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Activation of IGF-2R stimulates cardiomyocyte hypertrophy in the late gestation sheep fetus

Kimberley C. W. Wang[1], Doug A. Brooks[2], Kent L. Thornburg[3] and Janna L. Morrison[1]

[1]*Early Origins of Adult Health Research Group and* [2]*Mechanisms in Cell Biology and Diseases Research Group, School of Pharmacy and Medical Sciences, Sansom Institute for Health Research, University of South Australia, Adelaide, SA, Australia*
[3]*Heart Research Center, Oregon Health Science University, Portland, OR, USA*

Key points

- This study investigates the impact that insulin-like growth factor 2 receptor (IGF-2R) activation has on the fetal heart, by infusing Leu^{27}IGF-2 into the left circumflex coronary artery of the sheep fetus, to specifically activate IGF-2R and it's downstream signalling pathway.
- Activation of cardiac IGF-2R resulted in cardiomyocyte hypertrophy, but with no changes in heart weight, cardiomyocyte proliferation, binucleation or apoptosis. This hypertrophy was mediated via protein kinase A activation.
- Infusion of Leu^{27}IGF-2 increases atrial natriuretic peptide abundance, a marker of cardiac pathological hypertrophy.
- Cardiac IGF-2R activation may alter important regulators of cardiac contractility and relaxation by decreasing sarcoplasmic reticulum Ca^{2+}-ATPase and phospho-troponin I abundance.
- This study places the interaction between the IGF-2R and Gαs signalling pathway as a potential mechanism that can contribute to cardiomyocyte growth in fetal life, but which may result in pathological cardiac hypertrophy in postnatal life.

Abstract *In vitro* studies using rat and fetal sheep cardiomyocytes indicate that, in addition to its role as a clearance receptor, the insulin-like growth factor 2 receptor (IGF-2R) can induce cardiomyocyte hypertrophy. In the present study, we have determined the effect of specific activation of the IGF-2R in the heart of the late gestation fetus on cardiomyocyte development. Leu^{27}IGF-2, an IGF-2R agonist, was infused into the fetal left circumflex coronary artery for 4 days beginning at 128.1 $\pm$ 0.4 days gestation. Ewes were humanely killed at 132.2 $\pm$ 1.2 days gestation (term, 150 days). Fetuses were delivered and hearts dissected to isolate the cardiomyocytes and to collect and snap-freeze tissue. Leu^{27}IGF-2 infusion into the left circumflex coronary artery of fetal sheep increased the area of binucleated cardiomyocytes in the left, but not the right, ventricle. However, this infusion of Leu^{27}IGF-2 did not change fetal weight, heart weight, blood pressure, blood gases or cardiomyocyte proliferation/binucleation. The increase in cardiomyocyte size in the Leu^{27}IGF-2-infused group was associated with increased expression of proteins in the Gαs, but not the Gαq, signalling pathway. We concluded that infusion of Leu^{27}IGF-2 into the left circumflex coronary artery causes cardiac IGF-2R activation in the left ventricle of the heart, and this stimulates cardiomyocyte hypertrophy in a Gαs-dependent manner.

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Corresponding author J. L. Morrison: Early Origins of Adult Health Research Group, School of Pharmacy and Medical Sciences, Sansom Institute for Health Research, University of South Australia, GPO Box 2471, Adelaide, SA, Australia 5001. Email: janna.morrison@unisa.edu.au

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Abbreviations ANP, atrial natriuretic peptide; CaMKII, Ca^{2+}–calmodulin-dependent protein kinase II; CREB, cAMP response element-binding; DBP, diastolic blood pressure; Erk1/2, p44/42 MAP kinase; Gαq, G protein-coupled αq; Hb, haemoglobin; HDAC, histone deacetylase; HR, heart rate; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor-1 receptor; MAP, mean arterial pressure; NFATc3, nuclear factor of activated T-cells; p21, cyclin-dependent kinase inhibitor 21; PCNA, proliferating cell nuclear antigen; PKA, protein kinase A; PKC-α, protein kinase C-α; RPP, rate pressure product; SBP, systolic blood pressure; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; S_{O_2}, oxygen saturation.

Introduction

Pathological cardiac hypertrophy is associated with reduced left ventricular function and often leads to heart failure and death (Kannel *et al.* 1987; Levy *et al.* 1990). Pathological cardiac hypertrophy can be caused by increased pressure overload due to increased blood pressure or myocardial injury (Oakley, 1971). In the absence of pressure overload, cardiac hypertrophy can be mediated by the activation of specific receptor signalling pathways (Botting *et al.* 2011).

The insulin-like growth factor (IGF) system plays an important role in physiological cardiac hypertrophy, as well as the maturation of cardiomyocytes in late gestation. The IGF-1 receptor (IGF-1R) can be activated by either IGF-1 or IGF-2, and in the heart signalling from this receptor has been associated with normal growth and physiological hypertrophy. IGF-2 can also bind directly to the IGF-2R, but this has previously been thought to act as a clearance mechanism (Kornfeld, 1992), due to this receptor's association with the endosome–lysosome system, which has a function in degradation. The degradation of IGF-2 limits its interaction with the IGF-1R (Kornfeld, 1992), and in fetal life this appears to be an important regulatory system for cardiac growth (Powell *et al.* 2006; Wang *et al.* 2011). However, *in vitro* studies in rat cardiomyocytes have shown that IGF-2 can play a role in cardiomyocyte proliferation (Reini *et al.* 2009) and has the ability to induce cardiac hypertrophy in fetal (Wang *et al.* 2012) and adult (Chu *et al.* 2008) cardiomyocytes. This hypertrophic response is thought to occur through the activation of an IGF-2R signalling pathway (Huang *et al.* 2002; Chu *et al.* 2008).

The IGF-2R has been shown to activate phospholipase C-β via a heterotrimeric G protein-coupled receptor, involving αq subunits (Gαq). This Gαq signalling mechanism activates protein kinase C-α (PKC-α), Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII) and p44/42 MAP kinase (ERK), all of which appear to have a role in cardiac hypertrophy (Chu *et al.* 2008; Wang *et al.* 2012). In contrast, *in vitro* activation of the IGF-2R has been shown to induce cardiomyocyte apoptosis via the Gαq–calcineurin pathway (Chen *et al.* 2009; Chu *et al.* 2009*b*). This can involve a reduction in Gαs binding and decreased protein kinase A (PKA) phosphorylation (Chu *et al.* 2009*a*), changes that have been implicated in regulating cardiac contractility (Noland *et al.* 1995). Thus, there is now little doubt that the function of the IGF-2R is not limited to that of IGF-2 clearance (Kornfeld, 1992), but that this receptor also has critical involvement in signalling processes, which may contribute to adult cardiac pathogenesis.

Interestingly, we have shown that when growth is reduced *in utero*, the IGF-2R plays a role in signalling, which results in cardiac hypertrophy and this response extends at least into neonatal life (Wang *et al.* 2011, 2012). Altered signalling from the IGF-2R in cardiac development may provide an explanation for the link between sub-optimal growth in fetal life and increased risk of heart disease in adulthood (Barker, 1995; Rich-Edwards *et al.* 1997). Therefore the aim of this study was to investigate the impact that IGF-2R activation has on the fetal heart, by selectively infusing Leu^{27}IGF-2 into the left circumflex coronary artery in the sheep fetus, to specifically interrogate IGF-2R signalling. We hypothesized that activation of cardiac IGF-2R would not induce cardiomyocyte proliferation, binucleation or apoptosis, but would cause cardiomyocyte hypertrophy.

Methods

Animals and surgery

All procedures were approved by The University of South Australia Animal Ethics Committee. The authors have read, and the experiments comply with, the policies and regulations of *The Journal of Physiology* given by Drummond (2009). Vascular surgery was performed in 25 fetal sheep at 125.2 ± 0.3 days gestation (term, 150 ± 3 days), under aseptic conditions. Anaesthesia was induced with intravenous diazepam–ketamine ($0.3\ mg\ kg^{-1}$–$5\ mg\ kg^{-1}$) and maintained with 1–2.5% isoflurane in oxygen. Catheters (Critchley Electrical Products, Siverwater, NSW, Australia) were inserted in the maternal jugular vein, the fetal left circumflex coronary and carotid arteries, jugular vein, trachea and the amniotic cavity, as previously described (Edwards *et al.* 1999; Morrison *et al.* 2001; Danielson *et al.* 2005). At surgery, antibiotics were administered to the ewe (3.5 ml, $150\ mg\ ml^{-1}$ procaine penicillin; $112.5\ mg\ ml^{-1}$ benzathine penicillin; 2 ml, $250\ g\ ml^{-1}$ dihydrostreptomycin, Lyppard, Keysborough, VIC, Australia) and fetus (1 ml, $150\ mg\ ml^{-1}$ procaine penicillin; $112.5\ mg\ ml^{-1}$ benzathine penicillin; 1 ml, $250\ g\ ml^{-1}$ dihydrostreptomycin, Lyppard) and then

post-operatively to ewes intramuscularly for 3 days and to fetuses intraamniotically for 4 days (500 mg ampicillin, Lyppard).

Experimental protocol

After 3 days recovery from surgery, fetuses were randomly assigned to different treatment groups: vehicle (100 mM of acetic acid (Sigma-Aldrich, Australia; $n = 4$) or Leu^{27}IGF-2 (an analogue of IGF-2 that binds to the IGF-2R but not to the IGF-1R (Sakano *et al.* 1991; Oh *et al.* 1993); Novozymes GroPep, Adelaide, SA, Australia; 0.4 μg h^{-1}, $n = 8$; 0.8 μg h^{-1}, $n = 2$) and infused via the left circumflex coronary artery for 4 days. This effect was expected to be specific to the left ventricle because the left circumflex coronary artery directly perfuses the left ventricle and a posterior portion of the interventricular septum, but not the right ventricle (Rudolph *et al.* 1999). Furthermore, this approach was designed to specifically activate the IGF-2R in myocardium without promoting haemodynamic effects that may occur with a systemic infusion (Bauman *et al.* 1987). Some fetuses underwent the surgical procedure, but the coronary artery catheter was not patent (sham; $n = 10$). There were no differences between the sham and vehicle infused fetuses (Giraud *et al.* 2006); and there were no differences between the fetuses that received different doses of Leu^{27}IGF-2; for any of the physiological or cardiomyocyte measurements. Therefore the data presented was for Control and Leu^{27}IGF-2 groups.

Fetal arterial blood gas measurements

Fetal arterial blood samples (1 ml) were collected daily to measure P_{O_2}, P_{CO_2}, pH, oxygen saturation (S_{aO_2}) and haemoglobin (Hb) using an ABL CO-OX 80 FLEX analyser (Radiometer Pacific Pty Ltd, Mount Waverley, Australia) and temperature corrected to 39°C.

Fetal blood pressure and heart rate measurements

Fetal carotid artery, jugular vein and amniotic catheters were connected to displacement transducers and a quad-bridge amplifier (ADInstruments, Australia, Bella Vista, NSW) to record fetal arterial blood pressure and amniotic fluid pressure, for 1 day prior to infusion and during the 4 day infusion period. Each day, blood pressure was measured in the left circumflex coronary artery for 10 min to ensure patency of the catheter. All data were digitized and recorded at a rate of 1000 Hz using LabChart 7 (ADInstruments). Blood pressure data were selected over 20 min each morning and averaged for analysis. Fetal arterial blood pressure was calculated by subtracting amniotic fluid pressure from fetal resting arterial blood pressure. Fetal systolic (SBP) and diastolic (DBP) blood pressures were calculated as the mean maximum and minimum pressures, respectively. Mean arterial pressure (MAP) was calculated using the formula $MAP = DBP + 0.4(SBP - DBP)$ (Morrison *et al.* 1997; Danielson *et al.* 2005). Heart rate (HR) was derived from the blood pressure signal using the beats per minute function in LabChart 7 (ADInstruments, Australia). Rate pressure product ($(RPP) = SBP \times HR$) was used as an indirect measure of myocardial mechanical work (Hawkins *et al.* 2000).

Postmortem and tissue collection

All animals were humanely killed with an overdose of sodium pentobarbitone (Lethobarb, Lyppard) at 132.2 ± 0.2 days gestation. Fetuses were delivered by hysterotomy and the fetal and heart weights recorded. Before dissection of the heart, the placement of the catheter tip in the left circumflex coronary artery was confirmed by injecting methylene blue dye. A sample of the left ventricle that had been perfused by the left coronary circumflex artery was removed and snap frozen. The remainder of the heart was then dissociated to isolate cardiomyocytes as previously described (Sundgren *et al.* 2003; Morrison *et al.* 2007; Wang *et al.* 2011).

Determination of proportion of mononucleated cardiomyocytes, their proliferation rate and the size of binucleated cardiomyocytes

The relative proportion of mononucleated and binucleated cardiomyocytes was determined for a total of 300 cardiomyocytes (Morrison *et al.* 2007). The length, width and area of binucleated cardiomyocytes were determined as previously described (Wang *et al.* 2011). Immunohistochemistry was performed with a Ki67 antibody (Dianova, Hamburg, Germany) on fixed cells and analysed as previously described (Giraud *et al.* 2006; Morrison *et al.* 2007).

Protein extraction and Western blotting

Left and right ventricle tissue (~50 mg) was sonicated separately (John Morris Scientific, Australia) in 500 μl of sonication buffer (MilliQ water, 1 mM Tris HCl pH 8, 5 M NaCl, 1% NP-40, 1 mM sodium orthovanadate, 30 mM NaF, 10 mM sodium tetrapyrophosphate, 10 mM EDTA, a protease inhibitor tablet). The suspensions were centrifuged (Eppendorf Centrifuge 5415, Crown Scientific, Kingston, Australia) for 14 min at 14,300 *g* and 4°C. Protein content of extracts was determined using a Micro BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific Inc., USA) as previously described (Wang *et al.* 2011).

The abundance of the proteins present in samples between each well and the linearity of the density measurements were tested as previously described (Muhlhausler *et al.* 2009). Protein was transferred onto a PolyScreen polyvinylidene difluoride hybridization transfer membrane (PerkinElmer, USA). The membranes were blocked with 5% BSA in Tris-buffered saline with 1% Tween (TBS-T) for 1 h at room temperature. The membranes were then washed 3 times for 5 min with TBS-T and then incubated with the respective primary antibody. These included proliferation markers: proliferating cell nuclear antigen (PCNA, Cell Signaling Technology, Inc., Danvers, MA, USA), cyclin-dependent kinase inhibitor 21 (p21, Cell Signalling Technology), p27 (Cell Signaling Technology); apoptosis markers: calcineurin A (Abcam, Cambridge, UK), nuclear factor of activated T-cells (NFATc3, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), phospho-NFATc3 (Ser265; Santa Cruz Biotechnology), cleaved-caspase 3 (Cell Signaling Technology); proteins in the Gαq signalling pathways: CaMKII (Cell Signaling Technology), phospho-CaMKII (Thr286; Santa Cruz Biotechnology), PKC-α (Cell Signaling Technology), phospho-PKC-α (Thr638; Santa Cruz Biotechnology), histone deacetylases (HDAC) 4 (Cell Signaling Technology), HDAC 5 (Cell Signaling Technology), phospho-HDAC 4(Ser632)/HDAC 5 (Ser498; Cell Signaling Technology), downstream proteins of both the IGF-1R (Lavandero *et al.* 1998) and Gαq (Yue *et al.* 2000) pathway: Erk1/Erk2 (Cell Signaling Technology) and phospho-Erk1/2 (Thr202/Tyr204; Cell Signaling Technology); cardiomyocyte hypertrophy markers: atrial natriuretic peptide (ANP; Abcam); contractility markers: sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA; Pierce, Thermo Fisher Scientific), phospho-troponin I (cardiac; Ser23/24; Cell Signaling Technology); proteins in the Gαs signalling pathways: PKA $\alpha/\beta/\gamma$ catalytic subunits (Santa Cruz Biotechnology), phospho-PKA $\alpha/\beta/\gamma$ catalytic subunits (Thr198; Santa Cruz Biotechnology), phospho-PKAII α regulatory subunit (Ser96; Santa Cruz Biotechnology), cAMP response element-binding (CREB; Cell Signaling Technology) and phospho-CREB (Ser133; Cell Signalling Technology) overnight with agitation at 4°C. These membranes were washed 3 times for 5 min in TBS-T. Membranes were then incubated with their respective horse radish peroxidase labelled secondary IgG antibodies for 1 h. The membranes were washed with TBS-T 3 times for 5 min and the antigen–antibody complexes were detected by enhanced chemiluminescence. Protein abundance was quantified by densitometry using software AlphaEaseFC 4.0 (Cell Biosciences/Protein Simple, Santa Clara, CA, USA). Protein abundance was not measured in all animals for all proteins because background interference on some blots prevented analysis of some samples.

Cell culture protocol

Cardiomyocytes from the left and right ventricle of hearts ($n = 5$) from a separate group of fetuses that did not undergo surgery were isolated at 132.2 ± 0.2 days gestation and cultured in serum-free culture medium with 4×10^5 cells per coverslip in a final volume of 2 ml per culture plate well and incubated for 24 h before drug treatment commenced as previously described (Sundgren *et al.* 2003; Wang *et al.* 2012).

PKA involvement in Leu^{27}IGF-2 induced hypertrophy. Serum-free medium was replaced with either 2 ml of fresh serum-free medium, or 2 ml of medium with serum (to increase cardiomyocyte size), or 2 ml of serum-free medium containing an analogue of IGF-2 which binds to the IGF-2R but not IGF-1R (1 nM Leu^{27}IGF-2; Novozymes, GroPep) (Sakano *et al.* 1991; Oh *et al.* 1993) and/or a PKA inhibitor (2.5 μM H-89; Cell Signaling Technology) (Chijiwa *et al.* 1990; Zou *et al.* 1999). For cardiomyocytes that were exposed to both inhibitor and agonist, the inhibitor was added 30 min prior to the addition of the agonist. After 48 h incubation, cardiomyocytes were fixed and stained and analysed as previously described (Sundgren *et al.* 2003; Wang *et al.* 2012).

Statistical analysis

Basal blood pressure was calculated for each fetus by averaging every 30 s of a 20 min recording period between 09.00 and 10.00 h. The effects of treatment on blood pressure and blood gases were compared using multifactorial ANOVA with repeated measures (STATA 11, StataCorpLP, College Station, TX, USA). All cultured cardiomyocyte cross-sectional area measurements were expressed relative to the serum-free culture medium control for each animal. Comparisons were tested with a one way ANOVA (factor = treatment) followed by Duncan's *post hoc* test using SPSS 18 for Windows. For all cardiomyocyte size and protein expression measures, multiple comparisons were tested with ANOVA using SPSS 18 for Windows. Data were presented as the mean ± SEM. A probability of less than 5% ($P < 0.05$) was considered statistically significant.

Results

Leu^{27}IGF-2 infusion into the left circumflex coronary artery did not change fetal blood gases, blood pressure or organ/body weight of fetal lambs

The infusion of Leu^{27}IGF-2 into the left circumflex coronary artery of fetal sheep did not cause any

Table 1. Daily arterial blood gas values for control and Leu^{27}IGF-2-treated fetuses before and during the infusion period

		Day of infusion					
		−1 day	0 day (prior to infusion)	+1 day	+2 days	+3 days	+4 days
P_{aO_2} (mmHg)	Control	18.8 ± 1.3 (15)	20.1 ± 1.2 (14)	19.0 ± 1.2 (15)	19.3 ± 1.2 (14)	18.8 ± 1.2 (14)	19.1 ± 1.5 (10)
	Leu^{27}IGF-2	18.7 ± 1.1 (10)	18.6 ± 1.3 (10)	19.8 ± 0.9 (10)	19.5 ± 1.2 (10)	19.7 ± 0.9 (10)	20.5 ± 0.9 (10)
P_{aCO_2} (mmHg)	Control	44.9 ± 1.1 (15)	48.3 ± 0.8 (14)	49.6 ± 1.1 (15)	50.9 ± 1.1 (14)	52.0 ± 1.3 (14)	53.2 ± 1.8 (10)
	Leu^{27}IGF-2	45.9 ± 1.4 (10)	47.0 ± 1.4 (10)	47.7 ± 1.6 (10)	49.6 ± 1.1 (10)	49.5 ± 1.3 (10)	48.5 ± 1.2 (10)
pH	Control	7.380 ± 0.006 (15)	7.385 ± 0.008 (14)	7.379 ± 0.004 (15)	7.383 ± 0.013 (14)	7.366 ± 0.004 (14)	7.360 ± 0.007 (10)
	Leu^{27}IGF-2	7.366 ± 0.015 (10)	7.393 ± 0.008 (10)	7.392 ± 0.005 (10)	7.385 ± 0.003 (10)	7.379 ± 0.003 (10)	7.375 ± 0.005 (10)
S_{aO_2} (%)	Control	59.7 ± 4.0 (15)	63.3 ± 3.4 (14)	57.5 ± 3.4 (15)	57.5 ± 3.7 (14)	56.5 ± 3.4 (14)	51.8 ± 4.8 (10)
	Leu^{27}IGF-2	64.2 ± 3.7 (10)	65.43 ± 3.8 (10)	67.5 ± 3.3 (10)	63.8 ± 3.6 (10)	64.5 ± 3.2 (10)	66.0 ± 2.8 (10)
Hb (g l^{-1})	Control	92.0 ± 3.4 (15)	92.1 ± 2.7 (13)	91.6 ± 3.2 (15)	91.5 ± 3.2 (13)	91.5 ± 3.1 (14)	91.1 ± 3.0 (9)
	Leu^{27}IGF-2	74.4 ± 6.4 (10)	73.5 ± 6.5 (10)	72.8 ± 6.3 (10)	74.4 ± 5.2 (10)	76.5 ± 4.9 (10)	79.0 ± 4.5 (10)

Values are mean ± SEM (*n*). P_{aO_2}, fetal arterial partial pressure of oxygen; P_{aCO_2}, fetal arterial partial pressure of carbon dioxide; S_{aO_2}, oxygen saturation; Hb, haemoglobin.

Table 2. Mean arterial blood pressure, heart rate and rate pressure product over a 20 min period on each day of the experimental protocol in control and Leu^{27}IGF-2-infused sheep fetuses

		Day of infusion					
		−1 day	0 day (prior to infusion)	+1 days	+2 days	+3 days	+4 days
SBP (mmHg)	Control	44.7 ± 4.7 (8)	42.5 ± 2.6 (11)	41.7 ± 3.9 (12)	38.9 ± 2.8 (12)	39.2 ± 2.5 (13)	39.8 ± 3.3 (9)
	Leu^{27}IGF-2	44.9 ± 4.3 (8)	47.0 ± 3.6 (9)	49.6 ± 3.2 (8)	49.5 ± 2.9 (8)	50.9 ± 3.7 (8)	48.9 ± 3.3 (9)
DBP (mmHg)	Control	30.0 ± 2.7 (8)	29.8 ± 1.6 (11)	29.2 ± 2.2 (12)	28.7 ± 2.0 (12)	29.8 ± 1.7 (13)	32.2 ± 3.0 (9)
	Leu^{27}IGF-2	31.6 ± 3.6 (8)	32.5 ± 3.3 (9)	34.8 ± 3.4 (8)	36.5 ± 3.2 (8)	38.0 ± 3.6 (8)	36.7 ± 2.9 (9)
MAP (mmHg)	Control	35.9 ± 3.4 (8)	34.8 ± 1.9 (11)	34.2 ± 2.9 (12)	32.8 ± 2.2 (12)	33.6 ± 1.9 (13)	35.3 ± 3.1 (9)
	Leu^{27}IGF-2	36.9 ± 3.8 (8)	36.7 ± 3.7 (9)	40.7 ± 3.2 (8)	41.7 ± 3.0 (8)	43.2 ± 3.2 (8)	41.6 ± 3.0 (9)
HR (beats min^{-1})	Control	168.3 ± 3.8 (8)	172.8 ± 4.6 (11)	171.2 ± 4.0 (12)	171.8 ± 3.9 (12)	172.5 ± 2.6 (13)	167.4 ± 4.0 (9)
	Leu^{27}IGF-2	179.5 ± 6.7 (8)	180.5 ± 4.4 (9)	176.7 ± 3.9 (8)	174.9 ± 4.0 (8)	170.3 ± 3.5 (8)	159.9 ± 2.2 (9)
RPP (mmHg× beats min^{-1})	Control	7504.6 ± 775.5 (8)	7302.6 ± 435.4 (11)	7126.7 ± 664.1 (12)	6651.2 ± 468.5 (12)	6756.5 ± 427.5 (13)	6706.6 ± 670.9 (9)
	Leu^{27}IGF-2	8149.4 ± 921.7 (8)	8493.7 ± 680.7 (9)	8774.1 ± 605.1 (8)	8698.4 ± 626.2 (8)	8668.3 ± 638.2 (8)	7826.6 ± 558.3 (9)

Values are mean ± SEM (*n*). SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate; RPP, rate pressure product.

significant change in arterial blood gases (Table 1), blood pressure, heart rate or rate pressure product (Table 2), either before or during the infusion period, when compared to the Control group. There were also no changes in fetal body weight, absolute organ weight or relative organ weight, following Leu^{27}IGF-2 infusion, when compared to the Control group (Table 3).

Table 3. Fetal body, absolute and relative organ weights at 130–134 days gestation

	Control ($n = 15$)	Leu^{27}IGF-2 ($n = 10$)
Gestational age (days)	131.9 ± 0.3	132.7 ± 0.4
Body weight (kg)	3.5 ± 0.2	3.8 ± 0.1
Absolute organ weight (g)		
Heart	25.9 ± 1.2	28.3 ± 1.2
Kidney	21.2 ± 1.2	21.6 ± 1.5
Liver	94.9 ± 6.2	109.2 ± 7.4
Pericardial fat	4.2 ± 0.3	5.0 ± 0.3
Organ weight relative to body weight (g kg^{-1})		
Heart	7.3 ± 0.2	7.4 ± 0.3
Kidney	6.0 ± 0.2	5.6 ± 0.3
Liver	26.7 ± 1.1	28.3 ± 1.5
Pericardial fat	1.2 ± 0.1	1.3 ± 0.1

Values are mean ± SEM.

Leu^{27}IGF-2 infusion into the left circumflex coronary artery of fetal sheep did not alter the amount of cardiomyocyte proliferation, apoptosis or binucleation in the left ventricle

Leu^{27}IGF-2 infusion did not alter the proliferation of cardiomyocytes in the left ventricle, when compared to the Control group, as defined by the proliferation markers Ki67 (a nuclear protein, which is associated with ribosomal RNA transcription; Fig. 1*A*), PCNA (a marker of cells in S-phase; Fig. 1*B*), p21 (an inhibitor of cell cycle progression; Fig. 1*C*) and p27 (an inhibitor of cyclin dependent kinase; Fig. 1*D*). Leu^{27}IGF-2 infusion did not cause any change in the amount of apoptosis in the left ventricle, when compared to the Control group, as defined by calcineurin A (Fig. 2*A*), its NFATc3 signalling pathway (Fig. 2*B* and *C*) or caspase 3 cleavage (Fig. 2*D*).

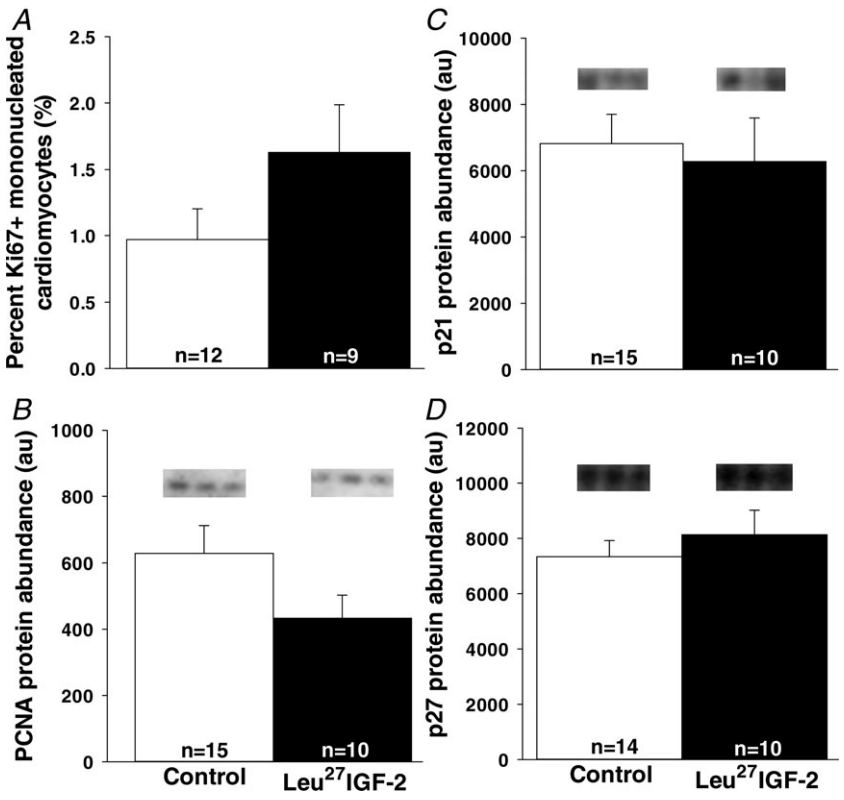

Figure 1. Activation of cardiac IGF-2R via Leu^{27}IGF-2 infusion into the coronary artery did not increase cardiomyocyte proliferation
There was no change in markers of proliferation such as the percentage of Ki67+ mononucleated cardiomyocytes (*A*) or PCNA (*B*) and regulators of cell cycle at G_1 phase such as p21 (*C*) or p27 (*D*) protein abundance in the left ventricle of control compared with Leu^{27}IGF-2-infused fetuses. Representative blots from 3 animals in each group for each protein. Sample size for each group is indicated in the bar.

There was also no change in the percentage mononucleated cardiomyocytes in the left ventricle (Fig. 3*A*), septum (Fig. 3*B*) or right ventricle (Fig. 3*C*) following Leu^{27}IGF-2 infusion, when compared with the Control fetuses.

Leu^{27}IGF-2 infusion into the left circumflex coronary artery in fetal sheep caused an increase in the cross-sectional area of cardiomyocytes in the left ventricle

The infusion of Leu^{27}IGF-2 resulted in increased cross-sectional area of binucleated cardiomyocytes in both the

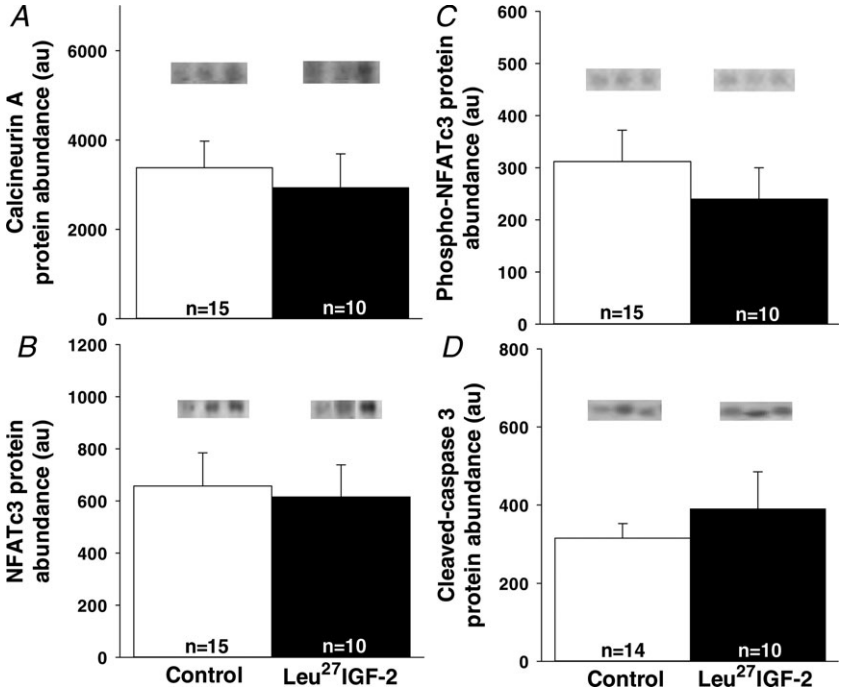

Figure 2. Activation of cardiac IGF-2R via Leu^{27}IGF-2 infusion into the coronary artery did not induce cardiomyocyte apoptosis
There were no changes in the protein abundance of calcineurin A (*A*), NFATc3 (*B*), phospho-NFATc3 (*C*) and cleaved-caspase 3 (*D*) in the left ventricle of control compared with Leu^{27}IGF-2-infused fetuses. Representative blots from 3 animals in each group for each protein. Sample size for each group is indicated in the bar.

left ventricle (Fig. 3*D*) and the septum (Fig. 3*E*), when compared with control fetuses. There was, however, no change in the area of binucleated cardiomyocytes in the right ventricle, following the infusion of Leu^{27}IGF-2 (Fig. 3*F*).

The increased left ventricular cardiomyocyte hypertrophy in response to Leu^{27}IGF-2 infusion was not associated with Gαq signalling

There were no differences in CaMKII (Fig. 4*A*), phospho-CaMKII (Fig. 4*B*), HDAC 4 (Fig. 4*C*), phospho-HDAC 4 (Fig. 4*D*), PKC-α (Fig. 4E), phospho-PKC-α (Fig. 4F), HDAC 5 (Fig. 4*G*) or phospho-HDAC 5 (Fig. 4*H*) protein detected in Control and Leu^{27}IGF-2 infused fetuses in either the left or the right ventricle. There were also no differences in the amount of ERK (Control, 53107 ± 2534 au; Leu^{27}IGF-2 infused, 58068 ± 3731 au) and phospho-ERK (Control, 2180 ± 519 au; Leu^{27}IGF-2 infused, 2098 ± 667 au) in the left ventricle (or the right ventricle, data not shown) for Control and Leu^{27}IGF-2 infused fetuses.

Leu^{27}IGF-2 infusion into the left circumflex coronary artery of fetal sheep induced the expression of markers of cardiomyocyte hypertrophy

The increased area of cardiomyocytes in the left ventricle of Leu^{27}IGF-2 infused fetuses was associated with an increase in the amount of ANP (Fig. 5*A*), and a concomitant decrease in the protein expression of SERCA (Fig. 5*C*) and phospho-troponin I (Fig. 5*E*), when compared to the control fetuses.

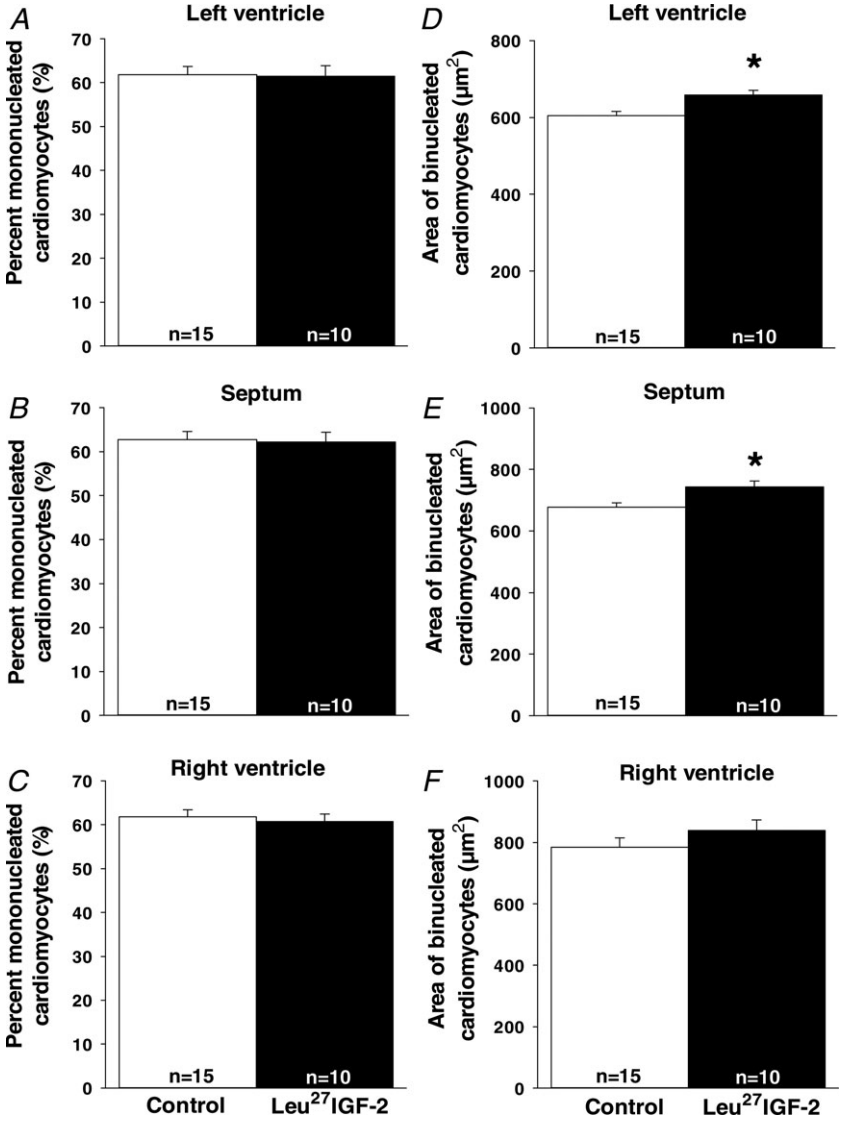

Figure 3. Activation of cardiac IGF-2R via Leu^{27}IGF-2 infusion into the coronary artery did not change the percentage of mononucleated cardiomyocytes but did increase the area of binucleated cardiomyocytes in only the left ventricle
There were no changes in the proportion of mononucleated cardiomyocytes in the left ventricle (*A*), septum (*B*) or right ventricle (*C*) in control and Leu^{27}IGF-2-infused fetuses. There was an increase in the area of binucleated cardiomyocytes in the left ventricle (*D*) and septum (*E*), areas perfused by the left circumflex coronary artery, but not the right ventricle (*F*) in control and Leu^{27}IGF-2-infused fetuses. Sample size for each group is indicated in the bar. *Significantly different from control fetuses ($P < 0.05$).

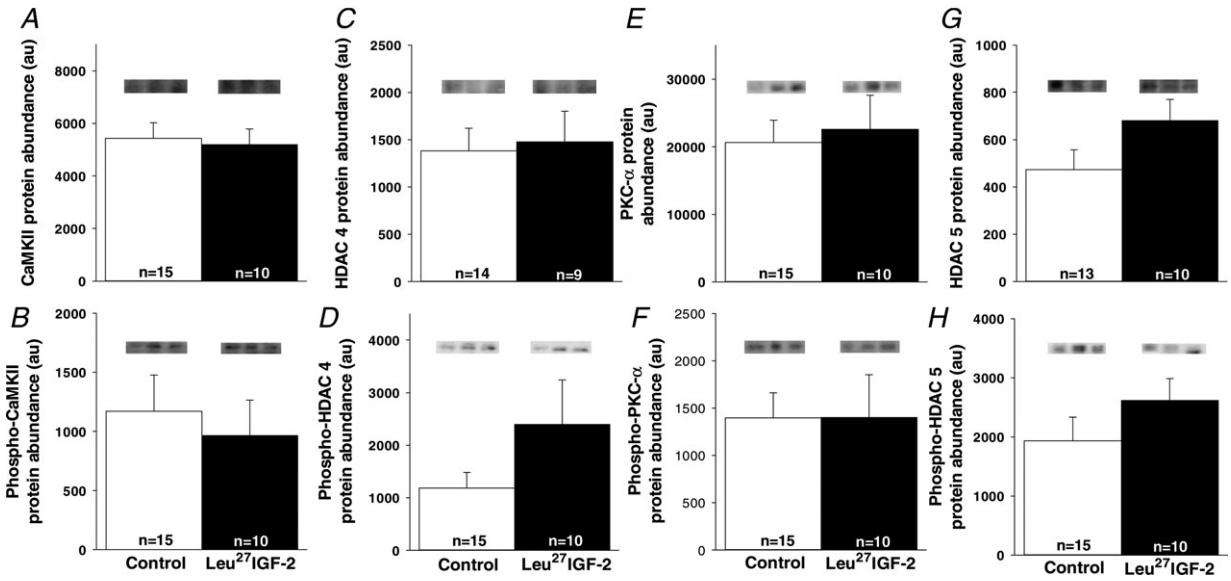

Figure 4. The increase in the area of binucleated cardiomyocytes in the left ventricle was not stimulated through activation of the Gαq signalling pathway
There were no differences in the amounts of CaMKII (*A*), phospho-CaMKII (*B*), HDAC 4 (*C*), phospho-HDAC 4 (*D*), PKC-α (*E*), phospho-PKC-α (*F*), HDAC 5 (*G*) and phospho-HDAC 5 (*H*) proteins in control compared with Leu^{27}IGF-2-infused fetuses. Representative Western blots from 3 animals in each group for each protein. Sample size for each group is indicated in the bar.

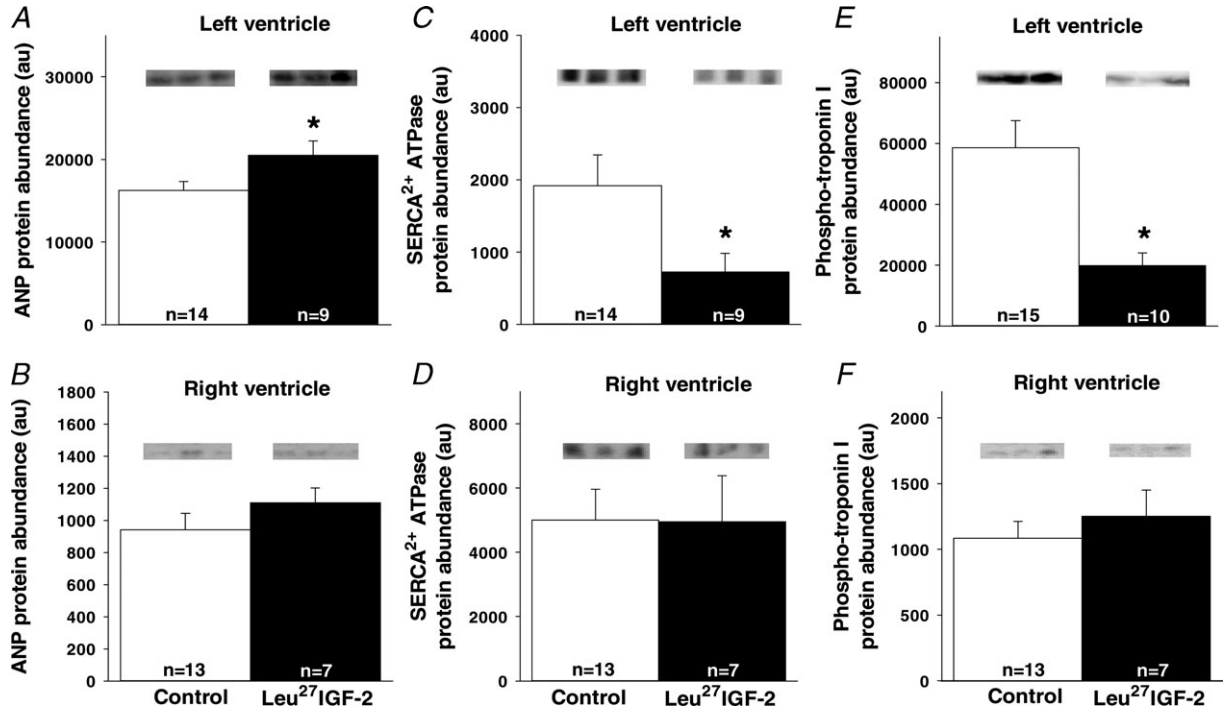

Figure 5. Activation of cardiac IGF-2R via Leu^{27}IGF-2 infusion into the coronary artery causes significant changes in the protein expression of markers of cardiac hypertrophy
The amount of ANP protein (*A*) was significantly increased in the left ventricle of Leu^{27}IGF-2-infused fetuses, while SERCA (*C*) and phospho-troponin I (*E*) were decreased. There were no changes in the amount of ANP (*B*), SERCA (*D*) and phospho-troponin I (*F*) proteins in the right ventricle of hearts from control compared with Leu^{27}IGF-2-infused fetuses. Representative Western blots from 3 animals in each group for each protein. Sample size for each group is indicated in the bar. *Significantly different from control fetuses ($P < 0.05$).

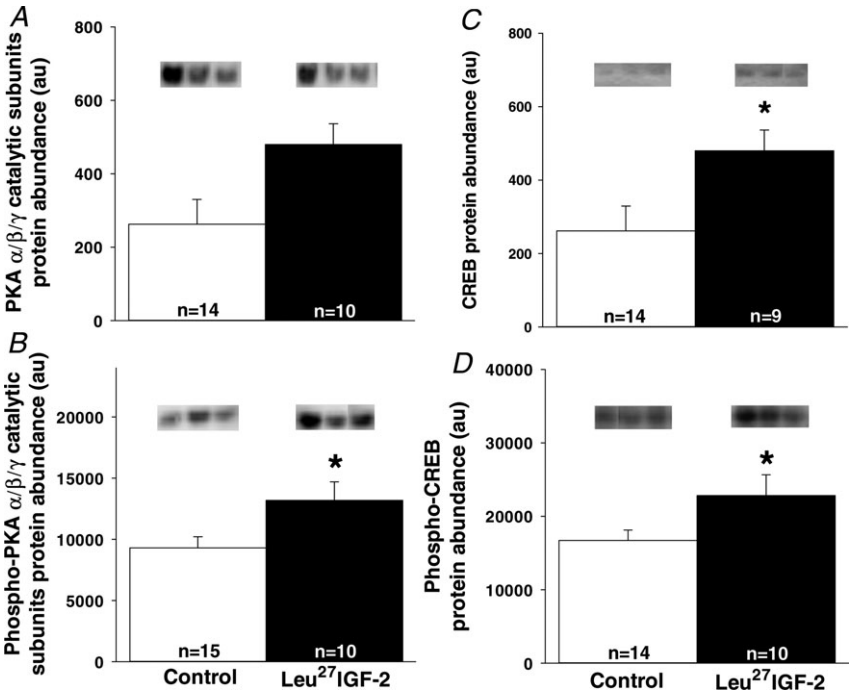

Figure 6. Cardiac activation of IGF-2R induced cardiomyocyte hypertrophy is mediated by the Gαs signalling pathway in normally grown fetuses
No change in the amount of PKA$\alpha/\beta/\gamma$ catalytic subunits (*A*) was observed in control and Leu^{27}IGF-2-infused fetuses. There were increased amounts of left ventricle phospho-PKA$\alpha/\beta/\gamma$ catalytic subunits (*B*), CREB (*C*) and phospho-CREB (*D*) in Leu^{27}IGF-2-infused fetuses. Representative blots from 3 animals in each group for each protein. Sample size for each group is indicated in the bar. *Significantly different from control fetuses ($P < 0.05$).

The increased left ventricular cardiomyocyte hypertrophy in response to Leu^{27}IGF-2 infusion was associated with Gαs signalling

Although there was no difference in the amount of PKA $\alpha/\beta/\gamma$ catalytic subunits (Fig. 6*A*), there was a significant increase in the amount of phospho-PKA $\alpha/\beta/\gamma$ catalytic subunits (Fig. 6*B*) in the left, but not right, ventricle (data not shown for right ventricle) of Leu^{27}IGF-2 infused compared with control fetuses. There was no difference in the amount of phospho-PKAII α regulatory subunit for control (749 ± 62 au) and Leu^{27}IGF-2 infused (738 ± 28 au) fetuses. There were significant increases in the amount of CREB (Fig. 6*C*) and phospho-CREB (Fig. 6*D*) in the left ventricle, but not the right ventricle (data not shown), for Leu^{27}IGF-2 infused compared to control fetuses. There was no change in the size of mononucleated cardiomyocyte after treatment with Leu^{27}IGF-2 (data not shown). Leu^{27}IGF-2 treatment of binucleated cardiomyocytes significantly increased the cell cross-sectional area relative to cardiomyocytes that had been cultured in serum-free medium. The PKA inhibitor H-89 (Chijiwa *et al.* 1990; Zou *et al.* 1999) prevented the increase in cardiomyocyte area caused by Leu^{27}IGF-2 (Fig. 7).

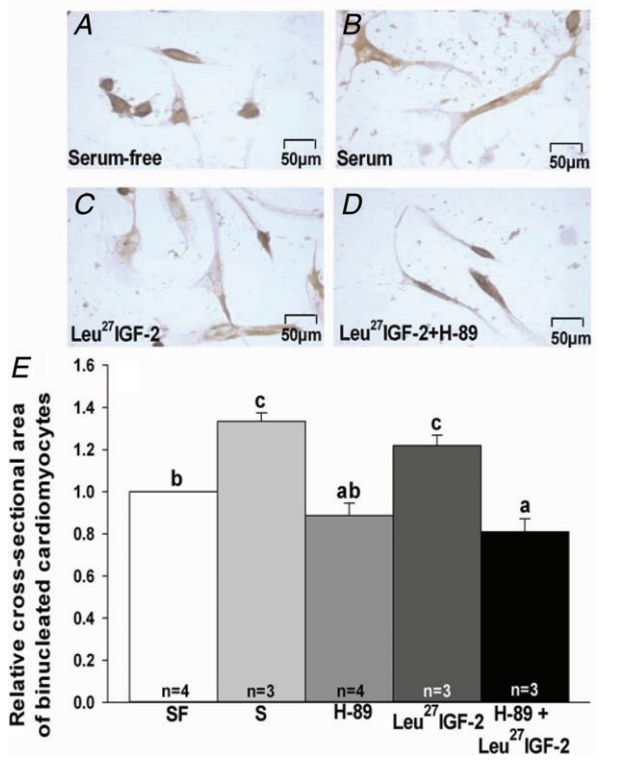

Figure 7. Activation of IGF-2R-induced cardiomyocyte hypertrophy in binucleated cardiomyocytes that is PKA dependent
Images of fetal sheep cardiomyocytes treated with serum-free (*A*), serum (*B*), Leu^{27}IGF-2 (*C*) and Leu^{27}IGF-2 + H-89, a specific inhibitor of PKA (Chijiwa *et al.* 1990; Zou *et al.* 1999) (*D*) (scale bar for all panels, 50 μm). Results (*E*) were expressed as the mean ± SEM. Different letters denote significant differences between treatment groups ($P < 0.05$). SF, serum-free; S, serum.

Discussion

The role of the IGF-2R and the signalling pathways that it activates during cardiac development are not well established. This is the first study to specifically activate the cardiac IGF-2R signalling pathways during fetal life, in an animal model where cardiomyocyte maturation occurs in

late gestation, as in the human (Woodcock & Matkovich, 2005). Our study demonstrated that cardiac IGF-2R activation resulted in hypertrophy via PKA activation that was independent of CaMKII, PKC or ERK signalling. Cardiac IGF-2R activation, however, did not result in cardiomyocyte proliferation, binucleation or apoptosis. This study places the IGF-2R-Gαs interaction as a potential contributor to cardiomyocyte hypertrophy.

The size of cardiomyocytes can be affected by changes in mechanical load (Jonker *et al.* 2007*a*); for example, right ventricular overload results in an increase in the percentage and size of binucleated cardiomyocytes (Barbera *et al.* 2000). The increase in cardiomyocyte size observed in this study was not due to changes in mechanical load because the intra-cardiac infusion of Leu^{27}IGF-2 did not change after load, heart rate or cardiac work load. Thus, the infusion of Leu^{27}IGF-2 into the left circumflex coronary artery to activate cardiac IGF-2R signalling demonstrated a specific effect on left ventricular cardiomyocyte hypertrophy, without any apparent systemic effects.

The increase in cardiomyocyte size in the absence of an increase in heart weight is consistent with our findings in the intrauterine growth restriction (IUGR) fetus (Morrison *et al.* 2007) where there is an increase in cardiac IGF-2R gene expression (Wang *et al.* 2011). In both cases, this may be due to a decrease in cardiomyocyte number. We did not observe a change in proliferation or apoptosis but it should be noted that the Leu^{27}IGF-2 treatment was specific to the left ventricle and septum. We were not able to weigh the ventricle and although there was no change in relative heart weight (right ventricle, left ventricle, septum, atria and some vessels), it is not known if there was an increase in relative left ventricular weight. Furthermore, to determine cardiomyocyte number, the weight of the left ventricle would be required (Brüel *et al.* 2005; Bensley *et al.* 2010); however, we do not have this information because we isolated the cardiomyocytes and thus did not dissect the left ventricle prior to isolating the cardiomyocytes. In addition, it was suggested that hypertrophic growth of binucleated cardiomyocytes plays a minor role in increasing heart weight during late gestation of fetal sheep (Jonker *et al.* 2007*b*), which may explain the absence of increase heart weight.

Pathological cardiac hypertrophy can be induced through the activation of IGF-2R signalling, via phospho-PKC-α and/or CaMKII, which are downstream of Gαq (Chu *et al.* 2008). While IGF-2R signalling can induce apoptosis in cultured neonatal rat ventricular myocytes via Gαq and calcineurin signalling (Chu *et al.* 2009*b*), there was no upregulation of proteins involved in Gαq or of apoptosis signalling pathways, following Leu^{27}IGF-2 infusion. There were, however, changes in proteins that are

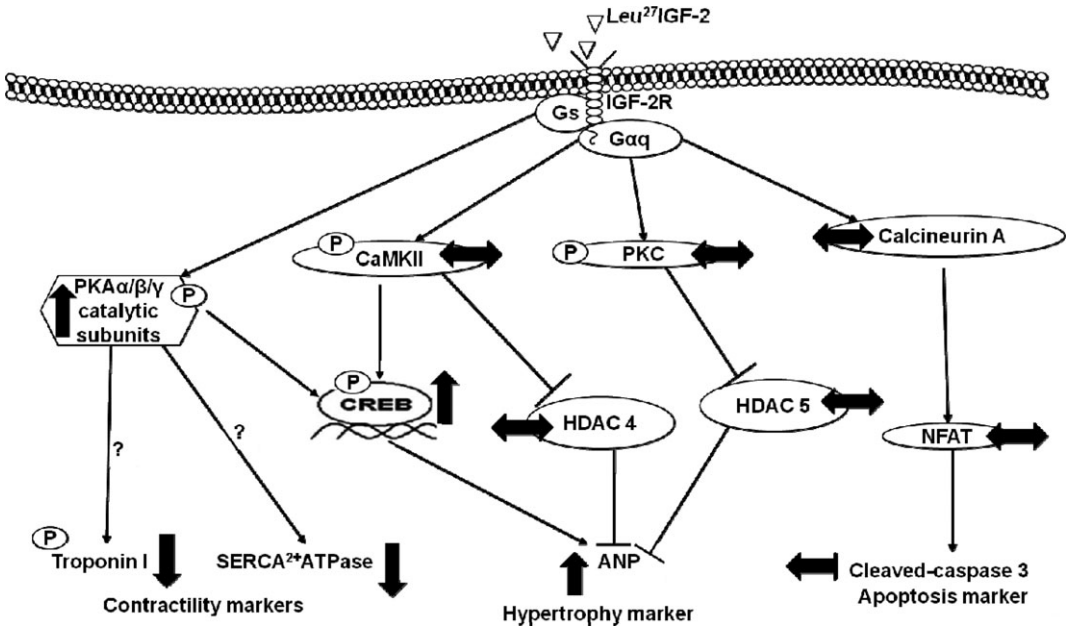

Figure 8. Infusion of Leu^{27}IGF-2 into the left circumflex coronary artery leads to cardiomyocyte hypertrophy in the normally grown fetus in late gestation
Activation of the IGF-2R with Leu^{27}IGF2 *in vivo* did not change the amount of downstream proteins in the Gαq signalling pathway. However, there was an upregulation of the Gαs pathway as indicated by increased amounts of phospho-PKA $\alpha/\beta/\gamma$ catalytic subunits. ↑, increased protein expression compared with control fetuses; ↓, decreased protein expression compared with control fetuses; ↔, no changes in protein expression compared with control fetuses; p, protein phosphorylation.

markers of cardiac pathological hypertrophy, including ANP, SERCA and phospho-troponin I. The increase in ANP and decrease in SERCA and phospho-troponin I suggested that an alternative signalling pathway may be involved. All of these markers can be affected by PKA, a protein that responds to Gαs (Skalhegg & Tasken, 2000). Gαs signalling has been linked to cardiac hypertrophy through its interaction with the β-adrenergic receptor (Barki-Harrington *et al.* 2004).

Gαs signals through the activation of adenylyl cyclase, resulting in the accumulation of cAMP, in turn resulting in activation of PKA (Skalhegg & Tasken, 2000); the PKA substrate then mediates a range of gene expression changes in response to cAMP, which can play an important regulatory role in cardiac function (Gonzalez *et al.* 1989). The IGF-2R has previously been linked to the Gi_2 (Nishimoto, 1993) and Gαq (Chu *et al.* 2008) subunit signalling, where both were associated with cardiac hypertrophy (Böhm *et al.* 1994; D'Angelo *et al.* 1997). In an *in vitro* cardiomyocyte study of apoptosis, IGF-2R activation resulted in decreased PKA protein expression (Chu *et al.* 2009*a*), but no previous studies have shown a link between the IGF-2R and cardiomyocyte hypertrophy involving Gαs signalling. Therefore our finding that IGF-2R activation can cause cardiomyocyte hypertrophy *in vivo*, in a Gαs signalling-dependent manner, is novel and this signalling could have potential implications for cardiac pathogenesis. Together, these findings suggest that IGF-2R activation can induce cardiomyocyte hypertrophy either through the Gi_2, Gαq or Gαs signalling pathways. Further studies investigating the trigger (e.g. glucose, oxygen, calcium levels) that regulates the specific activation of these different IGF-2R downstream signalling pathways are required.

An increase in cAMP and the phosphorylation of PKA leads to an increased amount of phospho-troponin I and SERCA and is suggestive of enhanced cardiac contractile ability (Noland *et al.* 1995). We have, however, observed a discrepancy between PKA and phospho-troponin I protein expression. A similar finding was also reported in 12- to 13-week-old spontaneously hypertensive rats, possibly due to local regulation of PKA activity or the existence of multiple subcellular pools of cAMP (Bokník *et al.* 2001). The discrepancy between phospho-troponin I and cAMP may be due to the dissociation of the total cellular cAMP levels and downstream activation of PKA-dependent substrate phosphorylation (McConnell *et al.* 1998). In addition, many studies have suggested that compartmentalization of cAMP or PKA in cardiomyocytes may account for the lack of correspondence between increased total cellular cAMP and phospho-PKA substrate (Buxton & Brunton, 1983). Overall, our findings suggest that cardiac IGF-2R activation may alter important regulators of cardiac contractility and relaxation. It is possible that these changes precede the onset of chronic heart failure and this also requires further investigation, for example investigating cardiac Ca^{2+} sensitivity for the contractile apparatus and maximum Ca^{2+}-activated force (Posterino *et al.* 2011).

In summary, Leu^{27}IGF-2 infusion into the left circumflex coronary artery activated cardiac IGF-2R and induced Gαs signalling that was associated with cardiomyocyte hypertrophy (Fig. 8). This is significant as it places the interaction between the IGF-2R and the Gαs signalling pathway as a potential mechanism that can contribute to cardiomyocyte growth in fetal life, but which may result in pathological cardiomyocyte hypertrophy in postnatal life. This link may be important to understand why individuals who are born with low birth weight have an increased vulnerability to cardiovascular disease in adult life (Barker, 1995; Rich-Edwards *et al.* 1997).

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Author contributions

K.W.C.W., D.A.B. and J.L.M. were responsible for the conception and design of the experiments. K.W.C.W., K.L.T. and J.L.M. were each involved in data acquisition. K.W.C.W., D.A.B. and J.L.M. were involved in analysis and interpretation of the data. K.W.C.W., D.A.B. and J.L.M. drafted the article and all authors contributed to the final version.

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