Betamethasone-exposed preterm birth does not impair insulin action in adult sheep

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Abstract

Preterm birth is associated with increased risk of type 2 diabetes (T2D) in adulthood; however, the underlying mechanisms are poorly understood. We therefore investigated the effect of preterm birth at ~0.9 of term after antenatal maternal betamethasone on insulin sensitivity, secretion and key determinants in adulthood, in a clinically relevant animal model. Glucose tolerance and insulin secretion (intravenous glucose tolerance test) and whole-body insulin sensitivity (hyperinsulinaemic euglycaemic clamp) were measured and tissue collected in young adult sheep (14 months old) after epostane-induced preterm (9M, 7F) or term delivery (11M, 6F). Glucose tolerance and disposition, insulin secretion, β -cell mass and insulin sensitivity did not differ between term and preterm sheep. Hepatic *PRKAG2* expression was greater in preterm than in term males ($P=0.028$), but did not differ between preterm and term females. In skeletal muscle, *SLC2A4* ($P=0.019$), *PRKAA2* ($P=0.021$) and *PRKAG2* ($P=0.049$) expression was greater in preterm than in term overall and in males, while *INSR* ($P=0.047$) and *AKT2* ($P=0.043$) expression was greater in preterm than in term males only. Hepatic *PRKAG2* expression correlated positively with whole-body insulin sensitivity in males only. Thus, preterm birth at 0.9 of term after betamethasone does not impair insulin sensitivity or secretion in adult sheep, and has sex-specific effects on gene expression of the insulin signalling pathway. Hence, the increased risk of T2D in preterm humans may be due to factors that initiate preterm delivery or in early neonatal exposures, rather than preterm birth *per se*.

Key Words

- ▶ sheep
- ▶ preterm birth
- ▶ glucose metabolism
- ▶ insulin signalling
- ▶ glucocorticoid

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Introduction

In developed countries the incidence of preterm birth, defined as delivery before 37 completed weeks' gestational age (GA), is ~7–10% and is progressively increasing

(Ananth *et al.* 2013, Li *et al.* 2013). Approximately 80% of preterm babies are born moderate to late preterm, at 32–36 completed weeks' GA, or 0.8–0.9 of term

(Li *et al.* 2013). In human epidemiological studies, preterm birth (including moderate and late preterm) is associated with increased risks of type 2 diabetes (T2D) in adult life (Lawlor *et al.* 2006, Kaijser *et al.* 2009, Kajantie *et al.* 2010, 2014, Pilgaard *et al.* 2010), with poorer insulin sensitivity implicated as an underlying mechanism (Tinnion *et al.* 2013). Direct measures of insulin sensitivity by hyperinsulinaemic euglycaemic clamp (HEC) are lower in young adult men born preterm than those born at term (Rotteveel *et al.* 2008, 2011, Mathai *et al.* 2012). Although there is evidence that insulin secretion increases to compensate and maintain insulin action in some preterm populations (Mathai *et al.* 2012, Kajantie *et al.* 2014), the overall increased risk of T2D after preterm birth implies that this is not always adequate and that insulin secretion is also impaired after preterm birth.

To explore the underlying mechanisms for effects of preterm birth on glucose metabolism, including on the pancreas and insulin-responsive tissues (skeletal muscle and liver), we have used a clinically relevant ovine model of preterm birth (De Matteo *et al.* 2009, 2010, Bensley *et al.* 2010). This also allows us to test causality for effects of preterm birth independent of confounding effects of other exposures. Importantly for studies of the effects of perinatal events on insulin action, the pancreas and β -cells develop at similar stages of gestation in sheep and humans (Gatford *et al.* 2010), and prenatal exposures therefore affect similar stages of pancreatic development in both species. Antenatal administration of the glucocorticoids, betamethasone or dexamethasone, to pregnant ewes improves lung maturation and survival of preterm-delivered lambs (Liggins 1969, De Matteo *et al.* 2010), consistent with clinical responses in humans (Liggins & Howie 1972, Roberts & Dalziel 2006, Brownfoot *et al.* 2013). It is therefore routine clinical practice to administer antenatal glucocorticoids to all women at risk of delivering preterm (Brownfoot *et al.* 2013), but there is some evidence for long-term effects on exposed progeny. Antenatal glucocorticoid exposure in sheep induces variable impairments in metabolic outcomes in progeny, which may be due to the different doses and compounds administered and/or timing of delivery. To date, however, there have been no reports in sheep of metabolic outcomes after combined exposure to preterm birth and antenatal betamethasone, which like dexamethasone is commonly administered to women in cases of threatened preterm labour (Brownfoot *et al.* 2013). We hypothesised that preterm birth after exposure to a clinical dose of betamethasone would impair glucose

tolerance, insulin sensitivity and insulin secretion in adult ovine progeny. Furthermore, we hypothesised that this exposure would decrease expression of insulin signalling pathway genes in two key insulin-responsive tissues, skeletal muscle and liver, and would decrease β -cell mass in pancreas of these adult progeny. Because morbidity after preterm birth is worse in males than females (Liggins & Howie 1972, De Matteo *et al.* 2010), and other perinatal exposures have sex-specific effects on ovine progeny (Owens *et al.* 2007), we assessed outcomes in adult offspring of both sexes.

Materials and methods

Animal cohort

All studies were jointly approved by the Monash University Animal Ethics Committee (MMCA-2011/01) and University of Adelaide Animal Ethics Committee (M-2013-173), and conducted in accordance with Australian guidelines (National Health and Medical Research Council of Australia 2004). Animal management during pregnancy and neonatal life, induction of preterm delivery and antenatal glucocorticoid treatment were as described previously (Nguyen *et al.* 2016). Briefly, Border Leicester $\times$ White Suffolk ewes were time-mated to White Suffolk rams. Pregnant ewes carrying singleton foetuses were randomly allocated to deliver either preterm at 132 ± 1 days' GA (~ 0.9 of term, term = 147 days) or delivered near term. As previously reported, 57% of preterm and 94% of control progeny survived to adulthood (Nguyen *et al.* 2016), and the present study reports outcomes in all adult progeny (16 preterm: 9M, 7F and 17 term: 11M, 6F). Ewes allocated to deliver preterm were treated with clinical doses of betamethasone (two doses of 11.4 mg i.m., Celestone Chronodose, Schering-Plough, North Ryde, Australia), at 130 ± 1 days' and at 131 ± 1 days' GA (Nguyen *et al.* 2016). Unlike dexamethasone (Bansal *et al.* 2015), clinical doses of betamethasone at 0.8–0.9 of gestation do not induce parturition in sheep. Preterm delivery at 132 ± 1 days was therefore induced by administration of epostane (50 mg in 2 mL 100% EtOH i.v.; Sanofi-Synthlabo, Winthrop, Guildford, UK) to pregnant ewes at 130 ± 1 days' GA, ~ 5 h after the first betamethasone injection (De Matteo *et al.* 2008, 2009, 2010, Nguyen *et al.* 2016). By inhibiting 3β -hydroxysteroid dehydrogenase, epostane rapidly reduces circulating progesterone concentrations in the ewe, which is followed by rapid upregulation of uterine prostaglandin $F_{2\alpha}$ production and induces parturition 30–40 h after administration in ewes near term at

137–142 days' GA (Silver 1988). Neonatal support of preterm lambs is described elsewhere (Nguyen *et al.* 2016). Control ewes were induced to deliver lambs vaginally near term by administration of epostane (dose as above) at 145 ± 1 days' GA (De Matteo *et al.* 2008, 2009, 2010, Nguyen *et al.* 2016).

Following birth, all lambs were housed in individual pens with their mothers for 4–6 weeks. After weaning at 12 weeks of age, male and female progeny were housed in separate paddocks (Nguyen *et al.* 2016); animals of both sexes were reproductively intact and oestrous cycles of females were not synchronised or manipulated during the study. The animals grazed natural pastures and were provided with 800–1000 g/sheep of lucerne/hay daily and 200 g/sheep of Rumevite pellets weekly (Ridley AgriProducts, Melbourne, Australia). At ~12 months of age, sheep were transported to an indoor animal facility where they were housed in individual metabolic cages on a 12 h light:12 h darkness cycle for the remainder of the experiment. During this period, sheep had *ad libitum* access to water and were fed 800–1000 g lucerne chaff twice daily, except when fasted before metabolic tests as described below.

In vivo measures of insulin secretion, sensitivity and action

After 5–8 days acclimatisation, and at least 3 days prior to metabolic tests, catheters were surgically implanted into the left femoral vein and femoral artery under aseptic conditions. Analgesia was provided by a pre-operative s.c. injection of 0.5% bupivacaine with adrenaline (10–15 mL Marcaine; Astra Zeneca) at the inguinal incision site, and then by administration of a transdermal fentanyl patch (7.5 mg Durogesic, Ortho-McNeil-Janssen Pharmaceuticals, Inc, Titusville, NJ, USA) at surgery to provide 3 days post-operative analgesia. General anaesthesia was induced by i.v. injection of thiopentone sodium (20 mg/kg) and maintained by isoflurane inhalation (1.5–2.5% in 70/30 O₂/N₂O); antibiotics were injected daily for 3 days post-surgery (ampicillin 500 mg i.v.; Aspen Pharmcare Australia Pty Ltd, St Leonards, NSW, Australia). Catheters were maintained by daily flushing with heparinised saline (100 IU/mL). Glucose tolerance and glucose-stimulated insulin secretion were measured during an intravenous glucose tolerance test (IVGTT, 0.25 g glucose/kg body weight) at 432 ± 2 days of age, and glucose tolerance indices and insulin secretion were calculated as previously described (Gatford *et al.* 2004, De Blasio *et al.* 2007, Owens *et al.* 2007). Two

days later, the whole-body insulin sensitivity of glucose metabolism was measured by HEC (2 U insulin.kg⁻¹.min⁻¹, 2 h, variable glucose infusion to restore/maintain euglycaemia) (Gatford *et al.* 2004). Insulin sensitivity_{glucose} and basal and maximal insulin-stimulated glucose disposition indices (DI) were calculated as previously described (Gatford *et al.* 2004).

Post-mortem

At least two days after completion of the *in vivo* studies, sheep were fasted overnight and then humanely killed by venous administration of an overdose of Lethobarb (Virbac, Milperra, NSW, Australia), and the pancreas, liver, heart, lungs, brain, kidneys, spleen, skeletal muscles (M. semitendinosus, M. soleus, M. vastus lateralis) and a dissectible adipose depot (perirenal fat) were removed and weighed (Nguyen *et al.* 2016). Tissue samples were snap frozen in liquid nitrogen and stored frozen at –80°C for studies of gene expression. Representative mixed aliquots of each pancreas were fixed in 10% buffered formalin prior to processing, embedding and sectioning as previously described (Gatford *et al.* 2008).

Analysis of plasma insulin and glucose

Plasma insulin concentrations were measured in duplicate by a double antibody, solid-phase radioimmunoassay using a commercially available kit (human insulin-specific RIA, HI-14K, Linco Research). The intra- and inter-assay coefficients of variation for the insulin assay were 5.1% and 12%, respectively, for an ovine plasma sample containing 14 U.L⁻¹ insulin ($n=15$ assays). Intra-assay coefficients of variation were 6.8% and 7.1%, and inter-assay coefficients of variation were 13.0% and 13.8% for human insulin quality control samples containing 9.1 and 49.2 U.L⁻¹ insulin, respectively. Plasma glucose concentrations were measured by colorimetric enzymatic analysis using a Hitachi 912 automated metabolic analyser and Roche/Hitachi Glucose/HK kits (Roche Diagnostics GmbH).

Immunostaining and morphometric analysis of pancreas

One pancreas section per block (5 µm) was immunostained to detect insulin-positive cells as described previously (Gatford *et al.* 2008). Stained slides were digitally captured at 40× magnification (NanoZoomer, Hamamatsu, Japan). β-Cell volume density (V_d) was

quantified by point counting (209 points/field), and numbers of islets, small islets (<5 β -cells) and β -cells per islet were counted in 30 fields per section, selected by random-systematic sampling (each field 0.217 mm²) and analysed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (Gatford *et al.* 2008). β -Cell mass was calculated by multiplying V_d by pancreas mass (Gatford *et al.* 2008). The number of fields counted was based on β -cell volume density and variation between fields and was validated in our previous studies to give a SEM of <10% within individuals in sheep at this age (Gatford *et al.* 2008).

Gene expression

We measured gene expression of components of the insulin signalling pathway in liver and M. vastus lateralis, as skeletal muscle accounts for >80% of insulin-stimulated glucose uptake (DeFronzo & Tripathy 2009). The M. vastus lateralis is a mixed-fibre type muscle, and we have previously reported that intra-uterine growth restriction (IUGR) impairs gene expression of determinants of insulin signalling in this muscle in sheep (De Blasio *et al.* 2012), consistent with changes seen at gene and protein levels in IUGR or small for GA humans (Jaquet *et al.* 2001, Ozanne *et al.* 2005, Vaag *et al.* 2006, Jensen *et al.* 2008).

RNA extraction Tissues were homogenised with 0.4–0.6 g of CB014 ceramic beads (Bertin-Technologies, Montigny le Bretonneux, France) using a PowerLyzer 24 (Mo Bio Laboratories, Carlsbad, CA, USA), and RNA extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The quantity and quality of total RNA was determined by spectrophotometry (NanoDrop spectrophotometer; Biolab, Mulgrave, Australia) and Experion RNA Analysis Kits (Bio-Rad).

cDNA synthesis RNA was DNase treated using TURBO DNA-free kit (Life Technologies) in accordance with the manufacturer's instructions and after DNase treatment, RNA was quantified with the NanoDrop. PCR was performed in a subset of treated RNA samples with genomic DNA-specific primers designed to cross an intron boundary (F: 5'CTGTCTGAGGTGCTGAATTG3', R: 5'GGTACCCAACATCAACAGTT3') to confirm successful DNase treatment before reverse transcription. Ovine genomic DNA was included as a positive control (12.5 ng/ μ L) and was extracted from liver samples

Table 1 Primer sequences, amplicon size and accession numbers for genes measured by quantitative real-time PCR.

Gene	Gene name	Predicted amplicon size (bp)	Forward primer 5'-3'	Reverse primer 5'-3'	GenBank accession no.
Target genes	INSR	154	CCTATGCCCTGGTGCTCACTT	CCCTGAGTGATGGTGAGGTT	XM_004008550
	AKT2	136	AGACTACAAGTGTGGCTCCC	CTTCATGGCAGTAGTAGCGGC	XM_004015692
SLC2A2	Solute carrier family 2 (facilitated glucose transporter), member 2	142	CGAAATTGGGACCACATCTCAC	TGAAGAGCACCCGATAGCACC	XM_004003162
SLC2A4	Solute carrier family 2 (facilitated glucose transporter), member 4	143	CCTCCTACGAGATGCTCAIT	GATGGCCAGTTGATTGAGTG	XM_004012643
PRKAA2	Protein kinase, AMP-activated, alpha 2 catalytic subunit	181	GGCATCTTGGAAATCCGAAGT	TTGTCAACCAGGTACAGCTG	NM_001112816
PRKAG2	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	173	GAATCCTCAAGTTCTCTCCAG	CAAGGCCGACACTCGTCTTT	XM_004008179
Reference genes	GAPDH	132	GCTGAGTACGTGGTGAGTC	CACGCCCATCACAAACATGG	NM_001190390
	SDHA	141	CCGAAGCAGGTTTCAACACG	TCACGGTGTCTAGAAAGTGC	XM_004017097
	YWHAZ	137	GACTACTCGCTACTTGGC	CCCAGTCTGATAGGATGTGT	NM_001267887
	RPL-P0	179	AATGGCAGCATCTACAACCC	TCCACAGACAAAGCCAGGAC	XM_004017413

homogenised in 500 µL of TES (10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8), 100 mM NaCl), followed by the addition of proteinase K and SDS (Miller *et al.* 1988). PCR specific for genomic DNA was performed on a Corbett RotorGene 6000 (QIAGEN) with the following cycling conditions: 95°C for 10 min, 95°C for 5 s and 55°C for 20 s for 40 cycles, with melt at 60–95°C, followed by agarose gel electrophoresis. DNase treated RNA from liver and muscle (500 ng/sample) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Controls consisted of samples without added reverse transcriptase and tubes with all reagents except RNA. All cDNA samples were diluted 1:5 before quantitative real-time PCR analysis.

Primer design Primers were designed using PrimerBlast (National Center for Biotechnology Information, Bethesda, MD, USA), Primer3 (Koressaar & Remm 2007, Untergasser *et al.* 2012) and UCSC In-Silico PCR (Genome Bioinformatics Group, UC Santa Cruz, CA, USA). Primers were designed across an exon boundary to amplify cDNA with a predicted amplicon size of 100–200 base pairs (bp, Table 1). Reaction efficiencies for primer pairs were determined by 1/5 serially diluted standards and ranged from 90% to 103%. The presence of a single PCR product of the expected size was confirmed by the presence of a

single peak on the melting curve and of a single product at the predicted size on an agarose gel.

qPCR analysis Quantitative PCR (qPCR) analysis was carried out for insulin signalling and reference genes (Table 1) using 2 µL cDNA in a 10 µL PCR. The QIAgility automated PCR setup robot (QIAGEN) and Bio-Rad CFX384 well real-time qPCR machine (Bio-Rad) were used with SsoFast Evagreen Supermix (Bio-Rad) and with PCR cycling conditions as follows: 95°C for 30 s, 95°C for 5 s and 60°C for 5 s for 40 cycles, melt at 60–95°C. Data were quantified using Bio-Rad CFX Manager version 3.1 software (Bio-Rad). Gene expression of key signalling components in insulin-regulated glucose uptake was measured in liver (*INSR*, *AKT2*, *SLC2A2*, *SLC2A4*, *PRKAA2*, *PRKAG2*) and muscle (*INSR*, *AKT2*, *SLC2A4*, *PRKAA2*, *PRKAG2*). Reference genes *GAPDH*, *SDHA*, *YWHAZ* and *RPL-P0* (Table 1) were measured in all samples. The stability of the four reference genes was assessed using the CFX Manager v3.1 software (Bio-Rad), with the two most stable reference genes (*YWHAZ* (CV=0.2354, M=0.7034) and *RPL-P0* (CV=0.2354, M=0.7034)) used for normalisation (Vandesompele *et al.* 2002). Gene expression of *INSR*, *AKT2*, *SLC2A2*, *SLC2A4*, *PRKAA2*, *PRKAG2* (liver) and *INSR*, *AKT2*, *SLC2A4*, *PRKAA2*, *PRKAG2* (muscle) was

Table 2 Adult metabolic outcomes^a.

	Term		Preterm		P-Values		
	Male	Female	Male	Female	Group	Sex	Group × sex
Number of animals	11	6	9	7			
Body weight (kg)	60.3 ± 2.1	55.8 ± 1.4	53.1 ± 2.9	54.4 ± 1.2	0.076	0.5	0.2
Fasting plasma concentrations							
Glucose (mmol/L)	4.08 ± 0.08	3.88 ± 0.12	3.89 ± 0.09	4.31 ± 0.10	0.246	0.3	0.004 ^b
Insulin (U/L)	6.14 ± 0.28	7.41 ± 0.37	5.49 ± 0.36	7.38 ± 0.53	0.29	<0.001	0.3
Glucose tolerance (mmol.min.L ⁻¹)							
First phase	116 ± 3	131 ± 3	114 ± 5	119 ± 3	0.077	0	0.2
Second phase	202 ± 15	266 ± 20	189 ± 27	209 ± 31	0.145	0.1	0.4
Total	318 ± 16	398 ± 19	303 ± 30	328 ± 32	0.104	0.1	0.3
Absolute insulin secretion (U.min.L ⁻¹)							
First phase	264 ± 52	263 ± 43	278 ± 48	302 ± 61	0.731	0.7	0.9
Second phase	507 ± 81	638 ± 62	582 ± 96	502 ± 42	0.781	0.4	0.3
Total	771 ± 111	902 ± 86	860 ± 131	804 ± 79	0.961	0.4	0.5
Relative insulin secretion (U/mmol)							
First phase	2.2 ± 0.4	2.0 ± 0.3	2.5 ± 0.4	2.5 ± 0.5	0.53	1	0.7
Second phase	2.5 ± 0.4	2.4 ± 0.2	3.3 ± 0.6	2.9 ± 0.6	0.384	0.9	0.6
Total	2.4 ± 0.3	2.3 ± 0.2	3.0 ± 0.5	2.7 ± 0.3	0.428	0.9	0.8
Insulin sensitivity _{glucose} (mg.L.U ⁻¹ .kg ⁻¹ .min ⁻¹)	0.051 ± 0.004	0.043 ± 0.005	0.055 ± 0.005	0.055 ± 0.005	0.082	0.4	0.3
Insulin-stimulated glucose disposition indices (mg.mL.kg ⁻² .min ⁻²)							
Basal DI _{glucose}	10.4 ± 1.5	9.68 ± 1.25	10.5 ± 1.6	13.5 ± 2.4	0.379	0.5	0.4
Max DI _{glucose}	41.1 ± 6.3	36.5 ± 6.6	50.5 ± 11.7	52.8 ± 10.5	0.229	1	0.6

^aData are expressed as mean ± s.e.m. Insulin data were log-transformed before statistical analysis. DI, insulin-stimulated glucose disposition index. ^bFasting blood glucose concentration did not differ between term and preterm males (*P*=0.130) but was greater in preterm than in term females (*P*=0.020).

normalised to the geometric mean of the most stable reference genes *YWAHZ* and *RPL-P0* using the CFX Manager v3.1 software (Bio-Rad).

Statistical analysis

For single measures made on each animal, effects of group (preterm vs term), sex and their interaction were analysed by two-way ANOVA. For measures made repeatedly on the same animals, the effects of group, sex, time and their interactions were analysed using a repeated-measures ANOVA. Insulin data were log-transformed before statistical analysis. Data for relative gene expression were not normally distributed and were therefore analysed overall and separately in males and females by Mann–Whitney *U*-test. Relationships between whole-body insulin sensitivity and gene expression of individual insulin signalling components were analysed by Pearson's correlation. Statistical analyses were carried out using SPSS software version 21.0 (SPSS). Unless otherwise stated, data are expressed as mean $\pm$ s.e.m. $P < 0.05$ was accepted as statistically significant.

Results

The preterm lambs studied as adults were born at earlier GA (term: 145.9 ± 0.2 days, preterm: 131.7 ± 0.1 days;

$P < 0.001$), and had 35% lower birth weights than term lambs (term: 6.45 ± 0.28 kg, preterm: 4.19 ± 0.14 kg; $P < 0.001$). These outcomes did not differ between sexes and were similar to those reported for all lambs born in this study, which included animals that did not survive to adulthood (Nguyen *et al.* 2016).

Preterm birth did not impair glucose tolerance or insulin secretion in adulthood

Adult body weight at metabolic testing did not differ between groups ($P = 0.076$, Table 2). Effects of group on fasting blood glucose concentration measured prior to the IVGTT differed between sexes (interaction $P = 0.004$, Table 2). Blood glucose concentration did not differ between preterm and term males ($P = 0.130$) but was greater in preterm than in term females ($P = 0.020$, Table 2). Fasting insulin concentration prior to the IVGTT did not differ between groups ($P = 0.290$) and was higher in females than in males ($P < 0.001$, Table 2). Glucose tolerance did not differ between groups overall or during the first or second phases of insulin secretion (Table 2). Males had better glucose tolerance during the first phase of insulin secretion than females (lower AUC_{glucose} , $P = 0.016$, Table 2). Absolute and relative insulin secretion did not differ between groups or sexes (Table 2). Insulin sensitivity did not differ significantly between groups ($P = 0.082$) or sexes (Table 2); neither group nor sex affected insulin sensitivity (each $P > 0.2$) when current body weight was

Table 3 Pancreas morphology and β -cell function^a.

	Term		Preterm		P-Values		
	Male	Female	Male	Female	Group	Sex	Group $\times$ sex
Number of animals	11	6	9	7			
Pancreas morphology							
Pancreas mass (g)	62.3 ± 4.1	49.7 ± 5.0	63.2 ± 3.3	56.1 ± 2.0	0.4	0	0.5
Pancreas (%)	1.04 ± 0.08	0.90 ± 0.09	1.21 ± 0.08	1.03 ± 0.03	0.1	0.1	0.8
β -Cell volume density	0.0061 ± 0.0007	0.0048 ± 0.0008	0.0049 ± 0.0008	0.0052 ± 0.0010	0.7	0.5	0.4
β -Cell mass (g)	0.383 ± 0.054	0.233 ± 0.038	0.322 ± 0.062	0.294 ± 0.058	1	0.1	0.3
β -Cell mass (%)	0.0065 ± 0.0010	0.0041 ± 0.0007	0.0062 ± 0.0013	0.0054 ± 0.0011	0.7	0.2	0.5
Islet density (no.mm ⁻²)	7.62 ± 0.90	5.87 ± 1.03	5.64 ± 0.69	6.14 ± 0.92	0.4	0.5	0.2
β -Cells/islet (no)	7.62 ± 0.39	8.93 ± 0.96	8.63 ± 0.83	8.99 ± 1.58	0.6	0.4	0.6
% of islets with < 5 β -cells	53.5 ± 3.1	51.1 ± 5.6	42.7 ± 4.4	45.3 ± 5.6	0.1	1	0.6
β -Cell function							
Absolute insulin secretion per β -cell mass (U.min.L ⁻¹ .g ⁻¹)	2690 ± 657	5922 ± 2632	4304 ± 1308	3415 ± 675	1	0.2	0.3
Basal DI per β -cell mass (mg.mL.kg ⁻² .min ⁻² .g ⁻¹)	33 ± 5	68 ± 34	44 ± 13	54 ± 11	0.6	0.1	0.7
Maximal DI per β -cell mass (mg.mL.kg ⁻² .min ⁻² .g ⁻¹)	151 ± 42	277 ± 158	273 ± 133	192 ± 25	0.4	0.4	0.7

^aData are expressed as mean $\pm$ s.e.m. Pancreas and β -cell weights are shown in absolute terms and as a percentage of body weight. Measures of β -cell function calculated from insulin data were log-transformed before statistical analysis. DI, insulin-stimulated glucose disposition index.

Figure 1

Preterm birth had few effects on gene expression of key insulin signalling components in liver of 14-month-old sheep. Expression of target genes is normalised to reference genes. Each data point represents an individual animal; horizontal lines indicate means within that group and sex. Term-born are shown in solid symbols, preterm-born in open symbols, males by circles and females by squares. * $P < 0.05$.

added as a covariate in statistical analyses. Measures of insulin action (basal and maximal indices of insulin-stimulated glucose disposition) did not differ between groups or sexes (Table 2).

Pancreas morphology and β -cell function were normal in preterm-born sheep

Absolute pancreas weight did not differ between groups and was greater in males than that in females ($P = 0.019$, Table 3), and relative pancreas weights did not differ significantly between groups or sexes (Table 3) as reported previously (Nguyen *et al.* 2016). β -cell volume density, absolute and relative β -cell mass, average number of β -cells per islet and islet density, and measures of β -cell function did not differ between groups or sexes (Table 3).

Preterm birth had little effect on hepatic expression of genes important for insulin signalling

INSR, *AKT2*, *SLC2A2*, *SLC2A4* and *PRKAA2* gene expression in liver did not differ between groups or sexes (Fig. 1). Expression of these genes in liver was also similar in preterm and term animals within each sex when analysed separately (Fig. 1). Hepatic *PRKAG2* expression was greater in preterm than in term males ($P = 0.028$), but was similar in preterm and term females, and did not differ between sexes (Fig. 1).

Skeletal muscle expression of several genes important for insulin signalling was increased by preterm birth, particularly in males

INSR gene expression in skeletal muscle did not differ between groups overall or in females, but was greater

Figure 2

Preterm birth increased gene expression of key insulin signalling components in skeletal muscle, particularly in males. Expression of target genes in M. vastus lateralis is normalised to reference genes. Each data point represents an individual animal; horizontal lines indicate means within that group and sex. Term-born are shown in solid symbols, preterm-born in open symbols, males by circles and females by squares. * $P < 0.05$.

in preterm males than in term males ($P=0.047$, Fig. 2). Muscle *AKT2* expression did not differ between preterm and term groups overall ($P=0.074$) or in females ($P>0.1$), but was greater in preterm than in term males ($P=0.043$, Fig. 2). Muscle *SLC2A4* expression was greater in preterm than in term overall (64% higher, $P=0.019$) and in preterm compared to term males ($P=0.019$), but was similar in preterm compared to term females ($P>0.1$, Fig. 2). Muscle *PRKAA2* expression was greater in preterm than in term overall (63% higher, $P=0.021$) and in males ($P=0.009$), but was similar in preterm compared to term females ($P>0.1$, Fig. 2). Expression of *INS*, *AKT2*, *SLC2A4* and *PRKAA2* in muscle did not differ between sexes (Fig. 2). Muscle *PRKAG2* expression was greater in preterm than in term sheep overall (51% higher, $P=0.049$) and in males ($P=0.015$), but was similar in preterm compared to term females ($P>0.1$, Fig. 2), and was greater in males than in females ($P=0.031$).

Hepatic expression of *PRKAG2* correlated positively with insulin sensitivity, in males only

Hepatic *PRKAG2* expression correlated positively with whole-body insulin sensitivity in males ($R=0.573$, $P=0.025$, $n=15$) but not in females ($R=0.135$, $P>0.6$, $n=11$). Expression of the other individual genes studied in liver (*INSR*, *AKT2*, *SLC2A2*, *SLC2A4*, *PRKAA2*) and muscle (*INSR*, *AKT2*, *SLC2A4*, *PRKAA2*, *PRKAG2*) did not correlate with whole-body insulin sensitivity within either sex (each $P>0.1$).

Discussion

In this study, we investigated the effect of preterm birth at 0.9 of gestation after antenatal betamethasone treatment on glucose homeostasis and its key determinants, insulin sensitivity and secretion, in young adult sheep. We found that preterm birth induced by maternal epostane treatment did not impair glucose homeostasis, insulin sensitivity, insulin secretion or β -cell mass at adulthood, compared to a term-born group also exposed to epostane. Unexpectedly, skeletal muscle gene expression of determinants of insulin signalling was greater in the preterm sheep, particularly in males, demonstrating sex-specific programming by this exposure, but these findings require confirmation in subsequent studies. Our findings differ from the impaired insulin secretion observed in adult sheep after

preterm birth induced by antenatal dexamethasone treatment (Bansal *et al.* 2015), and impaired metabolic outcomes in adult sheep after antenatal exposure to either betamethasone or dexamethasone when followed by spontaneous term birth (Moss *et al.* 2001, Long *et al.* 2012). Lambs delivered at ~ 0.9 of term without prior antenatal glucocorticoids have poor survival (Liggins 1969, De Matteo *et al.* 2010), and ethical reasons therefore prevented inclusion of a non-betamethasone-exposed preterm group. Clinical practice is to treat women at risk of preterm birth with glucocorticoids (Brownfoot *et al.* 2013), but the majority of women delivering at term will not have received glucocorticoids. The two groups in our study, therefore, also reflect the main groups in human populations. It is, however, not possible to separate effects of preterm birth from those of antenatal glucocorticoids within the present cohort. An additional potential impact of our experimental design is that labour in both groups was induced by epostane. This is unlikely to alter glucocorticoid exposure in our preterm group, where epostane exposure occurs before the normal developmental increases in adrenal cortisol production and circulating cortisol at ~ 132 days' GA or 10–15 days before delivery in the foetal sheep (Magyar *et al.* 1980, Phillips *et al.* 1996), and exogenous betamethasone was given to our preterm group. Maternal epostane treatment is likely to have perturbed cortisol profiles in the term group, albeit probably transiently. This inhibitor of 3β -hydroxysteroid dehydrogenase crosses the placenta and suppresses foetal cortisol concentrations by $\sim 50\%$ within 1 h in late gestation foetuses, followed by a rapid rebound with elevated circulating cortisol concentrations from 24 h post-epostane persisting to at least delivery (Silver 1988). Inclusion of a spontaneously delivering term group in future studies is recommended to remove this potential confounder. Nevertheless, our findings together with those of previous studies suggest that the developmental programming of metabolic outcomes by glucocorticoids may differ between betamethasone and dexamethasone, and may also be dependent on the timing of exposure relative to delivery. The lack of impairment of glucose tolerance and its determinants after preterm birth and antenatal betamethasone exposure in the present study lead us to further suggest, in contrast to previous conclusions (Bansal *et al.* 2015), that increased rates of diabetes and impaired insulin sensitivity seen in epidemiological studies of preterm-born humans are not a consequence of preterm birth itself.

Glucose tolerance

Glucose tolerance did not differ between preterm-born adult sheep at 0.9 of gestation after antenatal glucocorticoid exposure and those born at term. This was observed both in the present cohort in which preterm sheep were exposed to antenatal betamethasone and induced to deliver at 132 days' GA by maternal epostane or in a slightly less preterm cohort where delivery at 137 days' GA was induced by maternal antenatal dexamethasone (Bansal *et al.* 2015). Sheep are ruminants and like other adult ruminants produce most of their circulating glucose via gluconeogenesis rather than direct absorption from the diet (Larsen & Kristensen 2013). Nevertheless, they exhibit similar glucose profiles and biphasic insulin secretion responses following a bolus of intravenous glucose (Gatford *et al.* 2004, 2012, Owens *et al.* 2007) as seen in humans (e.g. Kjems *et al.* 2001). Interestingly, fasting glucose was higher in preterm than term progeny in the present study, but only in females, suggesting a mild disturbance of glucose metabolism in this cohort. The present results together with previous outcomes in studies of sheep (Bansal *et al.* 2015) and humans (Dalziel *et al.* 2005) therefore suggest that moderate preterm birth after antenatal glucocorticoid exposure at clinical doses does not induce a clinically significant impairment of glucose tolerance in early adulthood. The increase in diabetes risk after preterm birth in human cohorts appears to be worse in those born at early GA (<28 weeks) than in those born moderately preterm, and effect sizes are also generally larger in older adults than in young adults (Lawlor *et al.* 2006, Kaijser *et al.* 2009, Kajantie *et al.* 2010, Pilgaard *et al.* 2010). It is, therefore, possible that earlier preterm birth might impair glucose tolerance or that this might become more evident with ageing in the sheep.

Insulin sensitivity

Surprisingly, when we investigated insulin sensitivity using the 'gold standard' method (HEC) there was no detectable impairment in this cohort of preterm-born adult sheep. Animal numbers may have limited our capacity to detect differences in *in vivo* insulin sensitivity; although not significant ($P=0.082$), mean insulin sensitivity was 12% higher in preterm than in term overall, possibly also reflecting increased insulin sensitivity with lower body size. These findings are in contrast with the reported elevated risk of T2D (Lawlor *et al.* 2006, Kaijser *et al.* 2009, Kajantie *et al.* 2010, 2014, Pilgaard *et al.* 2010) and impaired insulin sensitivity (Rotteveel *et al.* 2008,

2011, Mathai *et al.* 2012, Tinnion *et al.* 2013) in preterm-born adult humans. In three studies from two cohorts of young adults, where the HEC was used to measure insulin sensitivity, insulin sensitivity was lower in those born preterm compared to those born at term (Rotteveel *et al.* 2008, 2011, Mathai *et al.* 2012). Insulin sensitivity calculated by minimal modelling did not differ between preterm and term-born young adult men and women, however (Willemsen *et al.* 2009). Based on our findings, we hypothesise that the reduced insulin sensitivity in adult humans born preterm might be induced by factors associated with preterm birth, rather than the exposure to preterm birth itself. One candidate factor that might contribute to the association in human cohorts is foetal exposure to chorioamnionitis, or other states of maternal and placental inflammation. Chorioamnionitis is clinically associated with 25–40% of preterm births and *in utero* exposure impairs cardiorespiratory, neurological and renal outcomes in human babies (Galinsky *et al.* 2013), although metabolic consequences have not been studied in humans. In rats, however, systemic maternal inflammation during pregnancy programmed multiple symptoms of the metabolic syndrome, including decreased insulin sensitivity measured by HEC, in male but not female young adult progeny (Hao *et al.* 2014), consistent with this hypothesis.

Outcomes may differ between glucocorticoids and depend on timing of delivery

Differences in metabolic consequences of antenatal glucocorticoid exposure, with and without preterm birth, between this and previous ovine studies (Moss *et al.* 2001, Long *et al.* 2012, Bansal *et al.* 2015) suggest that the metabolic programming effects of glucocorticoids differ between betamethasone and dexamethasone, and also depend on the relative timing of glucocorticoid exposure and birth. The glucocorticoid diastereoisomers betamethasone and dexamethasone have similar binding affinities for the human and rat glucocorticoid receptors (Ponec *et al.* 1986, Tanigawa *et al.* 2002), induce similar downstream transactivation and transrepression glucocorticoid receptor signalling *in vitro* (Tanigawa *et al.* 2002), and undergo similar transfer and metabolism in perfused human placenta (Levitz *et al.* 1978), but differ in kinetics. The half-life of betamethasone after intravenous administration is approximately twice that of dexamethasone in non-pregnant and pregnant humans (Petersen *et al.* 1983a,b). The kinetics, placental transfer and receptor activation of these glucocorticoids have

not been directly compared in the sheep. When foetally administered at the same dose, however, betamethasone is a more potent inducer of parturition than dexamethasone in sheep (Derks *et al.* 1996), suggesting similar differences in kinetics. Exposure to antenatal maternal courses of either dexamethasone at sub-clinical doses or betamethasone at clinical doses at preterm ages each induce adverse metabolic consequences in ovine progeny born at term, although the specific outcomes differ. Four doses of dexamethasone (each injection 2 mg, $\sim 60 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) given 12 h apart at d103 and d104 GA reduced postnatal body weight and induced glucose intolerance with decreased insulin secretion in term-born female ovine progeny across two generations, although their insulin sensitivity has not yet been reported (Long *et al.* 2012). Glucose tolerance of term-born adult progeny was unchanged by a single maternal course of 0.5 mg/kg betamethasone given to pregnant sheep at d104 GA (~ 0.7 of term), similar to the dose used clinically in humans (Brownfoot *et al.* 2013), although the increased glucose-stimulated insulin secretion is suggestive of insulin resistance (Moss *et al.* 2001). The latter is similar to the findings of the follow-up study of 30-year-old men (Dalziel *et al.* 2005) born in the first human RCT of antenatal betamethasone for the prevention of neonatal respiratory distress syndrome (Auckland Steroid Trial, Liggins & Howie 1972). In that cohort, antenatal exposure to betamethasone increased early plasma insulin concentrations during an oral glucose tolerance test by 16%, suggesting development of insulin resistance, although these individuals had normal glucose tolerance (Dalziel *et al.* 2005). Interestingly, although $\sim 30\%$ of the cohort went on to be born at term, correcting for GA did not remove the effect of prior betamethasone exposure on glucose-stimulated insulin concentration (Dalziel *et al.* 2005). In contrast to the effects of betamethasone on insulin sensitivity, clinical doses of dexamethasone that induced preterm delivery at 137 days' GA did not impair glucose tolerance or insulin secretion during IVGTT in adult ovine progeny (Bansal *et al.* 2015). These did, however, the capacity for sustained *in vivo* insulin secretion and induced profound reduction in β -cell mass in juvenile and adult progeny (Bansal *et al.* 2015). In the present study, we found similar insulin secretion in preterm-born adult sheep at 0.9 of gestation after antenatal betamethasone treatment compared to controls born at term. Consistent with this, we also did not see differences in β -cell mass or function between preterm and term sheep in the present cohort. The available evidence in humans also suggests little effect of preterm birth on insulin secretion. This includes a lack of relationship between GA and basal and first-phase insulin release in children at 5–7 years of

age (Bazaes *et al.* 2004), similar disposition index in adults born moderate–late preterm or at term (Mathai *et al.* 2012), and increases in insulin secretion that compensated for insulin resistance in young adults born preterm at very low birth weight (Kajantie *et al.* 2014). Overall, these results suggest that antenatal glucocorticoid exposure before preterm birth adversely affects metabolic outcomes in progeny, although betamethasone may programme insulin resistance while dexamethasone impairs insulin secretion. Direct comparisons of metabolic outcomes after antenatal exposure to clinical doses of these two widely used glucocorticoids are needed to directly test whether there are long-term differences in the effects of betamethasone and dexamethasone, and whether these effects depend on the timing of delivery relative to exposure.

Sex-specific effects

In the present study, we also investigated the molecular mechanisms underlying effects of preterm birth on insulin sensitivity by assessing insulin signalling and glucose transporter genes. Consistent with the trend for increased, rather than decreased insulin sensitivity in preterm sheep, we found greater gene expression of *SLC2A4*, *PRKAA2* and *PRKAG2* in skeletal muscle in preterm than in term sheep. Furthermore, this effect was sex-specific, with greater effects observed in males, although whole-body insulin sensitivity measured by HEC did not vary between sexes, consistent with variable or no sex differences in this outcome reported in previous studies of adult sheep (Gatford *et al.* 2004, Owens *et al.* 2007, Liu *et al.* 2015, Donovan *et al.* 2016).

The sex-specific effects of preterm birth on gene expression of the insulin signalling pathway in the present study is consistent with the concept that preterm birth induces greater long-term impacts in males than females. Males are also more susceptible to the short-term adverse effects of preterm birth, with higher rates of morbidity than females after preterm birth in sheep after sub-clinical glucocorticoid doses (De Matteo *et al.* 2010) and clinically in humans (Liggins & Howie 1972). Nevertheless, neonatal outcomes did not differ between sexes in the present cohort of sheep following clinical doses of betamethasone (Nguyen *et al.* 2016).

Conclusions

Contrary to results of human epidemiological studies, our study showed that induced preterm birth at 0.9 of

gestation after antenatal betamethasone exposure did not impair insulin sensitivity or secretion in young adult sheep, compared to induced term-born progeny. Surprisingly, gene expression of insulin signalling pathway components were increased in skeletal muscle and particularly in males. These results suggest that preterm birth with antenatal glucocorticoids does not impair glucose control and insulin action at least in young adults. Whether defects might be exposed with ageing or with additional challenges such as obesity is unknown. Together with findings of previous studies of young adult progeny in sheep (Moss *et al.* 2001, Long *et al.* 2012, Bansal *et al.* 2015), our results suggest that effects of antenatal exposure may differ between betamethasone and dexamethasone, and also depend on timing of delivery. Direct comparisons of metabolic outcomes of antenatal treatment with betamethasone and dexamethasone, the two glucocorticoids in widespread clinical use (Brownfoot *et al.* 2013), in both preterm and spontaneously term-delivered progeny are required in preclinical models to test this hypothesis and allow detailed mechanistic investigations that are not possible within current clinical RCTs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

R De M, G P, M J B and K L G were responsible for study design. R De M, D J H, T B-M, V N, B J A, G P and K L G were responsible for data acquisition. R De M, D J H, T B-M and K L G contributed to data analysis. K L G wrote and all the authors revised and approved the final manuscript.

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