 ± 3.4</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Right ventricle 73.3 ± 4.7</td>
<td>78.0 ± 5.5</td>
<td>NS</td>
</tr>
<tr>
<td>Mononucleated width, μm</td>
<td>Left ventricle 11.7 ± 1.0</td>
<td>12.6 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Right ventricle 13.3 ± 1.3</td>
<td>14.0 ± 1.6</td>
<td>NS</td>
</tr>
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</table>

Values are means ± SD. NS, not significant.

pared with serum-free conditions (2,001.86 ± 186.10 μm²), but PE did increase cell size (2,618.73 ± 124.90 μm², P < 0.05, Fig. 3B).

We have previously shown that PE induces cell enlargement in an ERK-dependent manner (45). We tested the hypothesis that ERK signaling in the absence of PI3K signaling from IGF1R activation could induce hypertrophy. However, LR3 IGF-1 given in combination with LY-294002 did not increase binucleated cell size over baseline serum-free conditions in LV or RV cells (LV = 1,562.32 ± 368.12 μm², n = 3, RV = 2,120.06 ± 288.95 μm², n = 4, Fig. 4).

BrdU uptake assays in culture show that both ERK and PI3K signaling are required for LR3 IGF-1 to increase proliferation. To rule out a nonspecific effect of an inhibitor or cross talk between cascades as the reason for the dual signaling necessity, we measured p-ERK and p-AKT levels in response to LR3 IGF-1 stimulation. A representative blot of LR3 IGF-1 stimulation of p-ERK is shown in Fig. 5A. Differences across treatment were highly significant (F2,33 = 164.13; P < 0.01). Addition of UO-126 produced uniform decreases in median fold changes compared with LR3 IGF-1 stimulation at all time points (P < 0.01), and addition of LY-294002 produced a significant increase in baseline p-ERK (P < 0.05, Fig. 5B). Changes were also observed across the four time points (F3,33 = 5.21; P < 0.01). LR3 IGF-1 alone significantly increased stimulation over time relative to baseline (Fig. 5B). A representative blot of LR3 IGF-1 stimulation of p-AKT is shown in Fig. 5C. Median fold changes are significantly affected by treatment (F2,33 = 30.86; P < 0.01) but not by time (F3,33 = 1.33; P > 0.05, Fig. 5D). Addition of UO-126 resulted in median fold changes that were not significantly different from LR3 IGF-1 alone (P > 0.05 for all time points).

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suggesting that the administration of LR3 IGF-1 stimulated a negative feedback to reduce endogenous IGF-1 levels. The proportion of the total IGF-1 in the unbound form was not determined. However, we measured LR3 IGF-1 plasma levels at the conclusion of the infusion period to show that they were within the expected physiological range.

While we hypothesized that IGF1R stimulation would increase the percentage of myocytes that were binucleated, we found the opposite: a decrease in the percentage of cardiomyocytes that were binucleated (Table 2). The increase in heart weight, but not cell size, suggests an increase in the population of myocytes. Consistent with that interpretation, LR3 IGF-1 induced a fivefold increase in BrdU uptake in normal fetal cardiomyocytes in vitro (Fig. 2B). Increased BrdU uptake indicates that DNA replication was occurring. In rare cases, BrdU uptake can occur in the absence of cytoplasmic division in transformed cells (11). However, the combination of in vivo and in vitro data reported here in normal cells is consistent with the accepted index of BrdU as an indicator of proliferation. This increase in BrdU uptake was dependent on both ERK and PI3K signaling (Fig. 2B). While several IGF-1 effects require both signaling cascades (13, 17, 25), others do not (6, 18, 22, 33). In our studies, the cardiomyocyte proliferation response was dependent on both ERK and PI3K.

It is also possible that LR3 IGF-1 induces fibroblasts and perhaps other cells to proliferate in vivo, which could explain some of the common increase in both heart and kidney weight in our experiments. Because the fetal sheep heart is >80% cardiomyocyte by volume and <1% fibroblast by volume under normal conditions (2), the increase in nonmyocyte volume under the influence of LR3 IGF-1 would have to be enormous to increase heart weight by 35% that was found in this study (Fig. 1). In addition we did not notice an increase in any nonmyocyte cell type in the isolated cell suspensions. The mechanism for increased weight of the kidney after LR3 IGF-1 seen by us and others is unknown.

Cross talk between these two cascades has been described previously at the level of AKT (a downstream target of PI3K) and Raf-1 (downstream target of Ras and upstream of ERK) (27, 50). AKT can directly phosphorylate Raf-1 and decrease its activity in skeletal myotubes but not skeletal myoblasts (37, 50). In our studies, LR3 IGF-1 increased ERK phosphorylation over 20 min of stimulation (Fig. 5B). In the presence of PI3K inhibition by LY-294002, baseline ERK phosphorylation is increased (0 min, Fig. 5B), suggesting that in fetal sheep cardiomyocytes in vitro, PI3K has a tonic inhibition of ERK activity perhaps by AKT phosphorylation of Raf-1. However, if proliferation were solely dependent on ERK, then we would predict that PI3K inhibition would increase ERK activity and therefore proliferation. Instead, PI3K inhibition abol-

Fig. 3. Hypertrophy of cardiomyocytes in culture. Area of left ventricle (LV; A) and right ventricle (RV; B) mononucleated (gray bars) and binucleated (black bars) cardiomyocytes (mean ± SE). Doses were 10 μg/ml phenylephrine (PE) and 1 μg/ml LR3 IGF-1. *P < 0.05 compared with serum-free binucleated, n = 7, paired ANOVA. There are also significant differences between mononucleated and binucleated cardiomyocyte size under all treatments.

Fig. 4. Extracellular signal-regulated kinase (ERK)-forced stimulation by LR3 IGF-1 does not cause hypertrophy. Area of LV (A) and RV (B) mononucleated (gray bars) and binucleated (black bars) cardiomyocytes (mean ± SE). Doses were 1 μg/ml LR3 IGF-1 and 10 μM LY-294002 (LY). LV, n = 3; RV, n = 4; P > 0.05, paired ANOVA.
ished the increase in BrdU uptake stimulated by LR3 IGF-1 (Fig. 2B). It is also reported that ERK activation can inhibit PI3K activation (48). Blockade of ERK signaling by UO-126 in fetal sheep cardiomyocytes may change LR3 IGF-1-induced AKT phosphorylation, causing an increase in AKT stimulation at 5 min although this was not quite significant (P = 0.067, Fig. 5D). Here again, if proliferation depended only on PI3K, then ERK inhibition would be expected to increase BrdU uptake by increasing PI3K activity. However, as with PI3K inhibition, ERK inhibition abolished an increase in BrdU uptake (Fig. 2B). Therefore, it appears that the increased BrdU uptake from LR3 IGF-1 stimulation represented the synergistic activity of ERK and PI3K signaling.

Disassociated mononucleate and binucleate cells from LR3 IGF-1-treated fetal sheep were no larger than control cells (Table 2). This is consistent with the absence of cardiomyocyte enlargement in transgenic mice overexpressing IGF-1 (36). In contrast, others have reported hypertrophy of rat cardiomyocytes in culture in response to IGF-1 (15). LR3 IGF-1 stimulation did not increase the size of fetal sheep cardiomyocytes in culture, while PE stimulation did cause hypertrophy (Fig. 3). PE-induced hypertrophy is dependent on ERK signaling (45, 47, 49). Other work has proposed that IGF-1-induced hypertrophy was dependent on ERK but not on PI3K signaling (18). We hypothesized that PI3K signaling in combination with ERK signaling would lead to hyperplasia and blocked hypertrophy, while ERK signaling alone would cause hypertrophy. We found, however, that IGF1R stimulation in the presence of PI3K blockade did not increase cell size either (Fig. 4).

It is often assumed that neonatal rat cardiomyocytes are similar to late fetal cardiomyocytes of more mature species such as the sheep. However, neonatal rat cardiomyocytes lose their ability to increase BrdU uptake after just 48 h in culture (38) while we have shown that fetal sheep cardiomyocytes in culture retain their ability to increase BrdU uptake after a total of 96 h in culture (Fig. 2B). Therefore IGF-1 may only be able to induce hypertrophy of cardiomyocytes after some further maturation event that occurs later in gestation or after birth in the sheep.

Recent work has shown that PI3K and its downstream target AKT may be critical components of the regulation of heart size (5, 7, 40, 41). Transgenic mice expressing a constitutively active PI3K-α construct have an increased heart size due to an increase in myocyte size, and mice expressing a dominant negative PI3K-α construct have decreased heart size due to decreases in myocyte size (40). The same results are seen when constitutively active AKT and dominant negative AKT are expressed (5, 41). It is important to note that in these transgenic mice the transgene is on an α-myosin heavy chain promoter that induces expression in mice days after birth, which is past the time of mouse cardiomyocyte multinucleation (36, 40). What role PI3K may have on regulation of fetal heart size when the cardiomyocytes are still able to divide at a high rate cannot be deduced from these experiments. Curiously, transgenic mice expressing IGF-1 with the same α-myosin heavy chain promoter showed no increase in cell size while total heart size was increased (36). These transgenic mice showed very low, but increased, levels of BrdU uptake in culture, and it was concluded that the increase in heart weight was due to an increase in the total number of myocytes (36). While LR3 IGF-1 does stimulate PI3K in fetal sheep cardiomyocytes (Fig. 5, C and D), the stimulation is not sufficient to cause hypertrophy (Fig. 3). PE stimulates hypertrophy in fetal sheep cardiomyocytes (Fig. 3), but we have not detected by Western blot analysis any stimulation of PI3K by PE (Sundgren and Thornburg, unpublished data). ANG II does not stimulate cardiomyocyte hypertrophy in fetal sheep either (45), but IGF-1, PE, and ANG II all stimulate hypertrophy in rat cardiomyocytes. Thus the data presented here show another example of the dramatic differences between fetal sheep cardiomyocytes and neonatal rat cardiomyocytes.
Our in vitro experiments suggest that IGF1R stimulation during fetal life leads to an increase in heart weight due to hyperplasia but not hypertrophy. Contrary to our hypothesis, IGF1R stimulation does not lead to an increase in cardiomyocyte maturation as measured by the percentage of binucleate cardiomyocytes. It follows that those born with higher IGF-1 levels may have more heart myocytes than those born with lower IGF-1 levels. It is not known when or how the optimal number of cardiomyocytes is set. However, if that number is set during the antenatal period, regardless of the fact that new cardiomyocytes can be generated during adult life (28), the number of cells at birth may be important for life. One consequence of growth restriction during fetal life may, therefore, be a reduction in the total number of cardiomyocytes at birth with associated risks for disease in adult life.

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DISCLOSURES

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