

β_2 -Adrenergic receptor desensitization in perirenal adipose tissue in fetuses and lambs with placental insufficiency-induced intrauterine growth restriction

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Placental insufficiency-induced intrauterine growth restriction (IUGR) fetuses have chronic hypoxaemia and elevated plasma catecholamine concentrations. In this study, we determined whether adrenergic responsiveness becomes desensitized in the perirenal adipose tissue of IUGR fetuses and lambs by measuring adrenergic receptor (AR) mRNA and protein levels. We also tested the ability of adrenaline to mobilize non-esterified fatty acids (NEFAs) in young lambs. Perirenal adipose tissue was collected from IUGR and control fetuses at 133 days of gestational age (dGA) and lambs at 18 days of age (dA). β_2 -AR mRNA concentrations were 59% and 74% lower ($P < 0.05$) in IUGR fetuses and lambs compared to controls, respectively, which also resulted in lower protein levels ($P < 0.05$). No treatment differences were detected for α_{1A} -, α_{1B} -, α_{1D} -, α_{2A} -, α_{2B} -, α_{2C} -, β_1 - and β_3 -AR expression. mRNA concentrations were also determined for hormone sensitive lipase (HSL), perilipin (lipid droplet-associated protein), and two adipokines, leptin and adiponectin. Adiponectin and HSL were not different between treatments at either age. Compared to controls, perilipin and leptin mRNA concentrations were lower ($P < 0.05$) in IUGR fetuses but not in lambs. Because of the β_2 -AR results, we challenged a second cohort of lambs with exogenous adrenaline at 21 dA. The ability of adrenaline to mobilize NEFA was $55 \pm 15\%$ lower ($P < 0.05$) in IUGRs than controls. Collectively, our findings indicate that elevated catecholamine exposure *in utero* causes desensitization of adipose tissue by down-regulation of β_2 -AR, and this persists in lambs. This impairment in adrenergic stimulated lipolysis might partially explain early onset obesity in IUGR offspring.

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Abbreviations AR, adrenergic receptor; AUC, area under the curve; dA, days of age; dGA, days of gestational age; DMI, dry matter intake; HSL, hormone sensitive lipase; IUGR, intrauterine growth restriction; NEFA, non-esterified fatty acid; PEPCCK, phosphoenolpyruvate carboxykinase.

Introduction

Placental insufficiency restricts fetal nutrient and oxygen supply and slows growth, requiring nutrient sparing by the fetus (Barker *et al.* 1993). This could result in an adaptive response *in utero* that is mismatched to postnatal nutrient availability, increasing the risk for obesity later in life (Ravelli *et al.* 1976; Gluckman *et al.* 2005; Fernandez-Twinn & Ozanne, 2006; Yliharsila *et al.* 2008). For example, small for gestational age children exhibit catch-up growth and are more likely to develop central adiposity at 5 years of age compared to children

born with body weight appropriate for gestational age (Hokken-Koelega *et al.* 1995; Ong *et al.* 2000). Similarly, lambs that were subjected to intrauterine growth restriction (IUGR) exhibit greater visceral and perirenal adipose mass by 45 days (De Blasio *et al.* 2007). Together, these findings demonstrate an association between IUGR and obesity, but explanations for this relationship are lacking. Endocrine factors, like catecholamines, may play a major role in fetal nutrient redistribution and could impact postnatal metabolism (Green *et al.* 2010; Leos *et al.* 2010). Previous data from our laboratory show that fetal hypoxaemia, as may occur during

IUGR, chronically elevates catecholamine concentrations (Limesand *et al.* 2006; Leos *et al.* 2010). We postulate that this response causes adrenergic desensitization in adipose tissue, lowering adrenergic stimulated lipolysis, which might increase an individual's risk for childhood obesity.

The catecholamines, adrenaline and noradrenaline, act through G-protein coupled receptors (Altan *et al.* 2007; Schaak *et al.* 2007). There are three main types of adrenergic receptor (AR), α_1 -AR, α_2 -AR and β -AR, and each has multiple subtypes, which include α_{1A} -, α_{1B} - and α_{1D} -AR, α_{2A} -, α_{2B} - and α_{2C} -AR, and β_1 -, β_2 - and β_3 -AR. In adipocytes, β -AR activates hormone sensitive lipase (HSL) to stimulate lipolysis through a cAMP-dependent process, resulting in accelerated triglyceride hydrolysis into non-esterified fatty acids (NEFAs) and glycerol. At the same time, phosphorylation of perilipin by protein kinase A causes perilipin to facilitate the interaction between the fat droplet and HSL to promote lipolysis (Tansey *et al.* 2004; Carmen & Victor, 2006). Chronic exposure to high catecholamine concentrations has been shown to down-regulate AR by reducing mRNA synthesis and increasing protein degradation (Benovic *et al.* 1990; Hausdorff *et al.* 1990; Santala *et al.* 1990; Dohlman *et al.* 1991; Collins *et al.* 1992; Garcia-Higuera & Mayor, 1994). Therefore, if AR desensitization occurs in the fetus and persists postnatally, it could lead to dyslipidaemia and impaired lipid mobilization, which could partially explain the increased incidence of obesity in IUGR offspring. In the present study, we examined AR expression in perirenal adipose tissue, the major fat depot in fetal sheep (Symonds *et al.* 2003), in a model of placental insufficiency-induced IUGR created by exposing pregnant ewes to hyperthermic conditions (Bell *et al.* 1987; Thureen *et al.* 1992; de Vrijer *et al.* 2004). Expression levels were determined in near-term fetuses and young lambs prior to weaning to encompass the postnatal transition. In addition, we determined adrenaline's ability to mobilize NEFAs in young IUGR lambs to identify adrenergic desensitization during the perinatal period.

Methods

Ethical approval

The Institutional Animal Care and Use Committee at the University of Arizona approved all animal protocols used in these experiments. All animal experiments were conducted at the William J. Parker Agricultural Research Complex (Tucson, AZ, USA), which is accredited by the American Association for Accreditation of Laboratory Animal Care. All experimental procedures comply with the outlined policies and regulations of *The Journal of Physiology* (Drummond, 2009).

Animal preparation

Time-mated, pregnant Columbia–Rambouillet crossbred ewes were purchased from Nebekar Ranch (Lancaster, CA, USA). Control ewes were maintained in a thermoneutral environment throughout pregnancy, and placental insufficiency intrauterine growth restriction (IUGR) was induced by exposing pregnant ewes to environmental hyperthermia (40°C for 12 h and 35°C for 12 h with a relative humidity of $35 \pm 8\%$) (Bell *et al.* 1987; Regnault *et al.* 1999). Control ewes were pair-fed with hyperthermic ewes. Whenever ewes were carrying twins, only one offspring was randomly included in the study. The IUGR fetus group was generated from nine pregnant ewes ($n = 9$ fetuses), six carrying singletons and three carrying twins, which were exposed to hyperthermia from 39 ± 2 to 96 ± 5 days gestational age (dGA, term = 150 dGA). IUGR fetuses were compared to control fetuses from ewes carrying singletons ($n = 6$) and twins ($n = 5$). Fetal weight, placental weight, plasma noradrenaline concentrations and other parameters for these fetuses were described previously (Limesand *et al.* 2006; Leos *et al.* 2010). For the lamb treatment groups, 10 ewes (six carrying singletons and four carrying twins) were exposed to hyperthermic conditions from 40 ± 2 to 85 ± 3 dGA. We chose to shorten their exposure to lessen the severity of IUGR in order to produce viable offspring (Galan *et al.* 1999). Control lambs (six single and five twins) were from ewes maintained in a constant thermoneutral environment ($25 \pm 2^\circ\text{C}$; $\sim 25\%$ humidity). Lambs were delivered naturally and housed in individual pens. All lambs received four to six feedings of colostrum before being placed solely on milk replacer with *ad libitum* access (Advance[®] Lamb Milk Replacer, Milk Specialties Co., Dundee, IL, USA). Birth weights were measured within 3 h of birth, usually prior to suckling. Body weights were measured daily at 08.00 h and immediately before each adrenaline challenge study. Singleton lambs were used for gene and protein expression experiments, and twin lambs were used for *in vivo* experiments. Pregnant ewes (133.4 ± 0.7 dGA) and singleton lambs (17.6 ± 0.7 dA) were killed with an intravenous overdose of sodium pentobarbital (86 mg kg^{-1}) and phenytoin sodium (11 mg kg^{-1} ; Euthasol, Virbac Animal Health, Fort Worth, TX, USA). Perirenal adipose, brain, liver, skeletal muscle, spleen, kidney, lung and heart tissues were dissected and weighed. All tissues were snap frozen in liquid nitrogen and stored at -80°C until RNA and protein were extracted.

RNA extraction and cloning

RNA was extracted from perirenal adipose tissue and reverse transcribed as described previously (Cole *et al.* 2009). Synthetic oligonucleotide primers were designed

Table 1. Oligonucleotide primer sequences and description of PCR products cloned for ovine genes

Gene name	Forward (5'–3')	Reverse (5'–3')	Product size (bp)	GenBank accession no.	Identity <i>Bos taurus</i>
α_{1A} -AR	CTCTGGGCTCCTTCTACGTG	CGCAGACACTGGATTCTCAA	475	EU723257	98%
α_{1B} -AR	CGTGGGCAACATTCTAGTCAT	AGTAGCGCACCCCAATGTAG	269	EU851039	97%
α_{1D} -AR	CTTCTCCTCCCTGTGCTCTTT	AGCAGGGGTAGATGAGTGGAT	449	EU723258	96%
α_{2A} -AR	AACGAGGTCATGGGCTACTG	CTCGACGAGATGACGTACCA	326	EU726635	97%
α_{2B} -AR	AGAAGGAAGGGGTCTGTGGT	ACCCAGGCTGTAGCTGAAGA	317	EU741650	94%
α_{2C} -AR	ACACTGGTCATGCCCTTCTC	TGTACCAGTCTCGTCGTTG	328	EU723259	99%
β_1 -AR	ATCGAGACCCTGTGTGCATC	TGTCGATCTTCTCACCTGCT	343	AF072433	98%
β_2 -AR	TCTTCACGAACCAACCCATG	GATCTTCTGGAGCTGCCTTTT	122	EU723260	97%
β_3 -AR	TCTATCTCCGCTTCTGGTGA	GAGTGAAGGTTCCCATGATGA	270	AF314205.1	92%
HSL	AGCAGTGACACAACAGACACG	CAGATCCATCCTCAGATCCAA	106	EU741652	92%
Perilipin	CCTCTGAGCTGAAGGACACC	ACCATCTTCTCGACACCACC	226	NM_001113773.1	96%
Leptin	AGAGGGTCACTGGTTGGACT	TTGGATCACATTCTGGAAGG	125	OAU84247	98%
ADIPOQ	TGGAACCTCTATCTACCCAGA	GCACAGTCATCCCTAACCTCA	166	HM149341	97%

AR, adrenergic receptor; HSL, hormone sensitive lipase; ADIPOQ, adiponectin.

Table 2. Quantitative PCR parameters for ovine genes

Gene name	Forward (5'–3')	Reverse (5'–3')	Product size (bp)	Annealing temperature (°C)
α_{1A} -AR	ACGCACTTCTCCGTGAGACT	ACCCAATGGGCATCACTAAG	124	60.0
α_{1B} -AR	CGTGGGCAACATTCTAGTCAT	AGTAGCGCACCCCAATGTAG	269	60.0
α_{1D} -AR	CTTCTCCTCCCTGTGCTCTTT	AGCAGGGGTAGATGAGTGGAT	449	60.0
α_{2A} -AR	AACGAGGTCATGGGCTACTG	CTCGACGAGATGACGTACCA	326	60.0
α_{2B} -AR	AGGGAGAAGAGGGCTGTGAG	GCCAGCACGAAGGTGAAC	208	60.0
α_{2C} -AR	TCATGGCCTACTGGTACTTCG	AGTAGCGGTCCAGGCTGAT	114	60.0
β_1 -AR	ATCGAGACCCTGTGTGCATC	TGTCGATCTTCTCACCTGCT	343	62.0
β_2 -AR	TCTTCACGAACCAACCCATG	GATCTTCTGGAGCTGCCTTTT	122	60.0
β_3 -AR	TCTATCTCCGCTTCTGGTGA	GAGTGAAGGTTCCCATGATGA	270	60.0
HSL	AGCAGTGACACAACAGACACG	CAGATCCATCCTCAGATCCAA	106	60.0
Perilipin	CCTCTGAGCTGAAGGACACC	ACCATCTTCTCGACACCACC	226	60.0
Leptin	AGAGGGTCACTGGTTGGACT	TTGGATCACATTCTGGAAGG	125	60.4
ADIPOQ	TGGAACCTCTATCTACCCAGA	GCACAGTCATCCCTAACCTCA	166	60.4
S15	ATCATTCTGCCCGAGATGGTG	TGCTTACGGGCTGTAGGTG	134	60.0

AR, adrenergic receptor; HSL, hormone sensitive lipase; ADIPOQ, adiponectin.

with the aid of OligoPerfect Designer software (Invitrogen Life Technologies, Carlsbad, CA, USA; Tables 1 and 2) and purchased from Eurofins MWG Operon (Huntsville, AL, USA). Ovine perirenal adipose cDNA was used as a template to amplify PCR products for ovine $\alpha_{1(A,B,D)}$ -AR, $\alpha_{2(A,B,C)}$ -AR, $\beta_{(1,2,3)}$ -AR, HSL, perilipin, leptin and adiponectin for cloning and nucleotide sequencing as described previously (Cole *et al.* 2009). Nucleotide sequences were compared to *Bos taurus* orthologues with Basic Local Alignment Search Tool and submitted to GenBank (Table 1).

Quantitative real-time PCR (qPCR)

The relative expression of $\alpha_{1(A,B,D)}$ -AR, $\alpha_{2(A,B,C)}$ -AR, $\beta_{(1,2,3)}$ -AR, HSL, perilipin, leptin and adiponectin mRNA transcripts was measured by quantitative PCR using SYBR Green (Qiagen, Valencia, CA, USA) in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules,

CA, USA). After the initial denaturation (95°C for 15 min), all reactions went through 40 cycles of 96°C (30 s), annealing temperature (30 s; Table 2), and 72°C (10 s), at which point the fluorescence intensity was measured. Melt curve analysis, starting at 60°C with an increase of 0.2°C every 6 s to 96°C, was performed at the end of the amplification to confirm product homogeneity. Optimal annealing temperature for each primer set was determined using a temperature gradient (54–62°C), and products were examined on an agarose gel for specificity.

PCR efficiency was determined with gene-specific plasmid DNA, for which threshold cycles (C_t) were linear over concentrations varying by eight orders of magnitude. Samples were run in triplicate. The results were normalized to the reference gene (ribosomal protein S15) using the comparative ΔC_t method (C_t gene of interest – C_t reference gene), and fold change was determined by Pfaffl's and Livak's method (Pfaffl, 2001; Livak & Schmittgen, 2001).

Western immunoblot analysis

Plasma membrane fractions were enriched from perirenal adipose tissue of control and IUGR fetuses and lambs as describe previously (Limesand *et al.* 2004). Briefly, pulverized perirenal adipose was homogenized in 0.25 mol l⁻¹ sucrose, 50 mmol l⁻¹ Hepes (pH 7.7), 0.5 mmol l⁻¹ EDTA, 2 µg ml⁻¹ aprotinin, 5 µg ml⁻¹ leupeptin, 0.2 mmol l⁻¹ phenylmethylsulfonyl fluoride (PMSF), and 3 mmol l⁻¹ DTT. Three successive centrifugations at 1200 g (10 min), 9000 g (15 min), and 25,000 g (20 min) were performed on the supernatant before the plasma membranes were pelleted with a 90 min centrifugation at 100,000 g. Plasma membrane pellets were washed once and resuspended in 0.25 mol l⁻¹ sucrose, 50 mmol l⁻¹ Hepes (pH 7.7), 100 mmol l⁻¹ KCl, 5 mmol l⁻¹ MgCl₂, 2 µg ml⁻¹ aprotinin, 5 µg ml⁻¹ leupeptin, 0.2 mmol l⁻¹ PMSF, and 3 mmol l⁻¹ DTT. Protein concentrations were determined with the bicinchoninic acid protein assay (Thermo Fisher Scientific Inc., Rockford, IL, USA). Purity and enrichment of plasma membrane proteins from perirenal adipose tissue were confirmed by evaluating Na⁺/K⁺ ATPase (anti-Na⁺/K⁺ ATPase α-1 clone C464.6, Millipore, Temecula, CA, USA).

Plasma membrane enriched perirenal adipose proteins (100 µg) were separated by 10% SDS-PAGE along with Precision Plus Protein Standards (Bio-Rad Laboratories). The electrophoresed proteins were transferred to polyvinylidene fluoride membrane (Bio-Rad Laboratories) and blocked in 5% non-fat dry milk with Tris-buffered saline (10 mmol l⁻¹ Tris-HCl, 150 mmol l⁻¹ NaCl, pH 8) with 0.05% Tween 20 (TBST) at room temperature for 1 h. Immunoblot detection of β₂-AR and perilipin were accomplished, respectively, with a rabbit anti-β₂-AR and anti-perilipin affinity-purified polyclonal antibody (β₂-AR, sc-569; perilipin, sc-67164, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1 µg ml⁻¹ in TBST containing 5% non-fat dry milk at 4°C for 16 h. The membrane was then washed 3 times for 10 min in TBST buffer. Binding of the rabbit antiserum was detected with anti-rabbit immunoglobulin G horseradish peroxidase conjugated secondary antibody (1:10,000; Bio-Rad Laboratories) in TBST for 1 h at room temperature and detected using SuperSignal West Pico (Thermo Fisher Scientific) exposed to Kodak x-ray film. The antibody specificity was tested in competition experiments with the β₂-AR blocking peptide (Santa Cruz; 10 µg ml⁻¹), which was added to the diluted rabbit anti-β₂-AR antiserum (1 µg ml⁻¹) and incubated at 4°C overnight (Limesand *et al.* 2004). Equality of loading was determined with Ponceau S staining (Sigma-Aldrich, St Louis, MO, USA). Densitometry analysis was completed with ImageJ software v. 1.41 (National Institutes of Health, USA), and data are presented as the mean ± S.E.M. Multiple

exposure times were analysed to confirm that films were not saturated.

Adrenaline challenge in lambs

An adrenaline challenge was performed on the twin lambs at 21.1 ± 1.1 days of age (dA). Catheters were placed in a jugular vein of lambs 1–2 days prior to study. Catheters were stored in a 4 cm × 4 cm elastic pouch secured to the neck with Vetrap bandaging tape (3M Animal Care Products, St Paul, MN, USA) and glue (Tag Cement, The Ruscoe Co., Akron, OH, USA). All challenges were performed on lambs in the fed state. During the adrenaline challenge, eight venous blood samples from control (23.0 ± 0.7 dA; n = 5) and IUGR (21.5 ± 2.1 dA; n = 4) lambs were collected in syringes lined with EDTA (Sigma-Aldrich) at -40, -20, 2.5, 5, 10, 15, 30 and 50 min relative to the adrenaline bolus. Three different adrenaline doses (0.4, 0.8, 1.6 µg kg⁻¹) were administered in random order as a bolus to each lamb at time 0. The three independent studies were separated by at least 6 h. Plasma was separated by centrifuging (16,000 g) for 2 min at 4°C and then aspirated from the pelleted red blood cells and stored at -80°C for subsequent determination of insulin and NEFA concentrations. Samples collected at -40 and -20 min were considered baseline measurements and the responsiveness to adrenaline (from 2.5 min to 50 min) was determined by calculating the area under the curve (AUC) from baseline (Prism 5.01, GraphPad Software, La Jolla, CA, USA).

Biochemical analysis

Blood pH, oxygen saturation and haemoglobin concentrations were measured in whole blood collected in syringes lined with dried heparin (Elkins-Sinn, Inc., Cherry Hill, NJ, USA) on an ABL 725 (Radiometer, Copenhagen, Denmark). The body temperature of each lamb was measured prior to the study and used for temperature correction of pH and calculations. Plasma glucose and lactate concentrations were also measured immediately using a YSI model 2700 SELECT Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA). Plasma insulin concentrations were measured by an ovine insulin enzyme-linked immunosorbent assay (ALPCO Diagnostics, Windham, NH, USA) as previously reported (Leos *et al.* 2010). The inter- and intra-assay coefficients were 5.2% and 4.9%, respectively.

Plasma NEFA concentrations were measured by enzymatic colorimetric analysis using a commercial kit (Wako Chemicals USA, Richmond, VA, USA) as modified by Baumgard *et al.* (2000). The reactions were conducted in 96-well microplates (Thermo Fisher Scientific) and

Table 3. Lamb biometry measurements, gestational length, and dry matter intake

	16 days of age		21 days of age	
	Control (6)	IUGR (6)	Control (5)	IUGR (4)
Gestation (days)	152.2 ± 0.5	149.3 ± 1.0*	151.2 ± 0.9	148.5 ± 0.3*
Birth weight (kg)	4.40 ± 0.39	3.48 ± 0.47	3.66 ± 0.33	2.88 ± 0.46
Study weight (kg)	7.43 ± 0.64	5.86 ± 1.22	8.28 ± 0.76	6.65 ± 0.82
Average DMI (g)	168.72 ± 13.58	157.97 ± 17.18	245.01 ± 18.73	237.12 ± 9.08

Values are means ± S.E.M.; IUGR, intrauterine growth restriction; DMI, dry matter intake; *, $P < 0.05$.

absorbance read on a microplate spectrophotometer (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA). Briefly, the method relies upon the acylation of coenzyme A (CoA) by NEFAs in the presence of acyl-CoA synthetase. The acyl-CoA thus produced is oxidized by acyl-CoA oxidase with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase, permits the oxidative condensation of 3-methyl-*N*-ethyl-*N*-(β -hydroxyethyl)-aniline with a peak absorbance at 550 nm. The inter- and intra-assay coefficients were 4.6% and 4.5%, respectively.

Statistical analysis

Statistical analysis of the body and organ weights, qPCR (ΔC_t), and western immunoblot data were subjected to a one-way ANOVA, using the general linear model procedure in SAS Proc GLM (SAS v. 9.1, SAS Institute Inc., Cary, NC, USA), and differences were determined by Student's *t* test. Statistical analysis of qPCR data was performed on the ΔC_t values. In order to avoid a lack of independence between twin pairs, only one lamb from each ewe was evaluated. Biochemical, haematological and hormone measurements during the adrenaline challenge were analysed with a SAS Proc MIXED method that accounted for lamb as a random variable (SAS/STAT User's Guide, v. 6, 4th edn, SAS Institute Inc.). Values for dGA are presented as the mean ± S.D.; all other values are expressed as the mean ± S.E.M.

Results

Morphometric characteristic of fetuses and lambs

In IUGR fetuses at 133 dGA, the average body weight was lower than controls (singles 1.44 ± 0.15 kg versus 3.22 ± 0.18 kg, $P < 0.01$; twins 1.51 ± 0.11 kg versus 3.02 ± 0.17 kg, $P < 0.05$). Placental weight was also reduced in IUGR fetuses (singles 117 ± 14 g versus 285 ± 20 g, $P < 0.01$; twins 119 ± 20 g versus 187 ± 20 g, $P < 0.05$). In the independent lamb cohorts, IUGR birth weights and body weights at 16 and 21 dA (Table 3) were not significantly less than controls ($P < 0.1$), but when lamb birth weights were combined and pregnancy type

(single or twin) was used as a covariate in the statistical model, the consistent 20% reduction in birth weight in IUGR lambs was significant ($P < 0.05$). The length of gestation was approximately 3 days shorter ($P < 0.05$) in both IUGR singletons and twins than controls. In singletons, IUGR and control lambs grew at the same rate of 0.16 ± 0.02 kg day⁻¹ during the first 16 days of life. In twins during the first 21 days, growth rate was also similar; control lambs gained 0.22 ± 0.02 kg day⁻¹ and IUGR lambs gained 0.17 ± 0.02 kg day⁻¹, neither of which were different from the singleton average daily gain. When the singleton and twin average daily gain measurements were combined, no differences were found between control ($n = 11$) and IUGR ($n = 10$) treatments (Fig. 1). The average daily dry matter intake (DMI) was also not different between treatments (Table 3).

mRNA expression in perirenal adipose tissue

Adrenergic receptor cDNA for all the genes of interest were cloned from fetal sheep or lamb perirenal adipose mRNA. Nucleotide sequences for these cloned PCR products shared greater than 92% identity with the *Bos taurus* orthologues (Table 1).

The β_2 -AR mRNA concentrations were 59% lower ($P < 0.01$) in IUGR singleton fetuses and 74% lower ($P < 0.05$) in IUGR lambs compared to the respective

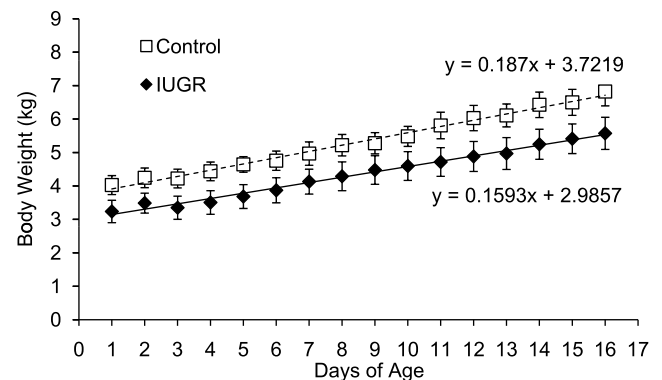


Figure 1. Growth rates in lambs

Body weights for control (\square , $n = 11$) and IUGR lambs (\blacklozenge , $n = 10$) are presented for their first 16 days of postnatal life. Values are means ± S.E.M.

controls (Fig. 2A and B). In singleton IUGR fetuses, perilipin mRNA concentrations were 43% less ($P < 0.05$) than in control fetuses, but there was no treatment effect in lambs. Perilipin protein concentrations were not different between treatments in fetuses or lambs (data not shown). In singleton IUGR fetuses, leptin mRNA concentrations were 49% lower ($P < 0.05$) than control fetuses and also tended to be lower ($P < 0.1$) in IUGR lambs. No differences in mRNA expression were observed for HSL, adiponectin and all other ARs (Fig. 2). The findings were similar for ARs in twins; β_2 -AR mRNA concentration were lower ($P < 0.05$) in IUGR twins compared to their controls. However, in twin fetuses, leptin and perilipin mRNA expression levels were not different between IUGR

and control treatments (data not shown). In control twin fetuses, leptin mRNA concentrations were less ($P < 0.05$) than singletons control.

β_2 -AR protein levels in perirenal adipose tissue

The antibody against β_2 -AR detected a single protein with an apparent molecular mass of 68 kDa, and this interaction was blocked with the immunizing peptide (data not shown). The average β_2 -AR protein level was $56 \pm 14\%$ ($P < 0.05$) lower in IUGR fetuses (Fig. 3A) and $48 \pm 12\%$ ($P < 0.05$) lower in IUGR lambs compared to controls (Fig. 3B).

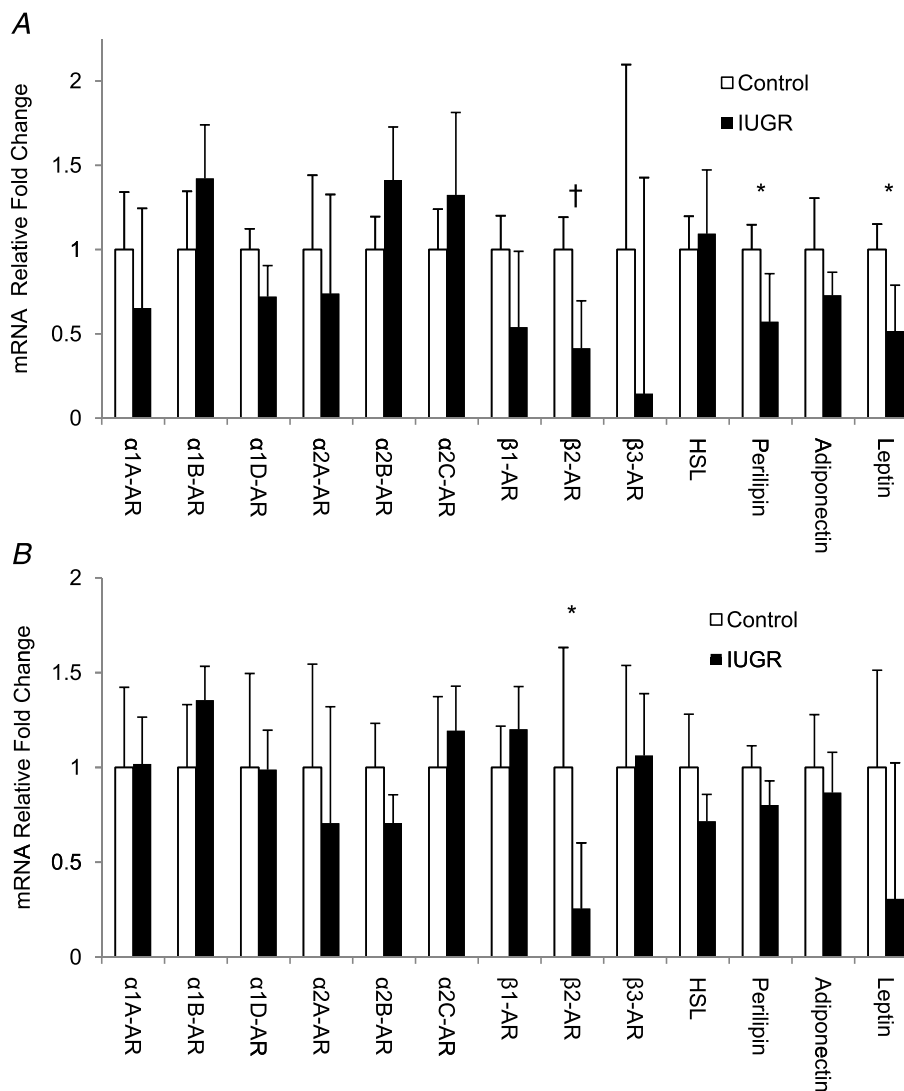


Figure 2. mRNA profiles in perirenal adipose tissue

mRNA concentrations for the genes listed on the abscissa are normalized to ribosomal protein S15 and expressed as the fold change from control calculated by the $2^{-\Delta\Delta C_t}$ in perirenal adipose tissue collected from singleton fetuses (A) and lambs (B). Mean \pm s.e.m. values for control (■) and IUGR (□) animals are presented. Significance is indicated by * ($P < 0.05$) and † ($P < 0.01$).

Table 4. Haematological values for lambs prior to and after the adrenaline challenge studies

Treatment condition	Control	IUGR
Prior to the adrenaline challenge		
pH	7.41 ± 0.02	7.43 ± 0.02
O ₂ (mmol l ⁻¹)	3.30 ± 0.18	3.35 ± 0.65
Haematocrit (%)	31.21 ± 1.32	33.63 ± 3.08
After adrenaline challenge		
pH	7.39 ± 0.01	7.42 ± 0.02
O ₂ (mmol l ⁻¹)	3.33 ± 0.24	3.58 ± 0.43
Haematocrit (%)	31.39 ± 1.29	32.7 ± 3.35

Values are means ± S.E.M.; IUGR, intrauterine growth restriction.

Table 5. Plasma metabolite and insulin concentrations in lambs prior to and after the adrenaline challenge studies

Treatment condition	Control	IUGR
Prior to the adrenaline challenge		
Glucose (mmol l ⁻¹)	6.84 ± 0.20	6.98 ± 0.44
Lactate (mmol l ⁻¹)	1.31 ± 0.10	1.33 ± 0.19
Insulin (μg l ⁻¹)	0.76 ± 0.19	1.34 ± 0.22
Response after adrenaline challenge (AUC)		
Glucose (mmol min l ⁻¹)	25.01 ± 6.67	51.83 ± 3.41*
Lactate (mmol min l ⁻¹)	8.81 ± 2.91	17.67 ± 1.82*
Insulin (μg min l ⁻¹)	25.12 ± 16.31	13.23 ± 4.87

Values are means ± S.E.M.; IUGR, intrauterine growth restriction; AUC, area under the curve; **P* < 0.05.

Adrenaline challenge in lambs

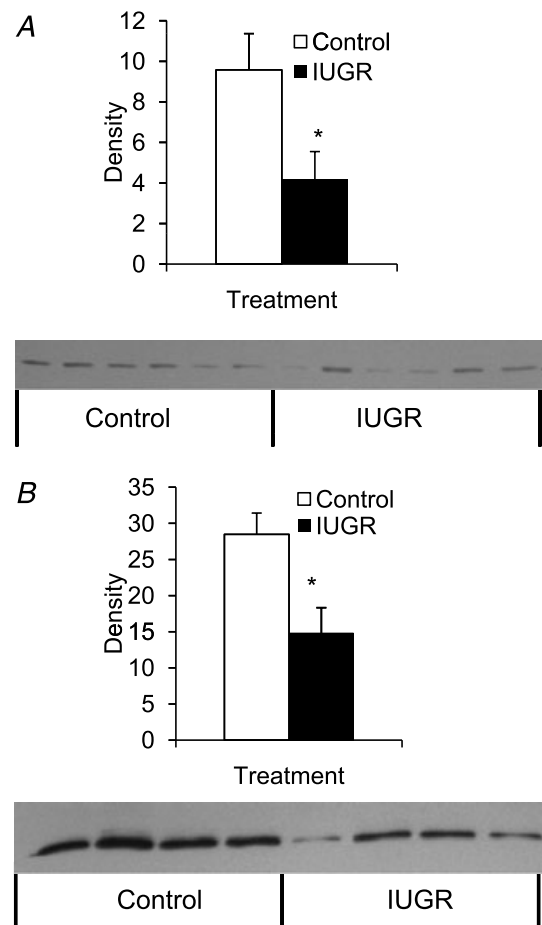
To determine adrenergic responsiveness, adrenaline was administered to control and IUGR lambs. The pH, oxygen content, haematocrit, glucose, insulin and lactate concentrations are presented for the highest dose (1.6 μg kg⁻¹ adrenaline; Tables 4 and 5). Haematological values were unchanged by adrenaline (Table 4). The baseline plasma glucose and lactate concentrations were similar between treatments; however, plasma glucose and lactate concentrations increased in response to the adrenaline challenge to a greater extent (*P* < 0.05) in the IUGR lambs compared with control lambs (Table 5).

All three adrenaline doses caused an elevation in plasma NEFA concentrations, and AUC values were not different between doses (data not shown). Therefore, the NEFA results from all three doses were combined to provide three replicate measurements per lamb. Baseline NEFA concentrations were similar between IUGR (468 ± 81 μEq l⁻¹) and control (338 ± 33 μEq l⁻¹) lambs (Fig. 4). However, the NEFA AUC was 55 ± 15% lower in IUGR lambs (*P* < 0.05) than in controls (Fig. 4).

Discussion

In the present study, we demonstrate that adrenergic desensitization occurs in response to placental

insufficiency-induced IUGR and can persist throughout the perinatal transition, causing impaired adrenaline induced lipolysis in lambs. Declines in β₂-adrenergic receptor mRNA and protein concentrations in perirenal adipose tissue appear to be the primary cause for the impaired adrenergic responsiveness. Previously, we showed that plasma noradrenaline concentrations are elevated in IUGR singleton and twin fetuses above threshold for all physiological aspects and are negatively associated with blood oxygen content (Padbury *et al.* 1987; Limesand *et al.* 2006; Leos *et al.* 2010). This indicates that hypoxaemia chronically raises fetal catecholamine concentrations during placental insufficiency, thus redistributing nutrients by suppressing insulin (Bassett & Hanson, 2000; Leos *et al.* 2010). Previous reports have shown that prolonged administration of catecholamines to fetal sheep causes adrenergic desensitization because

**Figure 3. Immunoblot analysis for β₂-AR in perirenal adipose tissue**

β₂-AR protein concentrations were measured in fetuses (A) and lambs (B). Representative immunoblots for plasma membrane enriched fractions from control (□) and IUGR (■) perirenal adipose tissue are presented below the bar graphs, which summarize the 68 kDa band intensities from each treatment group. Significance from Student's *t* test is depicted with an * (*P* < 0.05) for each age.

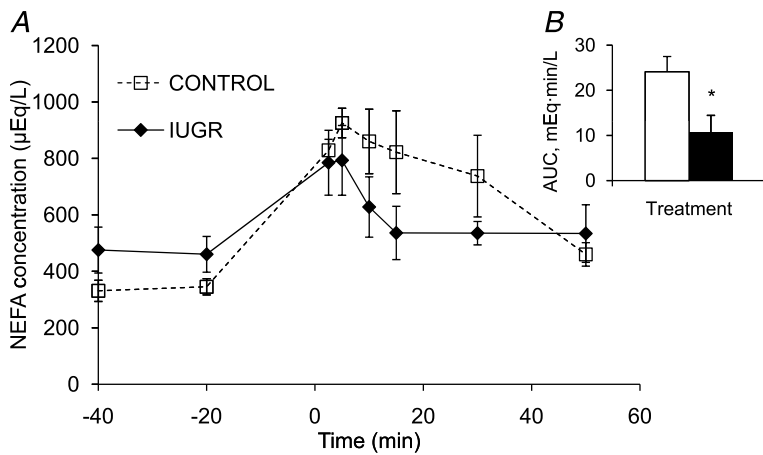


Figure 4. Adrenergic responsiveness in lambs at 21 days of age

During the adrenaline challenge, mean plasma NEFA concentrations (y-axis) for control (□) and IUGR (◆) lambs are presented for the duration of the study in the line graph. Time (x-axis) is presented relative to the administration of adrenaline, which occurred at time 0. Baseline values were taken as the average of samples prior to giving adrenaline, and area under the curve (AUC) post-adrenaline was calculated. AUC values are presented in the inserted bar graph with * indicating difference with $P < 0.05$.

several metabolite and hormone values returned to normal (Bassett & Hanson, 1998; Bassett & Symonds, 1998). Adrenergic desensitization is known to result from reductions in receptor mRNA concentration in a cAMP-dependent fashion due to either a suppression of receptor gene transcription or selective degradation of receptor mRNA (Bouvier *et al.* 1989; Collins *et al.* 1991; Hosoda *et al.* 1995; Kirigiti *et al.* 2001). Our current study in perirenal adipose tissue supports this assertion (Figs 2 and 3). The impairment in lipid mobilization is likely to leave IUGR offspring at greater risk for obesity and dyslipidaemia in postnatal life.

Catecholamines serve as regulators of intermediary metabolism and stimulate the mobilization of energy stores. IUGR lambs challenged with adrenaline at 21 dA had a lower NEFA response (Fig. 4), showing that lipid mobilization is impaired. In adipose tissue, adrenaline acts primarily through the β_2 -AR to stimulate HSL to hydrolyse stored triglycerides into free fatty acids and glycerol (Fig. 5; Carmen & Victor 2006). Signalling for lipolysis occurs via a cAMP-dependent protein kinase A pathway, where HSL and perilipin are activated by phosphorylation after catecholamines bind to the β_2 -AR. HSL is the rate-limiting enzyme for lipolysis, and perilipin undergoes conformational changes to facilitate HSL localization with the lipid droplet surface (Tansey *et al.* 2004). Therefore, reductions in β_2 -AR in the perirenal adipose tissue (Figs 2 and 3) will lead to decreased activation of downstream signalling events. Thus, the reduced adrenergic responsiveness observed in the present study is most likely due to lower mRNA and protein concentrations of β_2 -AR, because HSL and perilipin mRNA concentrations were not different between control and IUGR lambs.

Placental restricted lambs with IUGR created by uterine carunclectomy exhibited postnatal catch-up growth by 45 days (De Blasio *et al.* 2007). Although body weights were not different between controls and placental restriction lambs at autopsy, the ratios of visceral and peri-

renal adipose tissues relative to body weight were greater in placental restricted lambs (De Blasio *et al.* 2007). In our study, the weight of perirenal adipose tissue relative to body weight was not different between treatments in lambs at 18 dA. This may be too early to distinguish any treatment effects for fat mass, because differences in rate of gain and DMI were not observed (Fig. 1).

Global energy deficiency during hypoxia increases cAMP concentrations, which stimulates glycolysis and synthesis of pyruvate in muscle, leading to the

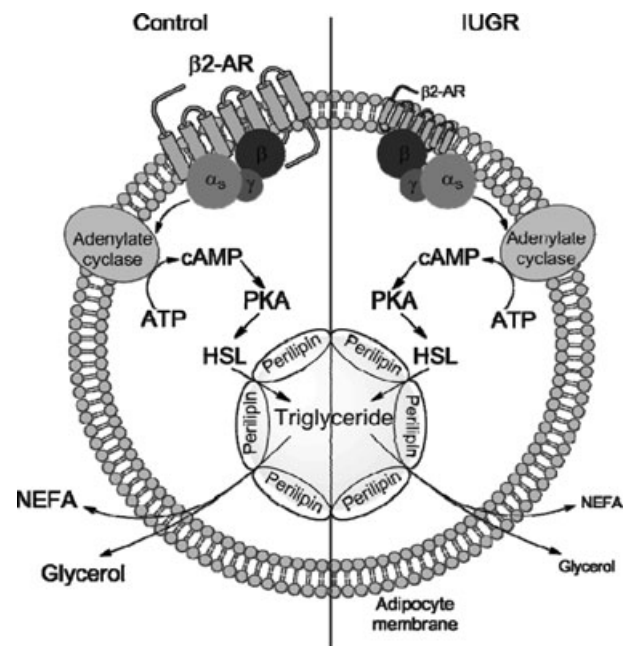


Figure 5. β_2 -AR signalling pathway of lipolysis in adipocytes

In control fetuses and lambs, stimulation of the β_2 -AR increases cAMP production to activate PKA to phosphorylate HSL and perilipin, which hydrolyses intracellular triglyceride stored in the lipid droplet. IUGR fetuses and lambs have lower β_2 -AR concentrations, which desensitizes them to catecholamine stimulate lipolysis, resulting in less NEFA and glycerol released from adipocytes. In the diagram, reductions in the IUGR animals are portrayed by smaller font and receptor size.

accumulation of cytoplasmic pyruvate and NADH. Because oxygen is lacking, lactate dehydrogenase converts pyruvate to lactate, which can be released into circulation and used as a substrate for hepatic gluconeogenesis. Interestingly, we have previously reported that IUGR fetuses have approximately twice as much skeletal muscle glycogen as controls (Limesand *et al.* 2007). Therefore, if elevated glycogen persists in lambs, it would promote glycogenolysis and lactate production. In IUGR lambs, a greater lactate response was found during the adrenaline challenge (Table 5). Thus, we postulate that increased lactate generation is caused by increased glycogenolysis in skeletal muscle. In addition to lowering NEFA mobilization, lower β_2 -AR will also reduce the efficiency of free fatty acid utilization as an energy source. Similar attributes in glycogenolysis would be expected in skeletal muscle, which also has lower β_2 -AR expression (X. Chen and S. W. Limesand, unpublished observation), but the increased glycogen content might partially compensate for the deficiencies in catabolic efficiency. Therefore, it appears that in order for IUGR lambs to appropriately meet metabolic demands, they may shift substrate metabolism from fat to glucose by increasing glycolysis and glycogenolysis. This shift is further supported by the observed elevation in plasma glucose concentrations post-adrenaline challenge in IUGR lambs (Table 5).

In addition to lipid storage, adipose tissue also has endocrine functions; it synthesizes and secretes adiponectin and leptin. These adipokines regulate glucose and fatty acids to control energy balance and metabolism. In our study, adiponectin expression was not different between IUGR and control fetuses or lambs, similar to findings in the uterine carunclectomy model (Muhlhausler *et al.* 2007*a,b*). Leptin expression, however, was reduced in perirenal adipose tissue of IUGR fetuses and tended to be lower in IUGR lambs. Interestingly, lower leptin expression was not specific to placental insufficiency-induced IUGR because twin fetuses, which are growth restricted compared to singletons, also had lower leptin mRNA concentrations. In near-term fetal sheep, leptin has been infused chronically to raise leptin concentrations for 5 days, where it was found to inhibit gluconeogenic enzymes and endogenous glucose production (Forhead *et al.* 2008). Furthermore, in rat hepatocyte cultures, exposure to higher leptin concentrations lowers gluconeogenesis by inhibiting phosphoenolpyruvate carboxykinase (PEPCK) (Anderwald *et al.* 2002). However, in our model of IUGR, hepatic gluconeogenesis is occurring, and gluconeogenic enzymes, glucose-6-phosphatase and PEPCK, are elevated (Limesand *et al.* 2007). Therefore, the reduction in leptin may also be playing an important role in IUGR nutrient redistribution by promoting gluconeogenesis. In the lamb, adequate nutrients are available, but if glucose rather than

fat utilization is occurring, lower leptin concentrations might prevail albeit to a lesser extent (Fig. 2).

In summary, we have shown that placental insufficiency-induced IUGR leads to β_2 -AR down-regulation in the perirenal adipose tissue of sheep fetuses, which most likely is caused by the chronic elevation of catecholamines in late gestation (Limesand *et al.* 2006; Leos *et al.* 2010). Furthermore, we show that reductions in β_2 -AR persist in IUGR lambs, causing AR desensitization that impairs adrenaline-stimulated lipolysis. Interestingly, plasma lactate and glucose concentrations were elevated in IUGR lambs following the adrenaline challenge, indicating that carbohydrate metabolism is enhanced while fat metabolism is spared. These data begin to explain how endocrine factors, like catecholamines, may facilitate prenatal adaptations to benefit survival but ultimately result in impaired metabolic function after birth, leading to dyslipidaemia and obesity.

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Author contributions

X.C. and S.W.L. designed the experiment. All authors participated in data collection, analysis, and interpretation. X.C. and S.W.L. wrote the manuscript. All authors revised the manuscript and approved the final version. The experiments were conducted at the William J. Parker Agricultural Research Complex, Department of Animal Sciences, University of Arizona, Tucson AZ, USA.

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