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Impaired β-Cell Function and Inadequate Compensatory Increases in β-Cell Mass after Intrauterine Growth Restriction in Sheep

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Poor growth before birth increases the risk of non-insulin-dependent diabetes mellitus (NIDDM) and impairs insulin secretion relative to sensitivity. We investigated the effects of intrauterine growth restriction in sheep on insulin secretion, β-cell mass, and function from before birth to young adulthood and its molecular basis. Pancreas was collected from control and placentally restricted sheep as fetuses (d 143 gestation), lambs (aged 42 d), and young adults (aged 556 d), following independent measures of *in vivo* insulin secretion and sensitivity. β-Cells and islets were counted after immunohistochemical staining for insulin. In lambs, gene expression was measured by RT-PCR and expressed relative to 18S. β-Cell mass correlated positively with fetal weight but negatively with birth weight in adult males. Glucose-stimulated insulin disposition and β-cell function correlated negatively with fetal weight but positively with birth weight in adult males. Placental restriction increased pancreatic expression of IGF-II and IGF-I but decreased that of voltage-gated calcium channel, α1D subunit (CACNA1D) in lambs. In male lambs, pancreatic IGF-II and insulin receptor expression correlated strongly and positively with β-cell mass and CACNA1D expression with glucose-stimulated insulin disposition. Restricted growth before birth in the sheep does not impair insulin secretion, relative to sensitivity, before birth or in young offspring. IGF-II and insulin receptor are implicated as key molecular regulators of β-cell mass compensation, whereas impaired expression of the voltage-gated calcium channel may underlie impaired β-cell function after intrauterine growth restriction. With aging, the insulin secretory capacity of the β-cell is impaired in males, and their increases in β-cell mass are inadequate to maintain adequate insulin secretion relative to sensitivity. (*Endocrinology* 149: 5118–5127, 2008)

SMALL SIZE AT birth consistently predicts increased risk of non-insulin-dependent diabetes mellitus (NIDDM) and impaired glucose tolerance in human cohorts (1), accounting for 18% of the lifetime risk of diabetes in 80-yr-old Swedish men (2). Whereas insulin resistance contributes to this increased risk of NIDDM, the extent of impairment in insulin secretion is less clear (1). This may reflect the need to assess insulin secretion relative to the individual's insulin sensitivity, termed insulin disposition (3). Several studies (4–6), although not all (7), in which insulin secretion and sensitivity were measured, report that intrauterine growth restricted (IUGR) children and young adults secrete less insulin than appropriate for their insulin sensitivity, implying that a defect at the pancreas contributes to their increased risk of NIDDM.

The capacity to secrete insulin reflects the β-cell mass and the insulin secretory capacity of each β-cell. There is some, albeit limited, evidence that severe human IUGR decreases β-cell mass at birth (8). Effects of IUGR on insulin secretion relative to β-cell mass have not yet been reported. Because of the limited capacity to address these questions in humans, models of prenatal growth restriction in nonhuman species have been used to investigate the mechanistic basis of impaired glucose homeostasis after IUGR. Poor fetal growth is usually the result of restricted fetal supply of oxygen and nutrients due to impaired placental growth and/or function (9). Restriction of placental and fetal growth during late pregnancy (PR) in rats produces progeny with normal circulating glucose and insulin levels at 1 wk of age but mild fasting hyperglycemia and hyperinsulinemia at 7–10 wk and frank diabetes with decreased β-cell mass by 26 wk (10, 11). Glucose-stimulated insulin secretion is already impaired in 1-wk-old PR rats, when β-cell mass and fasting glucose are normal (10, 11). Normal insulin secretory responses to arginine but not glucose imply that this defect in β-cell function is specific to glucose metabolism (10). This suggests that impaired β-cell function is the initial defect limiting insulin secretion after IUGR, with later reduction in β-cell mass.

Similarly, the molecular basis for changes in insulin secretion after IUGR is poorly understood. Manipulating the expression of IGF-II, IGF-I, the insulin and type 1 IGF-receptors, or the transcription factor pancreatic duodenal ho-

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Abbreviations: CACNA1D, voltage-gated calcium channel α1D subunit; IUGR, intrauterine growth restricted; IVGTT, iv glucose tolerance test; NIDDM, non-insulin-dependent diabetes mellitus; Pdx, pancreatic duodenal homeobox; PHIS, posthepatic insulin secretion rates; PR, restriction of placental and fetal growth during late pregnancy; V_d, volume density.

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meobox (Pdx)-1 in mice has major impacts on β-cell mass and function (12–16), and insulin receptor expression is downregulated in human diabetes (17). Slc2a2 (GLUT2), glucokinase, the voltage-gated L-type calcium channel, and the Kir 6.2 subunit of the ATP-regulated potassium channel have been identified as key determinants of β-cell function, based on effects of gene mutations in humans (18–20) and studies in the Goto-Kakizaki diabetic rat (21, 22). In rats, maternal protein-restriction and PR reduce progeny pancreatic expression of IGF-II and Pdx-1, respectively (23, 24), but the contribution of these other determinants to impaired insulin secretion after IUGR has not yet been assessed.

PR can be readily induced by surgical removal of the majority of placental implantation sites from the nonpregnant endometrium in sheep, with similar metabolic, endocrine, and growth consequences for the fetus and progeny as human IUGR (25–32). PR induces defects in insulin secretion from late fetal and early neonatal life onward, when basal and glucose-stimulated insulin secretion (although not corrected for sensitivity) are reduced in PR fetuses (26, 32, 33). This is followed by failure of basal and glucose-stimulated insulin disposition (*i.e.* inadequate insulin secretion relative to sensitivity) by young adulthood in lambs who were small at birth (34), as in humans (4). The mechanistic basis of impaired insulin secretion after PR is unknown. We hypothesized that restricted placental and fetal growth in sheep would decrease insulin disposition due to reduced β-cell mass and/or function from before birth, and that this would persist into adulthood. We further hypothesized that PR and restricted fetal growth would reduce expression of key molecular determinants of β-cell mass and/or function and that these would correlate with functional outcomes.

Materials and Methods

Animals and surgery

All procedures in this study were approved by the University of Adelaide Animal Experimentation and Ethics Committee and complied with the Australian code of practice for the care and use of animals for scientific purposes. Placental growth was restricted (PR) by removal of the majority of endometrial caruncles from the uterus before mating (25). Control ewes were unoperated. Delivery occurred naturally at term for those animals studied postnatally.

In vivo measures of insulin secretion, sensitivity, and action

We previously described the *in vivo* measurement of insulin secretion and sensitivity in these cohorts of late-gestation fetal sheep (33), lambs (32), and young adult sheep (34). The cohort of lambs described here includes all of the lambs that were euthanized at 43 d of age in our previous cohort (32) as well as additional animals. Insulin disposition data have previously been published for the earlier lamb cohort (32) and in adults (34), and in the present paper we add insulin disposition in fetuses as well as insulin disposition per β-cell mass for each age group. Briefly, glucose-stimulated insulin secretion was measured during an iv glucose tolerance test (IVGTT) at 139 ± 0 d gestation in fetuses, 35 ± 2 d in lambs, and 376 ± 1 d in young adults. Basal blood glucose and plasma insulin concentrations were calculated as the mean of concentrations measured in three samples collected 10, 5 (postnatal only), and 0 min before administration of glucose. A bolus of glucose (0.5 g/kg estimated body weight^{-1} in fetuses and 0.25 g/kg^{-1} in postnatal sheep) was infused over 0.5–2 min, and the start time was taken as the end of the bolus infusion. Blood samples were taken at intervals up to 120 min from the start of the experiment in fetuses and 210 min in postnatal animals. Insulin secretion was calculated as the area above baseline for the insulin profile, divided by the area above baseline for the glucose profile.

The insulin sensitivity of glucose metabolism was measured by hyperinsulinemic euglycemic clamp (HEC). Arterial blood was sampled 10, 5 (postnatal animals only), and 0 min before the start of the clamp for the determination of basal blood glucose and plasma insulin concentrations. Recombinant human insulin (Actrapid; Novo Nordisk, Baulkham Hills, Australia) was infused iv at 0.6 mU insulin/kg estimated body weight^{-1} per min^{-1} in fetuses and 2.0 mU insulin per kg^{-1} per min^{-1} in postnatal sheep for 120 min from 0 min. At 15 min, an iv infusion of glucose (25% dextrose) was commenced at 2 mg/kg^{-1}·min^{-1}, and this rate was adjusted every 5 min to restore and maintain euglycemia. Plateau rates of glucose infusion during the second hour of the clamp were termed steady-state glucose infusion rates. Arterial blood was sampled (2 ml) at 60, 75, 90, 105, and 120 min, and steady-state blood glucose and plasma insulin concentrations were calculated as the average concentrations in the second hour of the hyperinsulinemic euglycemic clamp. Calculations of insulin sensitivity, posthepatic insulin secretion rates (PHIS), and insulin disposition have been more fully described previously (35). Briefly, the insulin sensitivity of net whole body glucose uptake was calculated as the steady-state glucose infusion rates required to maintain euglycemia corrected for the steady-state plasma insulin concentration. The metabolic clearance rate of insulin was calculated as the insulin infusion rate throughout the hyperinsulinemic euglycemic clamp divided by the increase in plasma insulin from fasting to steady-state concentrations. PHIS and insulin disposition indexes for glucose metabolism were calculated as described previously (13). These calculations require independent measures of insulin secretion and clearance and/or sensitivity and were therefore calculated only for those animals in which intact catheters permitted both the IVGTT and hyperinsulinemic euglycemic clamp to be completed. Briefly, basal and maximal PHIS were calculated by multiplying the metabolic clearance rate of insulin with the basal insulin concentration (before IVGTT) or the maximal insulin concentration (during IVGTT), respectively. Basal and maximal insulin disposition were calculated by multiplying insulin sensitivity (from HEC) with basal or maximal PHIS, respectively. At least one measure of insulin secretion or sensitivity was obtained from 36 fetuses (19 control, 17 PR), 22 lambs (14 control, eight PR), and 49 adult (14 control, 25 PR) sheep.

Autopsy

All ewes and fetuses that survived to the completion of *in vivo* studies were killed with an overdose of sodium pentobarbitone at 143 ± 0 d gestation, and fetal sheep were delivered by hysterotomy. Lambs and young adults were similarly euthanized by overdose of sodium pentobarbitone at 42 ± 0 or 556 ± 1 d of age, respectively. At post-mortem, pancreas was rapidly dissected and weighed, and representative mixed aliquots were fixed for 48 h in 4% paraformaldehyde before embedding in paraffin wax. Additional aliquots of pancreas were snap frozen in liquid nitrogen and stored frozen at −80 C.

Immunostaining and morphometric analysis

Embedded pancreas blocks from fetal sheep [eight control (four males, four females), six PR (four males, two females)], lambs [14 control (eight males, six females), eight PR (four males, four females)], and young adult sheep [13 control (six males, seven females), 19 PR (5 males, 14 females)] were used in the present study. One section per block (5 μm) was immunostained to detect insulin-positive cells, using guinea pig antiporcine insulin (Dako Cytomation, Glostrup, Denmark) at 1:150 dilution as the primary antibody, goat biotin-conjugated F(ab)′ fragment of affinity purified anti-guinea pig IgG (Rockland Immunochemicals, Gilbertsville, PA) at 1:400 dilution as secondary antibody, with final staining using DAB Fast (Sigma-Aldrich Inc., St. Louis, MO). Negative controls were incubated without addition of the primary antiserum. Insulin-positive β-cells stained brown.

Stained images were visualized on an Olympus BH-2 microscope (×20 lens; Tokyo, Japan) and digitally captured with a GP-KR222 camera (Panasonic, Belrose, Australia). Morphometric analysis was performed using VideoPro version 3 software (Leading Edge Pty. Ltd., Adelaide, Australia). β-Cell volume density (V_d) was quantitated by point-counting (204 points/field, V_d equals the number of insulin-positive cells as a proportion of test points on pancreas), and numbers of islets, small islets (<5 β-cells), and β-cells per islet were counted in each field (0.102 mm^2/

field). Random-systematic sampling was used to select 10, 20, and 30 fields per section for the fetus, lamb, and young adult sheep, respectively. This sample size was calculated to give a SEM of less than 10% at each age. β-Cell mass was calculated by multiplying V_d and pancreas mass.

Real-time PCR

Total cellular RNA was isolated from 100 mg frozen pancreas, collected from 15 control (nine males, six females) and 13 PR (eight males, five females) lambs at 42 d postnatal age, using Trizol reagent (Invitrogen, Melbourne, Australia) according to the manufacturer's recommendations. Two micrograms of deoxyribonuclease-treated RNA was used for cDNA synthesis, according to the manufacturer's instructions using 500 ng random hexamers (GeneWorks, Adelaide, Australia), 10 nmol deoxynucleotide triphosphate mix, 20 U RNaseOUT, and 200 U Superscript III reverse transcriptase (Invitrogen). Negative controls were prepared by omitting Superscript III reverse transcriptase from the reaction mix.

Real-time PCR analysis was performed using a Corbett RotorGene 6000 (AdeLab, Adelaide, Australia) and real master mix (5 Prime; Quantum, Brisbane, Australia) with SYBR green as the fluorescence detector, according to the manufacturer's recommendations. An initial 2-min hold cycle at 95 C was followed by 40 cycles of 90 C for 15 sec and 60 C for 45 sec. Primers were designed across exon boundaries using Primer Express software (Applied Biosystems, Foster City, CA), and ovine or bovine sequences were used where available (Table 1). *Taq* polymerase amplified PCR products were cloned into pCRII-TOPO plasmid vector; plasmids were transformed into Top 10 F′ competent *Escherichia coli*. (Invitrogen). Plasmid DNA was purified using a NucleoSpin kit (Macherey Nagel, Düren, Germany) and sequenced to confirm the identity of the amplicon. Data were analyzed using RotorGene software (version 1.7) and the standard curve method of quantitation. Pancreatic expression of each gene was normalized to that of 18S and the gene to 18S ratio was divided by β-cell volume density to calculate gene expression per unit β-cell mass.

Statistics

Effects of PR and sex on morphology, insulin disposition, and β-cell function at each age were analyzed by two-way ANOVA. Gene expression data were nonnormally distributed and did not differ with sex, and effects of PR on gene expression were analyzed by Mann Whitney nonparametric test. Relationships between size at birth, gene expression, and other outcomes were analyzed by Pearson's correlation to test the *a priori* hypotheses base on studies in humans and our previous studies in sheep: that poor growth before birth will reduce β-cell mass and/or function, and expression of genes that regulate these and that expression of genes that regulate β-cell mass and/or function will correlate with measures of β-cell mass and/or function.

Results

Pancreatic morphology

In late gestation, PR decreased absolute mass of the pancreas and in females reduced the relative mass of the pancreas (Table 2). PR did not alter fetal β-cell mass, the proportion of small islets, or islet density (Table 2). PR lambs had increased β-cell volume density, which compensated for their decreased pancreas weight to maintain their β-cell mass (Table 2). PR did not alter the volume density or mass of β-cells in adults and increased the number of β-cells per islet in females but not males (Table 2).

In late gestation, fetal weight correlated positively with absolute ($r = 0.912$, $P < 0.001$, $n = 17$) but not with relative pancreas weight ($P > 0.6$). Fetal weight also correlated positively with β-cell mass in absolute but not relative terms (Fig. 1, $P > 0.6$) and tended to correlate negatively with the number of β-cells per islet ($r = -0.527$, $P = 0.078$, $n = 12$). In lambs, birth weight correlated positively with absolute ($r = 0.417$, $P = 0.031$, $n = 27$) but not relative pancreas weight ($P = 0.4$). Absolute and relative β-cell masses in lambs were not related to their birth weight (Fig. 1, $P > 0.6$ for each). In adults, absolute and relative pancreas weights, islet density, and number of β-cells per islet did not correlate with size at birth overall. In adult females, absolute pancreas weight ($r = 0.388$, $P = 0.061$, $n = 24$) and the proportion of small islets ($r = 0.396$, $P = 0.076$, $n = 21$) tended to correlate positively with birth weight. β-Cell volume density, absolute mass, and relative mass in adults were not related to size at birth overall or in females. In adult males, however, β-cell volume density ($r = -0.632$, $P = 0.037$, $n = 11$) and the absolute and relative mass of β-cells (Fig. 1) each correlated negatively with birth weight.

TABLE 1. Function and primer sequences for genes measured by RT-PCR

Gene	Function	Amplicon (bp)	Forward primer 5′–3′	Reverse primer 5′–3′	GenBank accession no.
18S, bovine	Housekeeper	90	agaaacggctaccacatccaa	cctgtattgttatttttcgt	DQ222453
IGF-II, ovine	Regulates β-cell mass; inhibits apoptosis	84	ggcggggagctggtggaca	tcggtttatgcggctggatggt	M89788
IGF-I, ovine	Regulates β-cell mass and function	64	gcttccggagctgtgatctg	gacttggcggccttgaga	NM_001009774
Type 1 IGF receptor, ovine	Regulates β-cell mass	104	aagaaccatgcctgcagaagg	ggattctcaggttctggccatt	AY162434
Type 2 IGF receptor, ovine	Regulates β-cell mass	90	atgaagctggactacaggcatca	gctcgccgtcctcagtttc	AF327649
Insulin receptor, ovine	Regulates β-cell mass	74	gcttcgaggctgcaccat	agctcagctgccaggttgtt	AY157728
PDX-1, bovine	Stimulates β-cell replication and β-cell function	97	cagagcccggaggagaacaag	cctggagatgtastttgttgaaaagg	XM_583722
Slc2a2, ovine	GLUT2, controls glucose uptake into β-cells	134	cgaaattgggaccatctcacat	caccgatagcacccctgagt	AJ318925
Glucokinase, bovine	Rate-limiting for glycolysis	115	aagaccacgcaccagatgtactc	cttcatctgatgcttatccaggaa	XM_868629
CACNA1D, bovine	Subunit of voltage-gated calcium channel	65	ttggcaagctgcaatcga	ggtgcggaggtgctcatagt	XM_876930
KIR6.2, human	Subunit of ATP-sensitive potassium channel	107	gatgccaacagcccactctac	ggtgatgccctggtttc	NM_000525

TABLE 2. Size at birth, body weight at postmortem, and placental morphology in control and PR, males and females at each age

	Control		PR		Significance		
	Male	Female	Male	Female	PR	Sex	PRsex[a]
Late gestation fetuses (143 d gestational age)							
Fetal (PM) weight (kg)	4.44 ± 0.58 (4)	4.56 ± 0.24 (5)	3.68 ± 1.06 (3)	2.91 ± 1.03 (2)	ns	ns	ns
Pancreas weight (g)	3.63 ± 0.49 (4)	4.32 ± 0.33 (5)	3.14 ± 0.55 (4)	2.59 ± 0.58 (4)	0.039	ns	ns
Pancreas weight (%)	0.082 ± 0.002 (4)	0.095 ± 0.005 (5)	0.090 ± 0.010 (4)	0.077 ± 0.003 (4)	ns	ns	0.038[a]
β-Cell volume density	0.104 ± 0.031 (4)	0.065 ± 0.023 (4)	0.095 ± 0.020 (3)	0.074 ± 0.042 (2)	ns	ns	ns
β-Cell mass (g)	0.362 ± 0.090 (4)	0.277 ± 0.081 (4)	0.334 ± 0.109 (3)	0.185 ± 0.141 (2)	ns	ns	ns
β-Cell mass (%)	0.0083 ± 0.0022 (4)	0.0043 ± 0.0009 (3)	0.0087 ± 0.0011 (3)	0.0053 ± 0.0030 (2)	ns	ns	ns
No β-cells/islet	10.5 ± 2.7 (4)	9.1 ± 0.8 (4)	10.5 ± 1.0 (3)	43 ± 31 (2)	0.082	ns	0.082[b]
Islets with <5 β-cells (%)	47 ± 14 (4)	50 ± 6 (4)	49 ± 11 (3)	13 ± 13 (2)	ns	ns	ns
Islet density (no/mm^2)	30.2 ± 6.1 (4)	22.4 ± 6.6 (4)	25.4 ± 4.2 (3)	5.7 ± 4.2 (2)	ns	0.061	ns
Lambs (43 d postnatal age)							
Birth weight (kg)	5.76 ± 0.20 (9)	4.60 ± 0.77 (6)	4.76 ± 0.30 (8)	3.5 ± 0.16 (5)	0.016	0.006	ns
PM body weight (kg)	20.1 ± 0.8 (9)	16.2 ± 1.4 (6)	19.2 ± 0.8 (7)	15.6 ± 0.7 (5)	ns	0.001	ns
Pancreas weight (g)	26.2 ± 3.5 (9)	16.3 ± 0.9 (6)	18.7 ± 1.9 (7)	13.7 ± 1.7 (5)	0.080	0.013	ns
Pancreas weight (%)	0.133 ± 0.020 (9)	0.103 ± 0.007 (6)	0.097 ± 0.008 (7)	0.088 ± 0.010 (5)	ns	ns	ns
β-Cell volume density	0.028 ± 0.002 (8)	0.029 ± 0.006 (6)	0.045 ± 0.011 (4)	0.040 ± 0.003 (4)	0.022	ns	ns
β-Cell mass (g)	0.725 ± 0.137 (8)	0.473 ± 0.110 (6)	0.771 ± 0.216 (4)	0.565 ± 0.098 (4)	ns	ns	ns
β-Cell mass (%)	0.0037 ± 0.0008 (8)	0.0030 ± 0.0007 (6)	0.0044 ± 0.0014 (4)	0.0034 ± 0.0006 (4)	ns	ns	ns
No β-cells/islet	15.0 ± 2.0 (8)	11.6 ± 2.5 (6)	15.7 ± 1.3 (4)	13.6 ± 2.5 (4)	ns	ns	ns
Islets with <5 β-cells (%)	31 ± 4 (8)	42 ± 8 (6)	20 ± 7 (4)	29 ± 6 (4)	0.077	ns	ns
Islet density (no/mm^2)	15.3 ± 2.3 (8)	16.7 ± 3.4 (6)	22.1 ± 5.6 (4)	19.6 ± 3.6 (4)	ns	ns	ns
Young adults (18 months postnatal age)							
Birth weight (kg)	5.55 ± 0.25 (13)	4.99 ± 0.14 (11)	4.08 ± 0.32 (9)	3.77 ± 0.30 (16)	<0.001	ns	ns
PM Body weight (kg)	61.8 ± 1.7 (12)	55.3 ± 1.9 (10)	61.3 ± 3.0 (6)	51.4 ± 2.1 (15)	ns	0.001	ns
Pancreas weight (g)	62.8 ± 3.0 (12)	55.1 ± 1.8 (10)	72.3 ± 4.0 (5)	54.6 ± 2.5 (14)	ns	<0.001	ns
Pancreas weight (%)	0.102 ± 0.005 (12)	0.100 ± 0.004 (10)	0.119 ± 0.004 (5)	0.107 ± 0.006 (14)	0.054	ns	ns
β-Cell volume density	0.022 ± 0.005 (6)	0.017 ± 0.004 (7)	0.025 ± 0.003 (5)	0.023 ± 0.002 (14)	ns	ns	ns
β-Cell mass (g)	1.47 ± 0.39 (6)	0.96 ± 0.27 (7)	1.86 ± 0.36 (4)	1.19 ± 0.17 (14)	ns	0.051	ns
β-Cell mass (%)	0.0023 ± 0.0005 (6)	0.0018 ± 0.0005 (7)	0.0029 ± 0.0006 (4)	0.0023 ± 0.0003 (14)	ns	ns	ns
No β-cells/islet	19.3 ± 3.3 (6)	11.2 ± 1.2 (7)	17.7 ± 1.6 (5)	17.7 ± 1.9 (14)	ns	0.098	0.097[c]
Islets with <5 β-cells (%)	31 ± 8 (6)	34 ± 6 (7)	24 ± 6 (5)	33 ± 6 (14)	ns	ns	ns
Islet density (no/mm^2)	6.0 ± 0.7 (6)	6.0 ± 1.0 (7)	6.8 ± 1.2 (5)	6.9 ± 0.8 (14)	ns	ns	ns

PM, Postmortem. Data are mean ± SEM (n) for each group.

[a] In late-gestation fetuses, pancreas weight (%) did not differ between control and PR males ($P = 0.42$) but was higher in control females than PR females ($P = 0.025$).

[b] In late-gestation fetuses, the number of β-cells per islet did not differ between control and PR males ($P = 1.0$) or females ($P = 0.15$).

[c] In young adult sheep, the number of β-cells per islet did not differ between control and PR males ($P = 0.6$) and was higher in PR than control females ($P = 0.032$).

Insulin disposition and β-cell function

Placental restriction did not alter insulin secretion (10.0 ± 1.3 mU·mmol^{-1}), basal or maximal PHIS (628 ± 156 and 2904 ± 900 μU·kg^{-1}·min^{-1}, respectively), or insulin disposition in absolute terms or per unit β-cell mass in late gestation fetuses ($P > 0.2$ for each). Male fetuses tended to have higher basal insulin disposition (Table 3, $P = 0.054$) and had higher basal insulin disposition per unit β-cell mass (320 ± 60, *cf.* 14 ± 14 mg·ml·kg^{-2}·min^{-2}, $P = 0.030$) than females. Maximal insulin disposition was higher in male than female fetuses in absolute terms (Table 3, $P = 0.045$) but not different per unit β-cell mass ($P > 0.7$). In lambs, the effect of PR on basal PHIS varied with sex, such that this did not differ between control and PR males (365 ± 40, *cf.* 429 ± 60 μU·kg^{-1}·min^{-1}, $P = 0.38$) but was higher in PR than control females (254 ± 46, *cf.* 538 ± 93 μU·kg^{-1}·min^{-1}, $P = 0.021$). PR and sex did not, however, alter insulin secretion (12.5 ± 2.3 mU·mmol^{-1}), maximal PHIS (2114 ± 247 μU·kg^{-1}·min^{-1}), or basal or maximal insulin disposition in absolute terms (23 ± 2 and 135 ± 22 mg·ml·kg^{-2}·min^{-2}, respectively) or per unit β-cell mass in lambs ($P > 0.15$ for each). In young adults, PR and sex did not alter insulin secretion or basal and maximal insulin disposition in absolute terms (12 ± 3 and 66 ± 7 mg·ml·kg^{-2}·min^{-2}, respectively) or per unit β-cell mass ($P > 0.15$ for each). Insulin secretion was higher in male than female young adults (Table 3, $P = 0.037$). Maximal PHIS was higher in male than female young adults (1586 ± 167, *cf.* 1129 ± 92 mU·mmol^{-1}, $P = 0.047$).

In late-gestation fetuses, insulin secretion (uncorrected for sensitivity) correlated positively with fetal weight overall (r = 0.600, $P = 0.014$, n = 14) and in males (r = 0.725, $P = 0.042$, n = 8) but not in females ($P > 0.2$). Basal insulin disposition in absolute terms or per unit β-cell mass did not correlate with fetal weight overall or in males (Fig. 2) or females. Maximal insulin disposition tended to correlate negatively with fetal weight overall (r = −0.526, $P = 0.097$, n = 11) and in males (Fig. 3). Maximal insulin disposition per unit β-cell mass correlated negatively with fetal weight overall (r = −0.789, $P = 0.012$, n = 9) and in males (Fig. 3). In lambs, insulin secretion did not correlate with birth weight. Basal insulin disposition tended to correlate negatively with birth weight overall (r = −0.387, $P = 0.068$, n = 23) and correlated negatively with birth weight in male lambs (Fig. 2). Basal insulin disposition per unit β-cell mass correlated negatively with birth weight in lambs overall (r = −0.493, $P = 0.038$, n = 18) but not significantly in males (Fig. 2) or females. Maximal

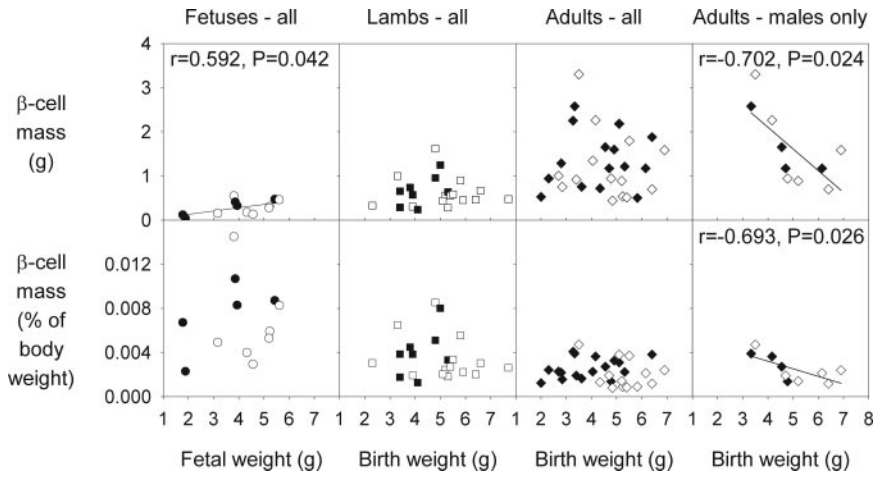

FIG. 1. Size in late gestation or at birth and β-cell mass in control (*open symbols*) and PR (*closed symbols*) sheep, in late-gestation fetuses (*circles*), as young lambs (*squares*), and in young adults (*diamonds*).

insulin disposition, in absolute terms or per unit β-cell mass, did not correlate with birth weight of lambs overall ($P > 0.2$ for each) or in males (Fig. 3) or females. In young adults, insulin secretion at 1 yr of age did not correlate with birth weight, overall or in males or females. Basal insulin disposition in adults correlated positively with birth weight overall ($r = 0.392$, $P = 0.036$, $n = 29$), and this relationship was stronger in males (Fig. 2) but not significant in females ($P > 0.4$). Basal insulin disposition per unit β-cell mass also correlated positively and strongly with birth weight in males (Fig. 2), although not overall or in females ($P > 0.3$ for each). Maximal insulin disposition in adults did not correlate with birth weight overall ($P = 0.14$) or in females ($P = 0.6$) but was strongly and positively correlated with birth weight in males (Fig. 3). Maximal insulin disposition per unit β-cell mass also correlated positively and strongly with birth weight in males (Fig. 3), although not overall or in females ($P > 0.6$ for each).

Expression of genes that regulate β-cell mass and function

In the present study, our initial results showed that enhanced glucose-stimulated insulin disposition in the low birth weight fetus was lost in lambs, although their basal insulin disposition was still elevated. Together with the observed switch from enhanced function in the growth-restricted fetus to impaired function in the young adult, this suggests the emergence of defective insulin secretion in early postnatal life. Furthermore, to maintain normal β-cell mass relative to body size, the pancreas in PR lambs must have undergone accelerated expansion of β-cell mass along with the catch-up in body weight, which is seen in early life in PR lambs who were small at birth. This resulted in similar body weights in PR and control lambs by postmortem at 43 d of age. This continues with compensatory expansion of β-cell mass evident in adult males who were small at birth. We therefore chose the young lamb to explore the molecular basis for effects of PR and restricted fetal growth on expression of genes that regulate β-cell function and mass on the basis of the emerging impaired function and ongoing compensation in mass of the β-cells.

PR increased pancreatic expression of IGF-II ($P = 0.025$) and IGF-I ($P = 0.028$) in young lambs and tended to increase expression of the insulin receptor ($P = 0.053$) and the transcription factor Pdx-1 ($P = 0.093$) but did not alter expression of the types 1 or 2 IGF receptors ($P > 0.4$ for each, Fig. 4). When corrected for β-cell mass, PR tended to increase expression of the type 1 IGF receptor ($P = 0.083$, Fig. 4). PR had variable effects on molecular determinants of β-cell function. PR tended to increase expression of Slc2a2 ($P = 0.083$, Fig. 5), did not alter expression of glucokinase or Kir6.2 ($P > 0.2$ for each, Fig. 5), and decreased expression of voltage-gated calcium channel α1D subunit (CACNA1D) ($P = 0.005$, Fig. 5). When corrected for β-cell mass, PR decreased the expression of CACNA1D ($P = 0.020$) but did not affect the expression of Slc2a2, glucokinase, or Kir6.2 ($P > 0.5$ for each, Fig. 5).

Expression of Kir6.2, when corrected for β-cell mass, correlated positively with several measures of size at birth overall

TABLE 3. Insulin secretion, insulin action, and β-cell function in males and females at each age

	Male	Female	Significance
Late-gestation fetuses (143 d gestational age)			
Insulin secretion ($mU \cdot mmol^{-1}$)	9.0 ± 1.7 (8)	11.0 ± 2.0 (8)	ns
Basal insulin disposition ($mg \cdot ml \cdot kg^{-2} \cdot min^{-2}$)	103 ± 25 (6)	29 ± 17 (5)	0.054
Maximal insulin disposition ($mg \cdot ml \cdot kg^{-2} \cdot min^{-2}$)	417 ± 75 (6)	126 ± 71 (5)	0.045
Lambs (43 d postnatal age)			
Insulin secretion ($mU \cdot mmol^{-1}$)	11.1 ± 2.3 (15)	14.3 ± 4.5 (11)	ns
Basal insulin disposition ($mg \cdot ml \cdot kg^{-2} \cdot min^{-2}$)	21.1 ± 1.6 (15)	27.1 ± 5.5 (8)	ns
Maximal insulin disposition ($mg \cdot ml \cdot kg^{-2} \cdot min^{-2}$)	110 ± 15 (15)	182 ± 56 (8)	ns
Young adults (18 months postnatal age)			
Insulin secretion ($mU \cdot mmol^{-1}$)	7.6 ± 1.2 (16)	5.1 ± 0.6 (19)	0.037
Basal insulin disposition ($mg \cdot ml \cdot kg^{-2} \cdot min^{-2}$)	17.1 ± 6.8 (12)	9.7 ± 1.6 (17)	ns
Maximal insulin disposition ($mg \cdot ml \cdot kg^{-2} \cdot min^{-2}$)	77.0 ± 12.1 (12)	57.6 ± 7.1 (17)	ns

Data are mean ± SEM (n) for each group.

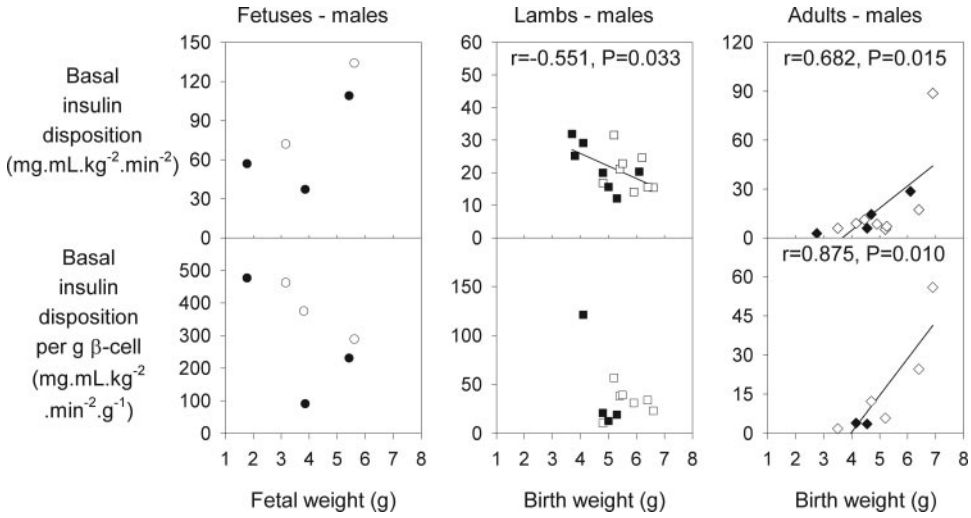

FIG. 2. Size in late gestation or at birth and fasting β-cell function in male control (*open symbols*) and PR (*closed symbols*) sheep, in late gestation fetuses (*circles*), as young lambs (*squares*) and in young adults (*diamonds*).

(radius-ulna length: r = 0.526, $P = 0.012$, n = 22; abdominal circumference: r = 0.518, $P = 0.014$, n = 22; thoracic circumference: r = 0.452, $P = 0.034$, n = 22), with a similar trend for birth weight (r = 0.387, $P = 0.075$, n = 22). Kir6.2 expression was not consistently related to size at birth in males. In females, pancreatic expression of Kir6.2 as well as corrected for β-cell mass, correlated strongly, positively, and consistently with size at birth, including birth weight (Fig. 6). Pancreatic expression of IGF-I correlated with size at birth in a sex-specific manner. In males, pancreatic expression of IGF-I correlated negatively with size at birth including crown-rump length and shoulder height (each $P < 0.05$), and similar but weaker correlations were seen between IGF-I expression corrected for β-cell mass and size at birth. In females, pancreatic expression of IGF-I corrected for β-cell mass correlated positively with size at birth, including crown-rump length, metacarpus length, radius ulna length, and abdominal circumference (each $P < 0.05$) with a similar trend for birth weight (r = 0.599, $P = 0.067$, n = 10). Expression of other molecular determinants of β-cell mass and function were not consistently related to measures of size at birth in either sex.

Gene expression and functional outcomes

Relationships between gene expression and functional outcomes differed markedly between sexes. In males only, pancreatic expression of IGF-II and the insulin receptor was strongly and consistently positively correlated with measures of β-cell mass (Fig. 7). Pancreatic expression of IGF-II in males also correlated strongly and positively with glucose-stimulated insulin secretion in absolute terms (r = 0.752, $P = 0.002$, n = 14). In contrast, pancreatic expression of IGF-II (r = −0.734, $P = 0.016$, n = 10) and insulin receptor (r = −0.655, $P = 0.040$, n = 10) in males correlated negatively with insulin secretion per unit β-cell mass. Also in males, expression of CACNA1D in pancreas and corrected for β-cell mass, correlated strongly and positively with maximal insulin disposition (Fig. 8). In females, pancreatic expression of the type 2 IGF-receptor (r = −0.672, $P = 0.047$, n = 9) and CACNA1D (r = −0.708, $P = 0.022$, n = 10) correlated negatively with β-cell volume density, and expression of the CACNA1D calcium channel, corrected for β-cell mass, correlated negatively with β-cell volume density (r = −0.752, $P = 0.012$, n = 10).

Discussion

Effects of IUGR on β-cell function and mass progress with aging

This study provides the first evidence that growth restriction before birth enhances rather than impairs β-cell function, with a β-cell mass appropriate for current fetal size in the small fetus.

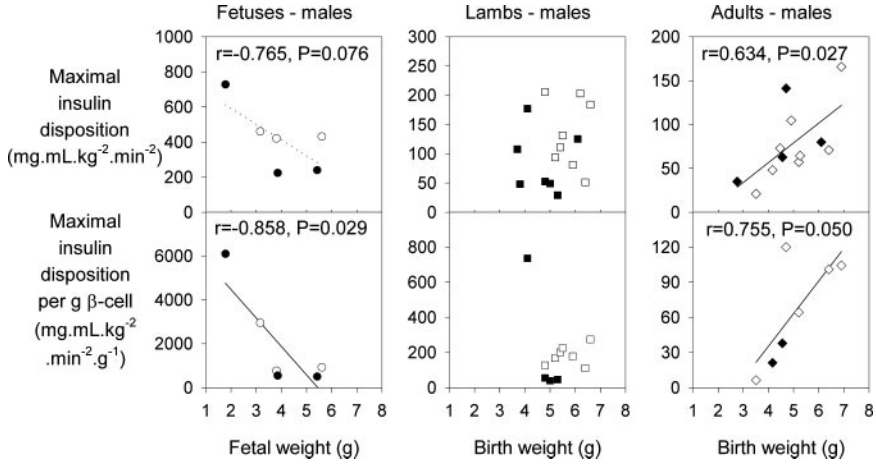

FIG. 3. Size in late gestation or at birth and glucose-stimulated β-cell function in male control (*open symbols*) and PR (*closed symbols*) sheep, in late gestation fetuses (*circles*), as young lambs (*squares*), and in young adults (*diamonds*).

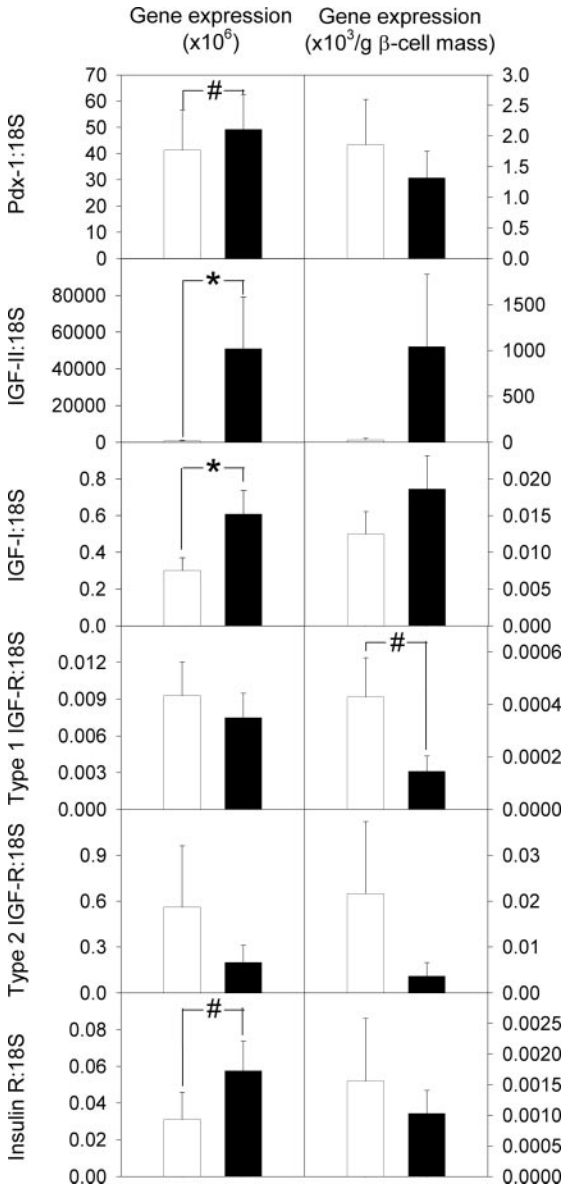

FIG. 4. Pancreatic expression of regulators of β-cell mass in control (*open bars*) and PR (*filled bars*) lambs. Data are mean ± SEM for each group. Gene expression was nonnormally distributed and did not differ with sex, and effects of PR on gene expression were analyzed by Mann Whitney nonparametric test. Differences between control and PR sheep are indicated as follows: *, $P < 0.05$; #, $P < 0.1$.

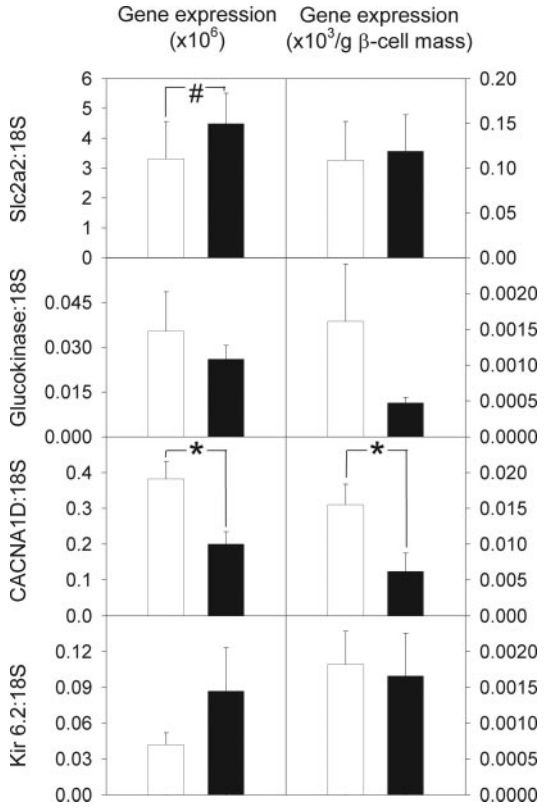

FIG. 5. Pancreatic expression of regulators of glucose-stimulated insulin secretion in control (*open bars*) and PR (*filled bars*) lambs. Data are mean ± SEM for each group. Gene expression was nonnormally distributed and did not differ with sex, and effects of PR on gene expression were analyzed by Mann Whitney nonparametric test. Differences between control and PR sheep are indicated as follows: *, $P < 0.05$; #, $P < 0.1$.

This reverses postnatally, and defects in β-cell function become apparent by adulthood in males who grew poorly before birth. This, together with inadequate compensatory increases in β-cell mass, decreases the insulin secretion relative to sensitivity of IUGR adult male sheep. Given the similarities in fetal and postnatal consequences of IUGR in humans and PR in sheep, we suggest that defects in β-cell function and inadequate compensatory increases in β-cell mass may also underlie reduced insulin disposition after human IUGR.

Fetus. In this study, PR and poor growth before birth did not impair insulin disposition before birth and glucose-stimulated β-cell function was in fact increased in small fetuses. Uncorrected glucose-stimulated insulin secretion and basal insulin concentrations were, however, reduced in proportion to fetal weight, consistent with effects of heat-induced IUGR in sheep (26, 36). In the present study, fetal sheep had a β-cell mass that was reduced in proportion to their body weight, consistent with previous reports in the PR rat and in heat-induced ovine IUGR (37, 38). β-Cell V_d was not reduced by PR or poor growth before birth, consistent with reduced β-cell V_d being seen only in the most severely restricted human neonates (8, 39). This relative mass of β-cells might, however, fall during the accelerated neonatal growth characteristic of IUGR humans and PR sheep (29, 31, 40, 41).

Young lamb. We previously reported that PR and small size at birth increased glucose-stimulated insulin secretion relative to sensitivity, as well as insulin sensitivity, in young lambs (32). In the present cohort, basal, although not glucose stimulated, insulin disposition was also increased in lambs that were born small, particularly males. Normal or improved insulin action and glucose tolerance in young IUGR sheep contrasts with the impairment seen in young and aged PR rats (10) and might reflect differences in the timing of restriction or in the maturity of the pancreas at birth. Like the human, the ovine pancreas is more mature at birth than in rodents (12, 42–44) so that a greater proportion of pancreatic development will be exposed to effects of an adverse fetal environment in sheep and humans.

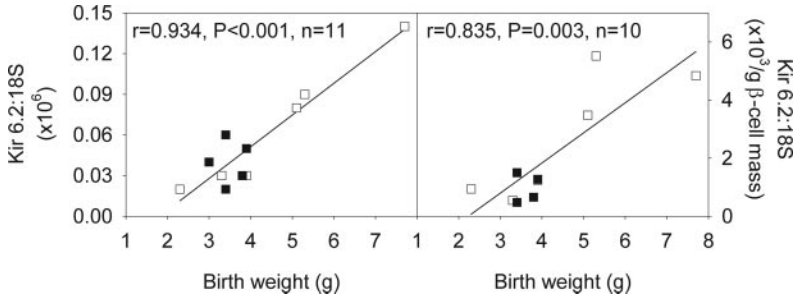

FIG. 6. Size at birth positively predicts pancreatic expression of the Kir 6.2 K-channel subunit in female lambs at 42 d of age. Control lambs are shown by *open squares* and PR lambs are shown by *filled squares*.

Young adult. By adulthood, we found evidence of compensatory increases in β-cell mass in male sheep, although this was insufficient to compensate for loss of β-cell function and maintain insulin disposition. IUGR in humans also increases diabetes incidence in adults (1), suggesting that the capacity of β-cells for compensatory increases in mass and function is impaired by exposure to an adverse fetal environment. This loss of β-cell plasticity seems to be a specific defect underlying the etiology of diabetes after IUGR. In contrast, most people who develop insulin resistance during obesity or pregnancy increase insulin secretion adequately to maintain insulin disposition (45). Intriguingly, maternal food restriction in late pregnancy and lactation prevents normal expansion of β-cell mass with aging or pregnancy in rat progeny (46–48). The effects of chronic restriction of placental and fetal growth and removal of restraint at birth, as in human IUGR, on plasticity of insulin secretion and β-cell mass have not yet been reported.

Molecular mechanisms underlying changes in β-cell function and mass after PR and IUGR

Maintenance of insulin action and normal β-cell mass in the young lamb implicates activation of compensatory mechanisms to maintain relative β-cell mass and function because PR and IUGR lambs underwent substantial accelerated or catch-up growth in the first month of life, challenging the capacity of the endocrine pancreas to similarly expand β-cell function and mass.

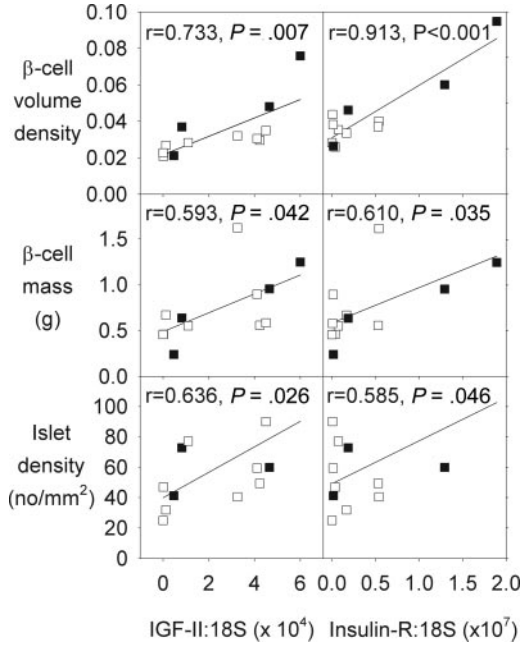

FIG. 7. Pancreatic expression of IGF-II and insulin receptor positively predict β-cell volume density and mass in male lambs at 42 d of age. Control lambs are shown by *open squares* and PR lambs are shown by *filled squares*.

Determinants of β-cell function. Pdx-1 and Slc2a2 were up-regulated in PR lambs, with the latter possibly contributing to increased basal insulin disposition in small lambs by increasing GLUT2-mediated glucose uptake into β-cells. Our results also identify Pdx-1 as an important regulator of adaptation of β-cell mass and function in large mammals, consistent with results from knockout studies in mice (13, 14). In rats, normalizing Pdx-1 expression after PR also normalizes β-cell mass and prevents their development of diabetes (49) and further suggests that interventions to increase expression of Pdx-1, or its capacity for up-regulation in response to increased demand for insulin, may be helpful in restoring insulin action after poor growth before birth. PR did, however, decrease pancreatic expression of the α1D subunit of the voltage-gated calcium channel in lambs. Expression of this calcium channel was also strongly positively correlated with glucose-stimulated insulin disposition and β-cell function, particularly in males, and implicates expression of this gene as a capacity-limiting factor in glucose-stimulated insulin secretion. Our results identify this as a candidate gene, and calcium channel function as a candidate process, for loss of β-cell function after IUGR.

Determinants of β-cell mass. Our findings that PR increased pancreatic expression of IGF-II and insulin receptor in PR lambs, together with the strong positive correlations between expression of these genes and markers of β-cell mass in males, implicates these genes as key regulators of compensatory increases in β-cell mass. IGF-I expression was also increased in PR lambs, although its expression levels were about 100,000-fold lower than IGF-II, consistent with IGF-II playing the major role in regulation of β-cell mass. We hypothesize that in older sheep, up-regulation of these genes and hence of β-cell mass may nevertheless be blunted after IUGR so that the increases in β-cell mass achieved are inadequate to compensate for impaired β-cell function and developing whole-body insulin resistance. Epigenetic changes before birth have been proposed as mechanisms by which changes induced before birth might persist and cause diseases in later life (24). PR in the rat reduces abundance of methyl donors and key methylating enzymes, decreases DNA methylation, and increases histone acetylation in liver before birth, and the latter persists postnatally (50). This directly affects regulation of β-cell mass and function, with epigenetic changes in PR rat fetuses leading to postnatal silencing of Pdx-1 (24). The IGF-II gene is also epigenetically

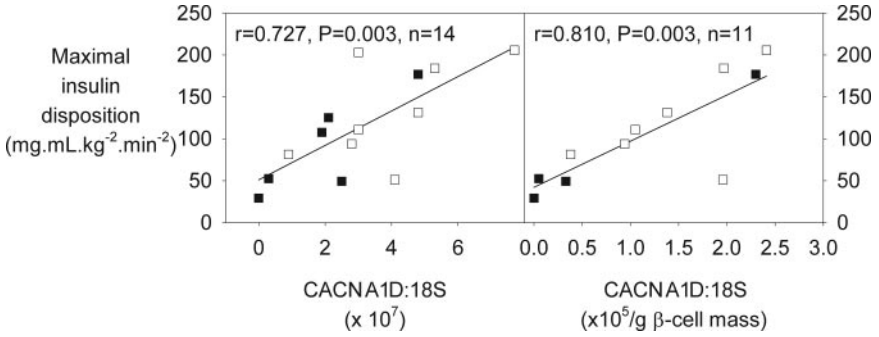

FIG. 8. Pancreatic expression of CACNA1D calcium channel subunit positively predicts maximal glucose-stimulated insulin disposition in male lambs at 42 d of age. Control lambs are shown by *open squares* and PR lambs are shown by *filled squares*.

regulated. Feeding mice a low folate diet for 60 d after weaning increases DNA methylation in the paternal IGF-II promoter, causes loss of imprinting of the IGF-II gene, and decreases total IGF-II gene expression, which persists (51). We suggest that PR in sheep may also reduce methyl donor abundance and alter epigenetic patterning of IGF-II before birth and that this persists and may limit later IGF-II expression or its up-regulation.

Sexually dimorphic effects of IUGR

Effects of poor intrauterine growth on insulin secretion and gene expression in the present study were sexually dimorphic. We previously reported that male but not female sheep developed impaired glucose tolerance as young adults if they grew poorly before birth (34). Consistent with this, young adult males but not females in the present study had impaired insulin secretion, relative to sensitivity, if they grew poorly before birth. Furthermore, PR induced changes in gene expression, associated with compensatory increases in β-cell mass and loss of β-cell function in the young lamb, that were most evident in males. Other studies have also reported that the effects of restricted growth before birth on insulin action become evident at earlier ages in males than females. In a study of 20-yr-old adults, men but not women were insulin resistant, and at this age glucose tolerance was maintained in men by compensatory increases in insulin secretion and glucose effectiveness (52). Similarly, in the rat whose mother was fed a low-protein diet in pregnancy and lactation, impaired glucose tolerance and hyperinsulinemia develop earlier in male than female progeny (53). This sexually dimorphic pattern in effects of restricted fetal growth on insulin action might be because male fetuses grow more rapidly than females, particularly in the second half of gestation when placental capacity to supply nutrients becomes limiting for fetal growth (54). Growth and development of male fetuses could therefore be restricted to a greater extent than females by poor placental function. Alternatively, the sexually dimorphic pattern in effects of restricted fetal growth on insulin action may be due to intrinsic sex differences in susceptibility to diabetes when challenged, possibly due to postnatal effects of gonadal steroids. Consistent with the latter, males are more susceptible to development of diabetes than females in many experimental rodent models of diabetes (55).

Implications

Maintenance of insulin secretion relative to sensitivity in the young PR lamb in concert with induction of genes that positively regulate β-cell mass suggests that interventions to further expand β-cell mass postnatally may delay onset of NIDDM after IUGR. Nevertheless, adult males failed to maintain adequate insulin secretion despite further increases in β-cell mass. We suggest that interventions to improve β-cell function or both β-cell function and mass are more likely to prevent NIDDM after IUGR than those that target β-cell mass alone. One putative target for intervention is the transcription factor Pdx-1, which positively regulates β-cell mass and function. Exciting studies have shown that neonatal treatment with the glucagon-like peptide-1 analog exendin-4, which up-regulates Pdx-1, prevents later development of diabetes in the PR rat (49). Because at least 18% of the population risk of diabetes can be accounted for by poor growth before birth (2), treatments to prevent or reverse the mechanisms underlying later development of diabetes in the IUGR individual would be of significant benefit. Whether neonatal interventions such as exendin-4 are effective in a species in which the pancreas undergoes most development before birth, rather than after birth, and is exposed to ongoing restraint during IUGR, is yet to be determined.

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