

# The transition from fetal growth restriction to accelerated postnatal growth: a potential role for insulin signalling in skeletal muscle

B. S. Muhlhausler<sup>1</sup>, J. A. Duffield<sup>1</sup>, S. E. Ozanne<sup>2</sup>, C. Pilgrim<sup>2</sup>, N. Turner<sup>3</sup>, J. L. Morrison<sup>1</sup> and I. C. McMillen<sup>1</sup>

<sup>1</sup>Early Origins of Adult Health Research Group, Sansom Research Institute, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide 5001, Australia

<sup>2</sup>Metabolic Research Laboratories, Institute of Metabolic Science, University of Cambridge, Cambridge, UK

<sup>3</sup>Diabetes and Obesity Research Program, Garvan Institute of Medical Research, 384 Victoria St Darlinghurst, NSW 2010, Australia

A world-wide series of epidemiological and experimental studies have demonstrated that there is an association between being small at birth, accelerated growth in early postnatal life and the emergence of insulin resistance in adult life. The aim of this study was to investigate why accelerated growth occurs in postnatal life after *in utero* growth restriction. Samples of quadriceps muscle were collected at ~140 days gestation (term ~150 days gestation) from normally grown fetal lambs (Control,  $n = 7$ ) and from growth restricted fetal lambs (placentally restricted: PR,  $n = 8$ ) and from Control ( $n = 14$ ) and PR ( $n = 9$ ) lambs at 21 days after birth. The abundance of the insulin and IGF1 receptor protein was higher in the quadriceps muscle of the PR fetus, but there was a lower abundance of the insulin signalling molecule PKC $\xi$  and GLUT4 protein in the PR group. At 21 days of postnatal age, insulin receptor abundance remained higher in the muscle of the PR lamb, and there was also an up-regulation of the insulin signalling molecules, PI3Kinase p85, Akt1 and Akt2 and of the GLUT4 protein in the PR group. Fetal growth restriction therefore results in an increased abundance of the insulin receptor in skeletal muscle, which persists after birth when it is associated with an upregulation of insulin signalling molecules and the glucose transporter, GLUT4. These data provide evidence that the origins of the accelerated growth experienced by the small baby after birth lie in the adaptive response of the growth restricted fetus to its low placental substrate supply.

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**Corresponding author** C. McMillen: Early Origins of Adult Health Research Group, Sansom Research Institute, University of South Australia, Adelaide 5000, Australia. Email: caroline.mcmillen@unisa.edu.au

**Abbreviations** Akt1, protein kinase B;  $\beta$ HAD,  $\beta$ -hydroxyacyl-CoA dehydrogenase; CCO, cytochrome *c* oxidase; CS, citrate synthase; GLUT1, glucose transporter type 1 (insulin independent); GLUT4, glucose transporter type 4 (insulin dependent); HK, hexokinase; IGF1R, insulin like growth factor 1 receptor; IR, insulin receptor; IR<sub>A</sub>, insulin receptor type A; IR<sub>B</sub>, insulin receptor type B; IUGR, intrauterine growth restriction; LDH, lactate dehydrogenase; p-Akt, phosphorylated Akt; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$ ; PFK, phosphofructokinase; PI3Kp85 $\alpha$ , phosphoinositide 3-kinase p85 $\alpha$  subunit; PKC $\xi$ , phosphokinase C  $\xi$  isoform; PPAR $\alpha$ , peroxisome proliferator-activated receptor- $\alpha$ ; PR, placental restriction; SDH, succinate dehydrogenase.

A world-wide series of epidemiological, clinical and experimental studies have demonstrated that there is an association between growing slowly before birth, accelerated growth in early postnatal life and the emergence of insulin resistance, type 2 diabetes and obesity in later life (Barker, 1992; McCance *et al.* 1994; Lithell *et al.*

1996; Leger *et al.* 1997; Bavdekar *et al.* 1999; Jaquet *et al.* 2000; McMillen *et al.* 2005). Infants who are small for gestational age have low circulating insulin (Economides *et al.* 1989, 1991) and insulin-like growth factor-1 (IGF1) (Enzi *et al.* 1981) concentrations at birth, and then undergo a period of accelerated postnatal growth during the first few years of life (Fitzhardinge & Steven, 1972; Albertsson-Wikland *et al.* 1993). This period of accelerated early growth is initially associated with an increased insulin

B. S. Muhlhausler and J. A. Duffield contributed equally to this work.

sensitivity, and precedes the subsequent emergence of insulin resistance (Curhan *et al.* 1996; Whincup *et al.* 1997; Bavdekar *et al.* 1999; Eriksson *et al.* 2001; Levy-Marchal & Jaquet, 2004). Experimental studies using a range of rodent models of intrauterine growth restriction (IUGR), including uteroplacental ligation (Siebel *et al.* 2008), global maternal nutrient restriction (Holemans *et al.* 1996) and maternal low protein diets (Ozanne *et al.* 1996, 2005; Ozanne, 2001) report that prenatal growth restraint is associated with an early phase of enhanced insulin sensitivity, followed by the later emergence of insulin resistance and type 2 diabetes. These animal models have provided important insights into the mechanisms which control the transition from a period of enhanced insulin sensitivity to insulin resistance.

Given that muscle represents the major site of postprandial glucose disposal, it is not surprising that changes in the functional characteristics of muscle fibres during the perinatal period are important in the programming of insulin resistance and type 2 diabetes. The offspring of rats fed a low protein diet during pregnancy have improved glucose tolerance at 3 months of age (Ozanne *et al.* 1996). Importantly, isolated muscle strips from these low-protein animals exhibit enhanced basal and insulin-stimulated glucose uptake, which is associated with a twofold increase in the abundance of insulin receptors in muscle membranes (Ozanne *et al.* 1996). By 15 months of age, however, there is a decrease in insulin sensitivity in skeletal muscle from the group exposed to the low-protein diet *in utero* (Ozanne *et al.* 2003). This impaired insulin action is associated with changes in the expression of the insulin receptor, and a decrease in the abundance of signalling molecules downstream of the insulin receptor, including the zeta ( $\zeta$ )-isoform of protein kinase C, an isoform that is positively involved in GLUT4-mediated glucose transport (Ozanne *et al.* 2003), and the p85 $\alpha$  regulatory subunit of phosphoinositide 3-kinase (Ozanne *et al.* 2005).

Rodent studies have also provided evidence that a reduced capacity for mitochondrial biogenesis and oxidative metabolism in growth restricted offspring may contribute to the impaired insulin sensitivity in the adult. In the uterine artery ligation model, the expression of mitochondrial proteins is lower in IUGR offspring at 3 weeks of postnatal age, suggesting that these offspring have a reduced capacity for oxidative metabolism (Lane *et al.* 2001). Furthermore, the mRNA expression and protein abundance of the master regulator of mitochondrial biogenesis, peroxisome proliferator activated receptor- $\gamma$  co-activator-1 $\alpha$  (PGC-1 $\alpha$ ), is decreased in male, but not female, IUGR offspring (Lane *et al.* 2003). In males, the reduced expression of PGC-1 $\alpha$  was associated with a reduced expression of downstream target genes involved in cellular oxidative metabolism, including mitochondrial transcription factor A (mtTFA)

and cytochrome oxidase (COX) III mRNA (Lane *et al.* 2003; Wadley *et al.* 2008). It would therefore appear that IUGR may programme mitochondrial function in a sexually dimorphic manner, and that a reduced oxidative capacity, which is known to be associated with impaired insulin sensitivity, may contribute to the development of insulin resistance in IUGR offspring.

Whilst these animal models provide insights into the mechanisms which govern the transition from the period of enhanced insulin sensitivity in early life to later insulin resistance, there are no studies which have determined the mechanisms which contribute to the transition from reduced growth *in utero* to enhanced insulin sensitivity in postnatal life, or which have adequately explained why it is that poor growth *in utero* should result in increased insulin sensitivity and accelerated growth after birth.

In the present study, we have utilised an experimental model of fetal growth restriction in the sheep. This model allows the collection of muscle samples from growth restricted animals before birth and during the period of accelerated growth in early postnatal life. The quadriceps muscle was selected because it has been shown that reduced size at birth is predictive of reduced individual and summed skeletal muscle mass, including the quadriceps muscle. Furthermore in placentally restricted lambs there is a positive relationship between the mass of the vastus lateralis and the postnatal growth rate (De Blasio *et al.* 2007). The quadriceps also represents a large muscle group in the postnatal sheep and hence makes a significant contribution to glucose metabolism. The aim of the present study was therefore to determine the impact of intrauterine growth restriction in the sheep on mRNA expression and protein abundance of insulin signalling molecules and activity of oxidative and glycolytic enzymes in quadriceps muscle before birth and in early postnatal life.

## Methods

### Animals and surgery

All procedures were approved by The University of Adelaide Animal Ethics Committee. Thirty-eight singleton bearing Merino ewes were used in this study. For all surgical procedures general anaesthesia was induced by intravenous injection of sodium thiopentone (1.25 g i.v., Pentothal, Rhone Merieux, Pinkenba, Qld, Australia) and maintained with 2.5–4% halothane (Fluothane, ICI, Melbourne, Vic, Australia) in oxygen. The analgesic xylazine (0.02 mg kg<sup>-1</sup>) was administered by intramuscular injection to all ewes in the immediate post-operative period.

Seventeen non-pregnant ewes underwent surgery to remove the majority of endometrial caruncles from the uterus, leaving 3–8 caruncles in each horn in order to

induce experimental restriction of placental and fetal growth (placental restriction group; PR) (Edwards *et al.* 1999; Morrison *et al.* 2007).

From around 109 days (d) gestation all ewes were housed in individual pens in rooms with a 12 h light–dark cycle and a daily temperature of  $\sim 20^{\circ}\text{C}$ . Each pregnant ewe was supplied with a diet which consisted of 1 kg lucerne chaff (85% dry matter, metabolisable energy (ME) content =  $8.3 \text{ MJ kg}^{-1}$ ) and 500 g concentrated pellets containing: straw, cereal, hay, clover, barley, oats, lupins, almond shells, oat husks and limestone (90% dry matter, ME content =  $8.0 \text{ MJ kg}^{-1}$ ; Johnsons and Sons, Kapunda, SA, Australia). The diet was calculated to provide 100% of the energy requirements for the maintenance of a pregnant ewe bearing a singleton fetus, as specified by the Ministry of Agriculture, Fisheries and Food, UK (Aldermann *et al.* 1975). After giving birth to her lamb, the ewe was fed 1 kg of lucerne chaff and 1 kg of concentrated pellets at 09.00–11.00 h each day in order to meet the additional energy demands of lactation (Aldermann *et al.* 1975). If the ewe consumed all of her morning feed ration before 15.00 h, then an additional 1 kg of lucerne chaff was provided to the ewe on that day. After birth, each ewe and her lamb were housed in an individual pen in an indoor housing facility which was maintained at a constant ambient temperature of  $\sim 20^{\circ}\text{C}$  and a 12 h light–dark cycle.

**Fetal study.** Between 109 and 124 d of gestation (term  $\sim 150$  d) surgery was performed on control ( $n = 7$ ) and carunclectomised ( $n = 8$ ) pregnant ewes as previously described (Edwards *et al.* 1999; Morrison *et al.* 2007) for the insertion of vascular catheters in a fetal and maternal jugular vein, fetal carotid artery and amniotic cavity. All ewes and fetuses received antibiotics (procaine penicillin ( $250 \text{ mg ml}^{-1}$ ), dihydrostreptomycin ( $250 \text{ mg ml}^{-1}$ ), and procaine hydrochloride ( $20 \text{ mg ml}^{-1}$ ); Penstrep Illium; Troy Laboratories, Smithfield, NSW, Australia) at the time of surgery. Animals were allowed to recover from surgery for at least 4 d. Fetal arterial blood samples (3.5 ml) were collected between 08.00 and 11.00 h throughout late gestation, for the measurement of arterial blood gas status (ABL 520 blood gas analyser; Radiometer, Copenhagen, Denmark).

Ewes were killed between 140 and 145 d of pregnancy with a lethal overdose ( $\sim 30 \text{ mg/kg}$ ) of sodium pentobarbitone delivered intravenously (Virbac Pty Ltd, Peakhurst, NSW, Australia). Fetuses were delivered by hysterectomy, weighed and killed by decapitation. Samples of quadriceps muscle were dissected from Control ( $n = 7$ ; 5 males, 2 females) and PR fetuses ( $n = 8$ ; 6 males, 2 females), frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**Lamb study.** Control ( $n = 14$ ) and PR ( $n = 9$ ) ewes lambed and were housed with their lambs throughout the experimental period. After birth, Control ( $n = 14$ , 8 males, 6 females) and PR ( $n = 9$ , 5 males, 4 females) lambs were weighed and crown-rump length measured daily between 10.00 and 14.00 h. Venous blood samples were collected in chilled tubes after approximately 60 min of non-suckling on alternate days between 09.00 h and 13.00 h, beginning on the day of birth (day 1). All blood samples were centrifuged at  $1500 g$  for 10 min, and plasma was stored at  $-20^{\circ}\text{C}$ . On postnatal day 21, lambs were killed with a lethal overdose ( $\sim 30 \text{ mg/kg}$ ) of sodium pentobarbitone delivered by intravenous injection (Virbac Pty Ltd, Peakhurst, NSW, Australia) and samples of quadriceps muscle were dissected, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### Plasma glucose and insulin assays

Plasma glucose was measured by an *in vitro* enzymatic colorimetric method (COBAS MIRA automated analysis system, Roche Diagnostica, Basel, Switzerland). The sensitivity of the assay was  $0.01 \text{ mmol l}^{-1}$ . Plasma insulin concentrations were measured using a radioimmunoassay (Linco Research, Inc., MO, USA) previously validated for sheep plasma (Muhlhausler *et al.* 2006). The sensitivity of the assay was  $0.1 \text{ ng ml}^{-1}$ . The intra- and inter-assay CV for both assays were each  $< 10\%$ .

### Isolation of RNA, production of cDNA and qRT-PCR analysis

RNA was extracted from 100 mg quadriceps muscle (Trizol reagent, Invitrogen Australia Pty Limited, Australia) from all fetuses and lambs. RNA was purified using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). The quality and concentration of the RNA were determined by measuring absorbance at 260 and 280 nm, and RNA integrity confirmed by agarose gel electrophoresis. cDNA was synthesised using the purified RNA ( $\sim 2 \mu\text{g}$ ) and Superscript 3 reverse transcriptase (Invitrogen Australia Pty Limited, Mount Waverley, Australia) with random hexamers.

The relative expression of mRNA transcripts of the insulin receptors A and B ( $\text{IR}_A$  and  $\text{IR}_B$ ), glucose transporter-1 (GLUT1), glucose transporter-4 (GLUT4), peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ) mRNA transcripts and the housekeeper gene acidic ribosomal protein large subunit P0 (ARP-P0) (Muhlhausler *et al.* 2007) were measured by quantitative real time reverse transcription-PCR (qRT-PCR) using the Sybr Green system in an ABI Prism 7500 Sequence Detection System (PE Applied Biosystems,

Foster City, CA, USA). The primer sequences used were (IR<sub>A</sub>, F: 5'CATCCCCAGGCCTTCGA3', R: 5'ACGGCTGCTGTCACATTCC3' (Y16093); IR<sub>B</sub>, F: 5'CATCCCCAGAAAATCATCTTCAG3', R: 5'CAAGGGCTCTGCGTTTCCT3' (Y16092); GLUT1, F: 5'TAACCGCAACGAGGAGAACC3' (U89029); GLUT4, F: 5'GTGGCCATCTTTGGCTTCGTG3', R: 5'CGGCTGA-ATCTGGTCAAAC3' (AY949177).

Each amplicon was sequenced to ensure the authenticity of the DNA product and qRT-PCR melt curve analysis performed to demonstrate amplicon homogeneity. Each qRT-PCR reaction well contained: 6  $\mu$ l Sybr Green Master Mix (PE Applied Biosystems, Foster City, CA), 1  $\mu$ l primer, 2.0  $\mu$ l molecular grade H<sub>2</sub>O and 1.0  $\mu$ l of cDNA (50 ng  $\mu$ l<sup>-1</sup>). Controls containing no reverse transcriptase were also used. The cycling conditions consisted of 40 cycles of 95°C for 15 min and 60°C for 1 min. At the end of each run dissociation melt curves were obtained.

The abundance of each mRNA transcript was measured and expression relative to ARP-P0 calculated using the comparative threshold cycle ( $C_t$ ) method (Q-gene qRT-PCR analysis software), which provides a quantitative measure of the relative abundance of a specific transcript in different tissues by the comparative threshold cycle ( $C_t$ ) method, which takes into account any differences in the amplification efficiencies of the target and reference genes. The  $C_t$  value was taken as the lowest statistically significant (>10 standard deviation (S.D.)) increase in fluorescence above the background signal in an amplification reaction.

### Insulin signalling protein analysis

The abundance of protein for the insulin signalling molecules and subunits of the mitochondrial respiratory chain were determined using western blotting as described in detail elsewhere (Forhead *et al.* 2008). Briefly, tissue samples (50 mg) were homogenised in lysis buffer, and centrifuged at 15 000  $g$  at 4°C for 5 min to remove lipid and insoluble material. Protein content of the clarified extracts was determined by a modification of the Lowry method. Equal volumes of protein (10  $\mu$ g and 15  $\mu$ g for insulin signalling and mitochondrial respiratory chain proteins, respectively) were subjected to SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membrane (Millipore, MA, USA), blocked overnight and then incubated with primary antibody against: insulin receptor  $\beta$  subunit, IGF1 receptor, PI3 kinase p85, Akt1, Akt2, Ser473 phosphoAKT, protein kinase C $\zeta$  and GLUT4 (Forhead *et al.* 2008) or with an anti-sera cocktail that cross-reacts with several subunits of the mitochondrial respiratory chain (MS601, Mitosciences, Eugene, OR, USA) which was validated for use in sheep tissues. Membranes were washed and bound antibody detected using horseradish peroxidase-conjugated

secondary antibodies and enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham/GE Healthcare, Little Chalfont, UK). Image Quant software (GE Healthcare) was used to quantify specific bands. To monitor the linearity of the density measurements, 10  $\mu$ g and 20  $\mu$ g of the same protein sample was loaded onto each gel to confirm that the chemiluminescent signal changed in a linear manner for all experiments. Prior to western blotting analysis samples (20  $\mu$ g protein) were subjected to SDS-PAGE and gels stained with coomassie blue. This revealed that there were no differences in abundance of the major proteins present in samples between the different experimental groups.

### Measurement of enzyme activity and mitochondrial proteins

Muscle samples (50 mg) were homogenized 1 : 19 (w/v) in 50 mmol l<sup>-1</sup> Tris-HCl, 1 mmol l<sup>-1</sup> EDTA, and 0.1% Triton X-100, pH 7.2, using a Polytron (Kinematica, Littau-Lucerne, Switzerland) and subjected to three freeze-thaw cycles. The activities of cytochrome *c* oxidase (CCO), citrate synthase (CS), succinate dehydrogenase (SDH),  $\beta$ -hydroxyacyl-CoA dehydrogenase ( $\beta$ HAD), hexokinase (HK), phosphofructokinase (PFK) and lactate dehydrogenase (LDH) were determined using a Spectra Max 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 37°C as described in detail elsewhere (MacArthur *et al.* 2008). Briefly, cytochrome *c* oxidase (CCO) activity was measured in a reaction mixture containing 100 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> and 0.1 mM cytochrome *c* reduced with sodium hydro-sulfite (Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>), pH 7.0. The rate of change in absorbance was monitored at 550 nm ( $e^{-19.1}$  mmol ml<sup>-1</sup> cm<sup>-1</sup>). Citrate synthase activity was measured in a reaction mixture containing 100 mM Tris-HCl, 1 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.2 mM dithio-bis(2-nitrobenzoic acid), 0.3 mM acetyl CoA and 0.5 mM oxaloacetate, pH 8.2. The rate of change in absorbance was monitored at 412 nm ( $e^{-13.6}$  mmol ml<sup>-1</sup> cm<sup>-1</sup>). Succinate dehydrogenase (SDH) activity of extracts was measured spectrophotometrically by assessing the rate of 2,6-dichlorophenol-indophenol (DCIP) reduction at 600 nm ( $e^{-21}$  mmol ml<sup>-1</sup> cm<sup>-1</sup>). SDH assays were conducted in 50 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> buffer, pH 7.0, 20 mM succinate, 2.5 mM antimycin A, 2.5 mM KCN, 0.45 mM phenazine methosulphate and 0.12 mM DCIP. For  $\beta$ -hydroxyacyl CoA dehydrogenase ( $\beta$ HAD), the reaction mixture contained 50 mM imidazole, 0.15 mM NADH and 0.1 mM acetoacetyl CoA, pH 7.4. Lactate dehydrogenase (LDH) assays were conducted in 10 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> buffer, 0.2 mM NADH and 2 mM pyruvate, pH 7.2. Phosphofructokinase activity was measured in a reaction mixture containing 50 mM

imidazole, 6 mM MgCl<sub>2</sub>, 60 mM KCl, 5 mM ATP, 0.4 mM NADH, excess aldolase (1 U), excess triosephosphate isomerase (50 U), excess  $\alpha$ -glycerophosphate dehydrogenase (8 U) and 5 mM fructose-6-phosphate, pH 7.4.  $\beta$ HAD, LDH and PFK activities were determined by monitoring the rate of NADH oxidation at 340 nm ( $e^{-6.22}$  mmol ml<sup>-1</sup> cm<sup>-1</sup>). Hexokinase assays were conducted in 50 mM imidazole, pH 7.4, 9 mM ATP, 9 mM MgCl<sub>2</sub>, 0.6 mM NADP, excess levels of glucose-6-phosphate dehydrogenase (4 U) and 5 mM glucose, by assessing the rate of NADP reduction at 340 nm ( $e^{-6.22}$  mmol ml<sup>-1</sup> cm<sup>-1</sup>). All assays were conducted in duplicate, and reaction rates were linear for >2 min.

### Statistical analysis and calculations

Data for protein expression/phosphorylation are presented as percentage of mean control value  $\pm$  S.E.M. All other values are presented as the mean  $\pm$  S.E.M.  $P < 0.05$  was considered statistically significant.

**Fetal study.** The effects of PR on fetal size, mRNA and protein expression and enzyme activities were determined using a Student's unpaired *t*-test. Relationships between variables were determined using linear regression and partial correlation analyses.

**Lamb study.** The classification of IUGR was determined using a frequency distribution curve of birth weights of control singleton lambs from a separate cohort ( $n = 45$ ) studied in this laboratory over the preceding 5 years. Newborn lambs in the current study were classified as IUGR when their birth weight was greater than 2 S.D. below the mean of this cohort, equivalent to the 3rd percentile (IUGR: < 4.3 kg), or normally grown if their birth weight was within 2 S.D. of the mean (Control: 4.5–6.7 kg). Two lambs in the PR group were excluded from the study due to the fact that their weight did not fall below the 4.3 kg cut-off point for IUGR. No control fetuses were excluded.

Daily growth rate (%) was calculated as body weight gained per day as a percentage increase from the previous day's body weight ( $(\text{body weight}_n / \text{body weight}_{n-1} \times 100) - 100$ )%.

The effects of placental restriction and sex on mRNA and protein levels and enzyme activity were determined using two-way analysis of variance (ANOVA). In the presence of an interaction between the effects of placental restriction and sex, the effects of PR were determined separately in male and female lambs. Relationships between variables were determined using linear regression and partial correlation analyses. All analyses were performed using SPSS for Windows v. 16 (SPSS Inc, Chicago, IL, USA).

## Results

### Placental and fetal growth restriction, fetal hypoxaemia and hypoglycaemia

Mean gestational arterial  $P_{O_2}$  was lower in PR fetuses compared to controls (Control,  $24.57 \pm 2.2$  mmHg; PR,  $14.39 \pm 0.8$  mmHg;  $P < 0.0001$ ). PR fetuses also had significantly lower plasma glucose concentrations during late gestation (Control,  $1.01 \pm 0.03$  mmol l<sup>-1</sup>; PR,  $0.68 \pm 0.07$  mmol l<sup>-1</sup>;  $P < 0.01$ ). Fetal weight was lower in PR fetuses at 141–144 d gestation (Control,  $5.14 \pm 0.2$  kg; PR,  $2.69 \pm 0.3$  kg;  $P < 0.0001$ ). The protein content of quadriceps muscle was also lower in the PR group (Control,  $57.5 \pm 2.6$  mg (g wet weight)<sup>-1</sup>; PR,  $48.5 \pm 1.0$  mg (g wet weight)<sup>-1</sup>,  $P < 0.01$ ).

### Placental restriction and the expression of the insulin and IGF1 receptor and post-receptor signalling proteins in fetal muscle

The mRNA expression of both the A and B subunits of the IR in quadriceps muscle was not different between Control and PR fetuses (IR<sub>A</sub> Control,  $0.038 \pm 0.006$ ; PR,  $0.055 \pm 0.01$ ; IR<sub>B</sub> Control,  $0.041 \pm 0.02$ ; PR,  $0.017 \pm 0.003$ ). The protein abundance of the IR and IGF1R in quadriceps muscle were each higher ( $P < 0.01$ ) in PR fetuses compared to Controls (Fig. 1A and B).

There was no effect of placental restriction on the abundance of protein for the PI3Kp85 $\alpha$  subunit, Akt1 or pAkt within the fetal quadriceps muscle (Figs 2A and 3A and C). The abundance of Akt2 ( $P < 0.02$ ) and PKC $\zeta$  ( $P < 0.05$ ) was lower in PR compared to Control fetuses (Figs 2B and 3B).

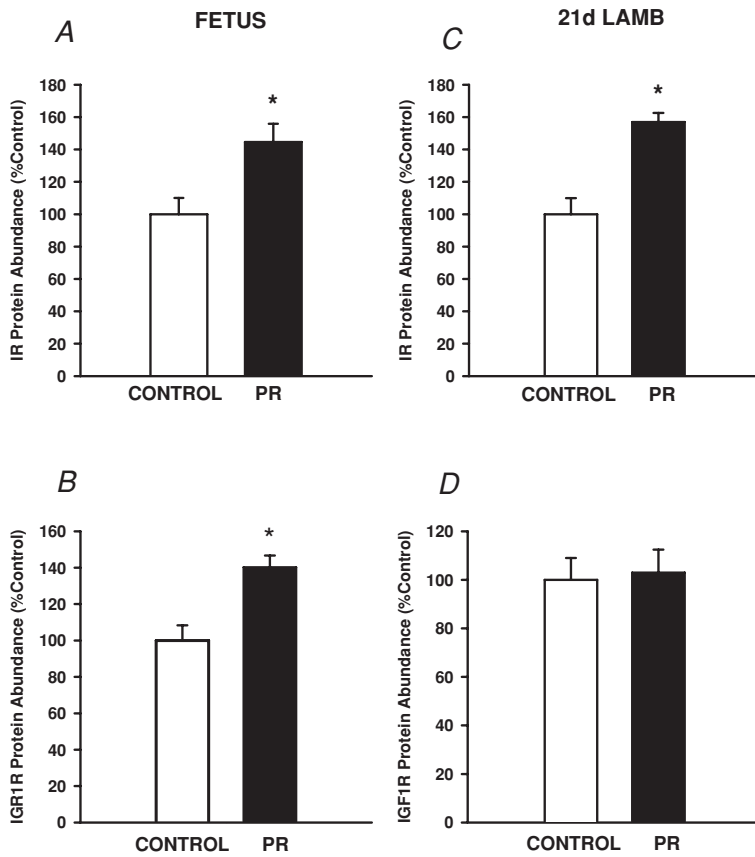
### Placental restriction and expression of glucose transporters, PGC-1 $\alpha$ and PPAR $\alpha$ in fetal muscle

The expression of GLUT4 mRNA and protein was each lower ( $P < 0.05$ ) in the quadriceps muscle of PR fetuses (Fig. 4A and B). There was no difference in the expression of GLUT1 mRNA between Control and PR fetuses (Fig. 4C). In PR fetuses, but not in Control fetuses, GLUT1 mRNA expression was inversely related to fetal body weight at  $\sim 140$  d gestation ( $r^2 = 0.65$ ;  $P < 0.01$ ).

Placental restriction resulted in a decrease in PGC-1 $\alpha$  mRNA (Control,  $0.032 \pm 0.005$ ; PR,  $0.019 \pm 0.004$ ,  $P < 0.05$ ) and PPAR $\alpha$  mRNA (Control,  $0.35 \pm 0.04$ ; PR,  $0.20 \pm 0.05$ ,  $P < 0.05$ ) expression in quadriceps muscle.

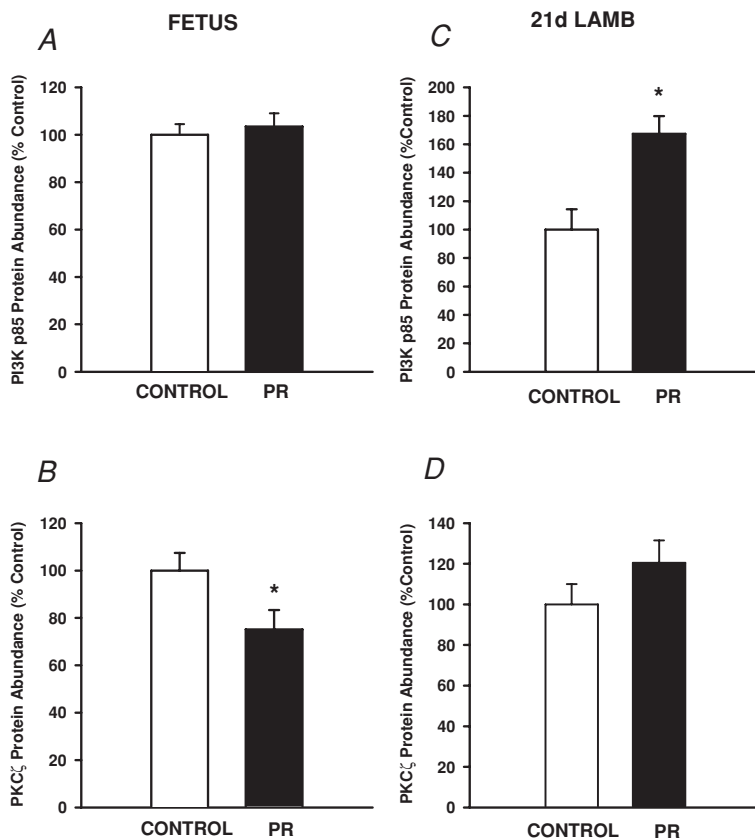
### Placental restriction and the activity of mitochondrial and glycolytic enzymes in fetal muscle

Citrate synthase (CS) ( $P = 0.08$ ) and succinate dehydrogenase activity (SDH) ( $P < 0.09$ ) tended to be lower in PR fetuses compared to Controls (Table 1), and



**Figure 1.** The abundance of protein for the insulin receptor (IR, *A* and *C*) and IGF1 receptor (IGF1R, *B* and *D*) in quadriceps muscle in the Control (open bars) and PR (filled bars) fetus (*A* and *B*) and 21 d lamb (*C* and *D*)

Protein abundance is expressed as a percentage of the mean value of the Control group. \*Significant effect of PR on protein abundance ( $P < 0.05$ ).



**Figure 2.** The abundance of protein for PI3K (*A* and *C*) and PKC $\zeta$  (*B* and *D*) in quadriceps muscle in the Control (open bars) and PR (filled bars) fetus (*A* and *B*) and 21 d lamb (*C* and *D*)

Protein abundance is expressed as a percentage of the mean value of the Control group. \*Significant effect of PR on protein abundance ( $P < 0.05$ ).

the expression of CS ( $r^2 = 0.62$ ;  $P < 0.01$ ,  $n = 15$ ) and SDH ( $r^2 = 0.57$ ;  $P < 0.01$ ,  $n = 15$ ) was each directly related to fetal body weight. There was no effect of placental restriction on the activity of  $\beta$ -HAD, CCO, HK, PFK or LDH in fetal quadriceps muscle (Table 1).

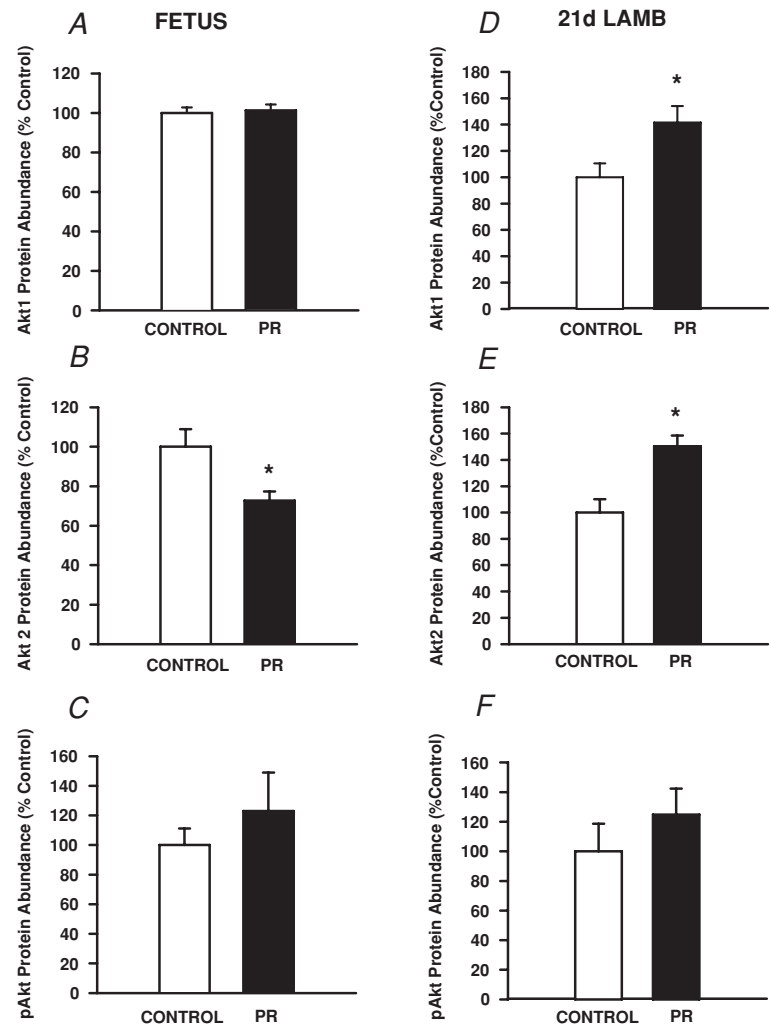
There was a significant reduction in the abundance of protein subunits from Complex III and Complex IV ( $P < 0.05$ , Table 1). There was no difference, however, in the abundance of protein subunits from Complex I ( $P = 0.07$ ) or Complex II in the muscle of PR fetuses compared to Controls (Table 1).

### Placental restriction, postnatal growth and expression of the insulin receptor and post insulin signalling proteins in muscle of the postnatal lamb

PR lambs were lighter at birth (Control,  $5.86 \pm 0.12$  kg; PR,  $3.76 \pm 0.17$ ,  $P < 0.001$ ). The fractional growth rate during the first 3 weeks of postnatal life was higher in PR lambs compared to Controls (Control,  $4.26 \pm 0.14\%$ ; PR,  $5.26 \pm 0.16\%$ ,  $P < 0.01$ ). The body

weight of PR lambs was, however, still lower than Control lambs at 21 d of age (Control,  $13.22 \pm 0.17$  kg; PR,  $10.00 \pm 0.40$  kg,  $P < 0.01$ ). Plasma glucose (Control,  $5.83 \pm 0.19$  mmol l<sup>-1</sup>; PR,  $5.50 \pm 0.11$  mmol l<sup>-1</sup>) and insulin (Control,  $2.08 \pm 0.16$  ng ml<sup>-1</sup>; PR,  $1.77 \pm 0.29$  ng ml<sup>-1</sup>) concentrations during the first 3 weeks of postnatal life were not different between Control and PR lambs.

There was no difference in the protein content of the muscle of Control and PR lambs at 21 d of age (Control,  $78.2 \pm 3.1$  mg (g wet weight)<sup>-1</sup>; PR,  $83.7 \pm 5.3$  mg (g wet weight)<sup>-1</sup>). There was also no effect of placental restriction or sex on IR<sub>A</sub> or IR<sub>B</sub> mRNA expression in the quadriceps muscle at 21 d (IR<sub>A</sub> Control,  $0.086 \pm 0.005$ , PR,  $0.095 \pm 0.009$ ; IR<sub>B</sub> Control,  $0.034 \pm 0.003$ , PR,  $0.035 \pm 0.005$ ). The abundance of IR protein was higher ( $P < 0.001$ ), however, in the muscle of both male and female lambs in PR lambs at 21 d (Fig. 1C). There was no effect of placental restriction on the abundance of IGF1R protein in the muscle of either male or female lambs (Fig. 1D).



**Figure 3.** The abundance of protein for Akt1 (A and D), Akt2 (B and E) and pAkt (C and F) in quadriceps muscle in the Control (open bars) and PR (filled bars) fetus (A, B and C) and 21 d lamb (D, E and F)

Protein abundance is expressed as a percentage of the mean value of the Control group. \*Significant effect of PR on protein abundance ( $P < 0.05$ ).

**Table 1. The effect of placental restriction on activity of glycolytic and oxidative enzymes and on the protein abundance of subunits from respiratory complexes I–IV in fetal quadriceps muscle at ~140 d gestation**

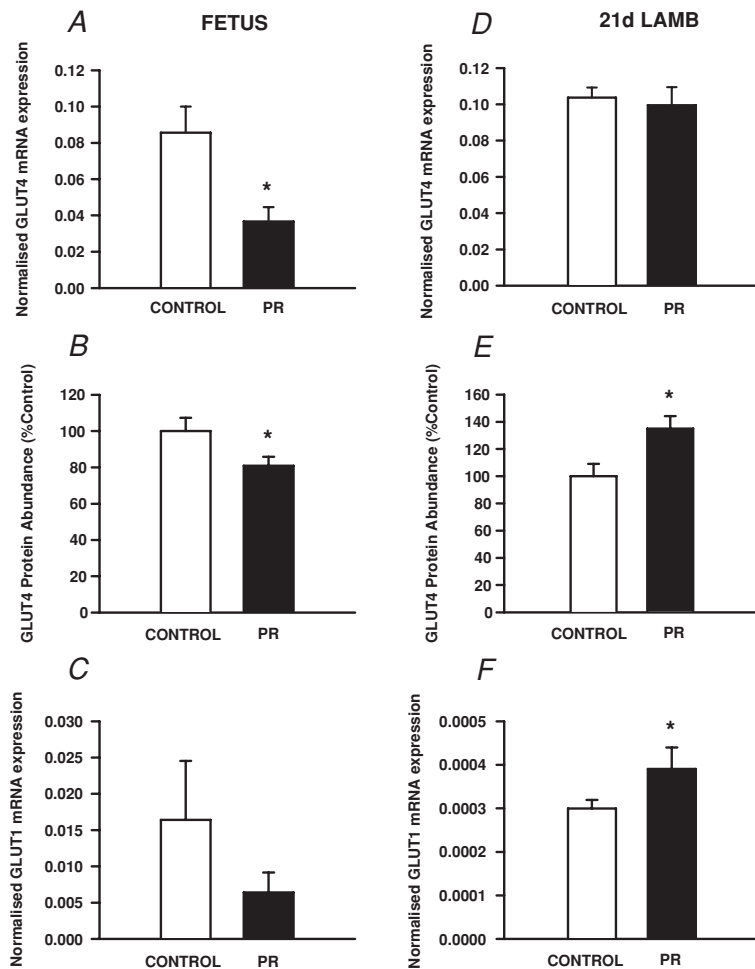
	Control (n = 7)	PR (n = 8)
Cytochrome c oxidase ( $\mu\text{mol min}^{-1} (\text{g wet wt})^{-1}$ )	4.91 $\pm$ 0.54	4.43 $\pm$ 0.74
Citrate synthase ( $\mu\text{mol min}^{-1} (\text{g wet wt})^{-1}$ )	3.13 $\pm$ 0.31	2.43 $\pm$ 0.21#
Succinate dehydrogenase ( $\mu\text{mol min}^{-1} (\text{g wet wt})^{-1}$ )	0.29 $\pm$ 0.03	0.22 $\pm$ 0.02#
$\beta$ -Hydroxyacyl CoA dehydrogenase ( $\mu\text{mol min}^{-1} (\text{g wet wt})^{-1}$ )	0.46 $\pm$ 0.04	0.40 $\pm$ 0.03
Hexokinase ( $\mu\text{mol min}^{-1} (\text{g wet wt})^{-1}$ )	0.19 $\pm$ 0.01	0.20 $\pm$ 0.02
Phosphofructokinase ( $\mu\text{mol min}^{-1} (\text{g wet wt})^{-1}$ )	6.8 $\pm$ 1.19	7.0 $\pm$ 0.76
Lactate dehydrogenase ( $\mu\text{mol min}^{-1} (\text{g wet wt})^{-1}$ )	45.7 $\pm$ 3.1	42.1 $\pm$ 3.5
Mitochondrial complex I abundance (a.u.)	140.9 $\pm$ 19.1	92.2 $\pm$ 15.1
Mitochondrial complex II abundance (a.u.)	32.3 $\pm$ 5.8	24.1 $\pm$ 4.4
Mitochondrial complex III abundance (a.u.)	88.7 $\pm$ 3.5	71.7 $\pm$ 4.4*
Mitochondrial complex IV abundance (a.u.)	77.4 $\pm$ 4.6	65.1 $\pm$ 2.9*

# $P < 0.09$ , \* $P < 0.05$  compared to Control fetuses. a.u., arbitrary units.

The abundance of PI3K p85 $\alpha$  subunit (Fig. 2C), Akt1 and Akt2 (Fig. 3D and E) in skeletal muscle was higher ( $P < 0.02$ ) in PR male and female lambs compared to their Control counterparts at 21 d of postnatal age. There was no effect of placental restriction on the abundance of pAkt (Fig. 3F) or PKC $\zeta$  (Fig. 2D) in lamb quadriceps muscle.

#### Placental restriction and expression of glucose transporters, PGC-1 $\alpha$ and PPAR $\alpha$ in lamb muscle

There was no difference in the expression of GLUT4 mRNA in the muscle between the PR and Control lambs (Fig. 4D). The abundance of GLUT4 protein, however, was higher ( $P < 0.02$ ) in the quadriceps muscle of the PR



**Figure 4. The relative expression of GLUT4 mRNA (A and D), abundance of GLUT4 protein (B and E) and expression of GLUT1 mRNA (C and F) in quadriceps muscle in the Control (open bars) and PR (filled bars) fetus (A, B and C) and 21 d lamb (D, E and F)**

The expression of GLUT1 and GLUT4 mRNA was normalised to the housekeeper gene ARP-P0. Protein abundance is expressed as a percentage of the mean value of the Control group. \*Significant effect of PR on normalised mRNA expression or protein abundance ( $P < 0.05$ ).



**Table 2.** There was no effect of placental restriction on the activity per mg wet weight of mitochondrial enzymes, and abundance of protein subunits from mitochondrial respiratory complexes I–IV in lamb quadriceps muscle at 21 d postnatal age

	Control ( <i>n</i> = 14)	PR ( <i>n</i> = 9)
Cytochrome <i>c</i> oxidase ( $\mu\text{mol min}^{-1}$ (g wet wt) $^{-1}$ )	8.2 $\pm$ 0.4	7.6 $\pm$ 0.6
Citrate synthase ( $\mu\text{mol min}^{-1}$ (g wet wt) $^{-1}$ )	6.9 $\pm$ 0.3	7.2 $\pm$ 0.6
Succinate dehydrogenase ( $\mu\text{mol min}^{-1}$ (g wet wt) $^{-1}$ )	0.32 $\pm$ 0.02	0.27 $\pm$ 0.03
$\beta$ -Hydroxyacyl CoA dehydrogenase ( $\mu\text{mol min}^{-1}$ (g wet wt) $^{-1}$ )	0.63 $\pm$ 0.02	0.67 $\pm$ 0.06
Phosphofructokinase ( $\mu\text{mol min}^{-1}$ (g wet wt) $^{-1}$ )	40.6 $\pm$ 3.2	36.9 $\pm$ 4.9
Lactate dehydrogenase ( $\mu\text{mol min}^{-1}$ (g wet wt) $^{-1}$ )	145.5 $\pm$ 7.1	133.3 $\pm$ 13.2
Complex I abundance (a.u.)	181.3 $\pm$ 31.6	182.4 $\pm$ 11.0
Complex II abundance (a.u.)	38.1 $\pm$ 9.5	47.8 $\pm$ 6.3
Complex III abundance (a.u.)	107.6 $\pm$ 6.3	112.2 $\pm$ 5.3
Complex IV abundance (a.u.)	101.8 $\pm$ 4.9	92.4 $\pm$ 3.8

a.u., arbitrary units.

lambs compared to Controls at 21 d in both males and females (Fig. 4E). The expression of GLUT1 mRNA was also higher ( $P < 0.05$ ) in muscle of PR lambs, in both males and females (Fig. 4F).

PGC-1 $\alpha$  mRNA expression was significantly higher in the skeletal muscle of male (Control, 0.031  $\pm$  0.004; PR, 0.063  $\pm$  0.009,  $P < 0.05$ ), but not female, PR lambs compared to Controls. The expression of PPAR $\alpha$  mRNA was not different, however, between either males or females or between PR and Control lambs.

### Placental restriction and the activity of mitochondrial and glycolytic enzymes in lamb muscle

The activity of hexokinase in skeletal muscle was increased in both male and female lambs in the PR group (Control, 0.19  $\pm$  0.02; PR, 0.26  $\pm$  0.02,  $P < 0.05$ ). There was no effect of PR or sex on the activity of any other mitochondrial enzymes at 21 d (Table 2). There was also no effect of placental restriction on the abundance of any of the subunits from complexes I–IV in the postnatal lamb (Table 2).

## Discussion

The present study has provided new insights into the molecular mechanisms underlying the transition from growth restriction *in utero* to postnatal catch-up growth. We found that whilst the abundance of insulin and IGF1 receptors were increased in the quadriceps muscle of growth restricted fetus, this was accompanied by a decrease in the abundance of the insulin signalling molecule, PKC $\zeta$ , and of the insulin responsive glucose transporter, GLUT4. Interestingly, however, the increase in the abundance of the insulin receptor in the IUGR lamb persisted after birth and was then associated with an up-regulation of Akt1 and 2 and GLUT4 protein. These data provide the first evidence for the *in utero* origins of the increased insulin sensitivity

in skeletal muscle which drives accelerated growth after birth.

### Placental restriction and insulin signalling in fetal skeletal muscle

Consistent with previous studies using the placentally restricted (PR) sheep model, PR fetuses in the current study were chronically hypoxaemic, hypoglycaemic and growth restricted (Simonetta *et al.* 1997; Duffield *et al.* 2008). A key finding of the present study was that fetal substrate deprivation was associated with an increased abundance of insulin and IGF1 receptor (IGF1R) protein in the quadriceps muscle before birth. It has consistently been reported that plasma concentrations of both insulin and IGF1 are reduced in growth-restricted fetuses (Kind *et al.* 1995; Cetin *et al.* 2001; Duffield *et al.* 2008). Previous studies have shown that insulin and IGF1 receptor abundance in muscle is increased proportionally in response to a decrease in availability of signalling hormone (Duckworth *et al.* 1998; Flati *et al.* 2008), which suggests that increases in IR and IGF1 receptor abundance in the growth restricted fetus may be a response to the low circulating concentrations of these hormones.

The increase in the abundance of IR protein in the PR fetus did not appear to lead to increased insulin action (as assessed by Akt phosphorylation), at least under basal conditions. We found that there was a downregulation of Akt2, PKC $\zeta$  and the insulin-responsive glucose transporter, GLUT4, in the muscle of the growth restricted fetuses. The IRS–PI3k–Akt pathway stimulates GLUT4 translocation and regulates fusion of GLUT4 containing vesicles with the plasma membrane (Kanzaki, 2006), whilst atypical PKC isoforms lambda and zeta (aPKC $\lambda/\zeta$ ) play a role as downstream targets for the IRS–PI3K signalling pathway (Kanzaki, 2006). Therefore, while growth restricted fetuses had an increased abundance of insulin receptors, they also had reduced abundance of signalling molecules

responsible for the translocation of GLUT4, and lower GLUT4 protein content.

Recent studies have reported that there was no difference in insulin sensitivity between placentally restricted and control fetal sheep in late gestation as measured by hyperinsulinaemic euglycaemic clamp (Owens *et al.* 2007). This suggests, therefore, that the reduced abundance of signalling proteins downstream of the insulin receptor in the growth restricted fetus is most likely due to the low level of activation of the receptor by insulin. A decrease in the insulin-mediated glucose uptake and protein synthesis in skeletal muscle would be an important adaptive response, enabling this tissue to maintain basal metabolic activity in the low insulin, low glucose environment of the growth restricted fetus.

### Placental restriction and insulin signalling in skeletal muscle of the postnatal lamb

As expected, the IUGR lambs in the present study were smaller and thinner at birth and had a higher daily fractional growth rate in the first 2 weeks of life compared with control lambs. Importantly, there was a greater abundance of insulin receptor protein in the skeletal muscle of the IUGR lamb at 21 d of postnatal age. Furthermore, and in direct contrast to the growth-restricted fetus, we found that by 21 d of postnatal life, there was an increase in protein expression of Akt1 and Akt2 and increased abundance of the insulin-sensitive glucose transporter, GLUT4, and the insulin-independent glucose transporter, GLUT1, in the skeletal muscle of growth restricted lambs. Therefore, we have shown that in growth restricted lambs, there is an increase in the proteins required for the promotion of GLUT4-containing vesicle translocation and fusion with the cellular membrane. This suite of molecular changes is consistent with an increase in both basal, i.e. insulin-independent, and insulin-stimulated glucose uptake in the skeletal muscle of the postnatal lambs undergoing accelerated growth (De Blasio *et al.* 2006).

We found that the protein abundance, but not mRNA expression, of the insulin receptor was increased in the IUGR postnatal lamb. This suggests that the stable increase in insulin receptor protein is likely to be a consequence of reduced protein turnover or production, rather than increased gene transcription. One possibility is that reduced clearance of the insulin receptor within the skeletal muscle of the growth restricted fetus occurs as a consequence of the low insulin concentrations before birth, and that this reduced clearance rate persists into postnatal life and results in an enhanced capacity for insulin signalling.

Experimental studies using a range of rodent models of intrauterine growth restriction (IUGR) have shown

that IUGR is associated with an early phase of enhanced insulin sensitivity, followed by the later emergence of insulin resistance and type 2 diabetes (Ozanne *et al.* 1996; Ozanne, 2001; Ozanne *et al.* 2005). It has also been shown that young healthy men who had a low birth weight have reduced skeletal muscle expression of the PI3K p85 $\alpha$  regulatory subunit, the p110 $\beta$  catalytic subunit and PKC $\zeta$  and GLUT4, changes which have been shown to precede the development of whole body insulin resistance and glucose intolerance (Ozanne *et al.* 2005). In a recent follow-up study, in the same cohort of LBW men there was a marked insulin-mediated up-regulation of the PI3K p85 $\alpha$  and p110 $\beta$  subunits and reduced PKC $\zeta$  and pAkt responses, both of which are required for insulin-stimulated glucose uptake (Jensen *et al.* 2008). Interestingly increased expression of p85 $\alpha$  has been implicated in insulin resistance as it has been proposed that excess p85 $\alpha$  may act to sequester IRS-1 and PI3K enzymatic activity into inert cellular foci incapable of PI-3,4,5-trisphosphate (PIP<sub>3</sub>) generation (Luo *et al.* 2005).

The data from the current study highlight that IUGR results in changes in the insulin signalling pathway which are present in fetal life and which may be the antecedents of the initial phase of enhanced insulin sensitivity which is associated with accelerated growth in early postnatal life and which precedes the later emergence of insulin resistance.

### Placental restriction and protein synthesis in fetal and lamb skeletal muscle

In the current study we found that growth-restricted fetuses were not only smaller in late gestation, but also had a lower skeletal muscle protein content. We found that fetal growth restriction resulted in a significant decrease in the abundance of Akt2 within the skeletal muscle. The Akt signalling pathway activates the mTOR complex which controls protein synthesis (Kimball, 2007). The reduced availability of Akt2 in the skeletal muscle of growth restricted fetuses may therefore reduce the capacity for insulin action through this pathway and explain the reduction in muscle protein content. By 21 d of postnatal age, however, we found that the abundance of both Akt1 and Akt2 proteins was significantly increased in the skeletal muscle of IUGR lambs and that myocellular protein content in IUGR lambs was restored to levels present in control lambs, suggesting a rapid recovery of muscle protein synthesis in the early postnatal period. It would be important to determine whether in the growth restricted lamb insulin action results in an increased pAkt response in order to determine whether enhanced activation of the Akt signalling proteins represents the molecular basis for the increased rate of muscle protein synthesis in IUGR offspring in the immediate postnatal period.

### Growth restriction and mitochondrial biogenesis in skeletal muscle

Mitochondria play a central role in the regulation of cellular energy metabolism, and impaired mitochondrial function in skeletal muscle has been associated with the onset of insulin resistance both in animal models and in human subjects (Højlund *et al.* 2008). Whilst there is clear evidence from previous studies that fetal growth restriction is associated with an increased incidence of insulin resistance in later life (Ozanne, 2001; Ozanne & Hales, 2002), there are few studies which have investigated the impact of IUGR on markers of mitochondrial biogenesis and activity of mitochondrial enzymes before or after birth (Peterside *et al.* 2003; Selak *et al.* 2003).

In the present study, we found that the mRNA expression of PGC-1 $\alpha$ , a key transcriptional activator of mitochondrial biogenesis, was lower in skeletal muscle of growth restricted fetuses. Furthermore, abundance of two respiratory complexes of the electron transport chain were lower, and the activity of citrate synthase and succinate dehydrogenase tended to be lower, in skeletal muscle in growth restricted fetuses. Citrate synthase catalyses the first step in the Krebs cycle, whilst succinate dehydrogenase plays a vital role in both the Krebs cycle and the electron transport chain. Correct functioning of both these enzymes is therefore essential for the complete oxidation of glucose. These changes may therefore represent an adaptation which reduces the capacity of the muscle for oxidative phosphorylation, and thus slows muscle growth, when there is a decrease in fetal substrate supply. These results are somewhat different from those in the rodent, in which fetal growth restriction induced by uterine artery ligation in late gestation was associated with increased abundance of PGC-1 $\alpha$  mRNA and protein in the skeletal muscle at birth and at 21 d of age (Lane *et al.* 2003). Other studies have also shown, however, that these animals have reduced PGC-1 $\alpha$  mRNA and protein and cytochrome oxidase III and IV mRNA at 6 months of age (Wadley *et al.* 2008), and that this precedes the development of insulin resistance and glucose intolerance in this animal model of fetal growth restriction (Siebel *et al.* 2008).

In addition to its role in mitochondrial biogenesis, PGC-1 $\alpha$  is also known to activate intracellular fatty acid oxidation through the activation of PPAR $\alpha$ , and the abundance of PPAR $\alpha$  mRNA in skeletal muscle was also significantly lower in growth restricted fetuses. Whilst experimental restriction of placental growth has been shown to decrease plasma glucose concentrations, it is not clear whether plasma concentrations of free fatty acids are also lower, and therefore the potential implication of placental restriction on fetal fatty acid metabolism is unknown.

At 21 d of postnatal age, expression of PGC-1 $\alpha$  mRNA was higher in skeletal muscle of growth restricted male,

but not female, lambs. It is interesting that the response of PGC-1 $\alpha$  in skeletal muscle was the only change in this study which was sex specific. This is consistent with previous work in rats, in which increases in PGC-1 $\alpha$  mRNA and protein in rat pups exposed to uterine artery ligation in late gestation were more marked in male offspring compared to females (Lane *et al.* 2003).

Interestingly, there appeared to be no effect of fetal growth restriction on the abundance of respiratory complexes or activity of key oxidative (SDH, CS, CCO,  $\beta$ HAD) and glycolytic enzymes (PFK, LDH) at 21 d of postnatal age, with the exception of a significant increase in the activity of the glycolytic enzyme hexokinase in both growth restricted male and female lambs. The principal role of hexokinase is to convert glucose to glucose-6-phosphate, which is trapped within the cell and used as a substrate for glycolysis (Arai *et al.* 1995). The increase in hexokinase activity may therefore result in greater efficiency of the muscle cell to retain glucose and facilitate its metabolic conversion.

### Summary

It is well established that an accelerated growth and increased peripheral insulin sensitivity are present in the early postnatal period following fetal growth restriction, but to date there have been no studies which have determined the mechanisms which explain the transition from growth restriction *in utero* to enhanced insulin sensitivity and catch-up growth in the early postnatal period. The present study has provided important and novel insights into the molecular mechanisms underlying the transition from growth restriction *in utero* to postnatal catch-up growth which commonly occurs in growth-restricted infants after birth.

We have shown that fetal growth restriction is associated with an up-regulation of insulin receptors before birth. Importantly, the increase in the abundance of the insulin receptor persists in the IUGR postnatal lamb and is then associated with an up-regulation of Akt1 and 2 and GLUT4 protein. The presence of higher insulin receptor abundance when the fetus emerges into the postnatal environment where nutrient supply is no longer constrained may explain the accelerated assimilation of nutrients into skeletal muscle, and accelerated growth rate in these infants. The findings of this study therefore suggest that catch-up growth has its origins in the fetal adaptive response to a low substrate and insulin environment. A greater understanding of the mechanism that determines the programmed increase in insulin receptor abundance in the IUGR offspring may contribute to a better understanding of the mechanisms which result in the subsequent emergence of insulin resistance and metabolic disease in adult life in growth restricted infants.

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### Author contributions

All authors contributed to the conception and design or analysis and interpretation of data. All authors have actively participated in the process of drafting the article or revising it critically for important intellectual content and have given final approval of the version to be published.

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