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Neonatal Exendin-4 Reduces Growth, Fat Deposition and Glucose Tolerance during Treatment in the Intrauterine Growth-Restricted Lamb

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Abstract

Background: IUGR increases the risk of type 2 diabetes mellitus (T2DM) in later life, due to reduced insulin sensitivity and impaired adaptation of insulin secretion. In IUGR rats, development of T2DM can be prevented by neonatal administration of the GLP-1 analogue exendin-4. We therefore investigated effects of neonatal exendin-4 administration on insulin action and β-cell mass and function in the IUGR neonate in the sheep, a species with a more developed pancreas at birth.

Methods: Twin IUGR lambs were injected s.c. daily with vehicle (IUGR+Veh, n = 8) or exendin-4 (1 nmol.kg^{-1}, IUGR+Ex-4, n = 8), and singleton control lambs were injected with vehicle (CON, n = 7), from d 1 to 16 of age. Glucose-stimulated insulin secretion and insulin sensitivity were measured *in vivo* during treatment (d 12–14). Body composition, β-cell mass and *in vitro* insulin secretion of isolated pancreatic islets were measured at d 16.

Principal Findings: IUGR+Veh did not alter *in vivo* insulin secretion or insulin sensitivity or β-cell mass, but increased glucose-stimulated insulin secretion *in vitro*. Exendin-4 treatment of the IUGR lamb impaired glucose tolerance *in vivo*, reflecting reduced insulin sensitivity, and normalised glucose-stimulated insulin secretion *in vitro*. Exendin-4 also reduced neonatal growth and visceral fat accumulation in IUGR lambs, known risk factors for later T2DM.

Conclusions: Neonatal exendin-4 induces changes in IUGR lambs that might improve later insulin action. Whether these effects of exendin-4 lead to improved insulin action in adult life after IUGR in the sheep, as in the PR rat, requires further investigation.

Introduction

Small size at birth or intrauterine growth restriction (IUGR) consistently predicts increased risk of type 2 diabetes mellitus (T2DM) in human studies [1,2], including independently of gestation length [3]. This relationship is consistent and significant, with ~18% of the lifetime risk of T2DM accounted for by poor growth before birth [4]. Impaired insulin sensitivity and inadequate insulin secretion are each implicated as contributing to this increased risk of T2DM in the IUGR human [1,5,6,7].

Poor fetal growth commonly reflects restricted fetal supply of oxygen and nutrients due to impaired placental growth and/or function [8]. In the sheep, surgically-induced restriction of placental growth (PR) from before mating, and small size at birth, increase insulin sensitivity in early neonatal life in association with catch-up growth and increased fat deposition [9,10]. PR

nevertheless impairs glucose-stimulated insulin disposition before weaning at 1 month of age, and this progresses to impaired insulin sensitivity and blunted basal and glucose-stimulated insulin disposition in young adult males at 1 year of age [11,12]. Impaired β-cell function is the primary cause of this inadequate insulin secretion, which occurs despite increases in β-cell mass in 1-year-old males [12]. Similarly, PR late in pregnancy in rats produces progeny with normal circulating glucose and insulin levels at 1 week of age, but mild fasting hyperglycemia and hyperinsulinemia at 7–10 weeks and frank diabetes by 26 weeks [13,14]. Impaired β-cell function with later reduction in β-cell mass is also implicated in decreased insulin secretion in the PR rat postnatally [13,14]. Excitingly, administration of the GLP-1 analogue exendin-4 to neonatal PR rats normalised subsequent β-cell mass and insulin secretion and prevented later development of T2DM [15]. Prevention of T2DM by neonatal exendin-4

treatment in PR rats is at least partially due to induction and normalisation of expression of the transcription factor *Pdx-1* [15,16], which regulates β-cell function as well as adaptive increases in β-cell mass [17,18], and is epigenetically down-regulated in PR rat progeny [19].

The timing of pancreatic development and maturation of β-cell function, and therefore developmental stages of exposure to IUGR and neonatal interventions, differs between species. In humans and sheep, most pancreatic development takes place before birth, with β-cells present by 0.25 gestation, islets present in mid-gestation and substantial remodelling to a mature endocrine pancreas by near term [20,21,22,23]. In both species, β-cell function is present and matures from mid-gestation onwards [24,25,26,27]. This functional maturation in humans and sheep may be driven in part by their pre-partum surge in cortisol. In contrast, rodents undergo later development of β-cells than sheep or humans, with β-cells first appearing in late gestation (0.6) and pancreatic remodelling at ~10-17 d postnatal age [28,29,30]. Neonatal surges in corticosterone and β-cell maturation in rodents are marked by increased expression of key molecular determinants of glucose-induced insulin secretion coupling [31] and mitochondrial enzymes of the NADH shuttle, essential for stimulation of insulin secretion by oxidative metabolism [32]. Exendin-4 may in part be effective in preventing PR programming of reduced β-cell mass and function in rodents, because it occurs before and during such maturation. In the present study, we have therefore treated neonatal IUGR sheep with exendin-4 and assessed whether it is able to induce changes in growth, insulin action and β-cell mass and function after IUGR in a species in which the pancreas undergoes most maturation before birth.

Materials and Methods

Ethics statement

All procedures in this study were approved by the University of Adelaide Animal Experimentation and Ethics Committee (approval M-84-2007) and complied with the Australian code of practice for the care and use of animals for scientific purposes [33].

Animal, treatments and surgery

Australian Merino ewes underwent a timed-mating program, and pregnancies were confirmed by ultrasound scanning at ~60 d gestational age (term~150 d). Delivery occurred naturally at term and the lambs were housed in floor pens with their mothers throughout the study and allowed to suckle freely, with access to their mother's feed and water, except during experimental protocols as described below. Natural twinning was used to induce IUGR. Sibling twin lambs were injected with vehicle (0.5% methanol in 0.9% saline s.c., IUGR+Veh) or exendin-4 (1 nmol.kg^{-1} s.c., IUGR+Ex-4, n = 8), with the first twin pair randomly allocated to treatments and then the heavier and lighter birth weight twin alternately allocated in order to balance birth weights between the two treatments. Exendin-4 (Bachem, Buberndorf, Germany) was prepared as a 5 nM stock in 0.5% methanol and 0.9% saline, and stored at $-20°$C in single use aliquots, which were thawed immediately prior to injection. Singleton lambs were injected daily with vehicle (CON, n = 7). All lambs (singletons and twins) were supplemented with whey protein (Resource Beneprotein instant protein powder, Nestle, Australia) given orally in two equal feeds (at 0900–1000 h and 1600–1700 h), commencing at 1.25 g.kg^{-1}.d^{-1} on d 4 and increasing to 5 g.kg^{-1}.d^{-1} on and after d 7. Feeding this supplement during this period of maximal catch-up growth in IUGR lambs [10] was intended to minimise the potential for limitation of neonatal growth by milk availability in twins [34] by providing ~25% of the protein expected to be available through milk, and allowing lambs to self-regulate their milk intake to appetite.

On d 4, catheters were inserted into the lamb's femoral artery and vein under general anaesthesia, induced and maintained by fluothane inhalation anaesthetic, as described previously [10]. Basal blood samples were collected from arterial catheters every second morning before supplement feeding. Lambs were weighed at birth and then every 2 d throughout the study. Lamb size was measured at birth and then every 4 d, and absolute (AGR) and fractional (FGR) growth rates from birth to d 16 fitted by linear regression [10].

In vivo measures of insulin secretion, sensitivity, and action

Glucose tolerance and glucose-stimulated insulin secretion were measured during an intravenous glucose tolerance test (IVGTT) at d 14, and indices of glucose tolerance and insulin secretion calculated as described previously [10,11,35]. The whole body insulin sensitivity of glucose metabolism was measured by hyperinsulinemic euglycemic clamp at d 12 [35]. Insulin sensitivityglucose, the metabolic clearance rate (MCR) of insulin, basal and maximal post-hepatic insulin delivery rates, and basal and maximal insulin disposition indices (IDI) were calculated as described previously [35].

Analysis of plasma insulin and metabolites

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 Inc., St Charles, MO, USA), which has 100% cross-reactivity with ovine insulin. The intra-assay coefficients of variation (CV) for the insulin assay were 7.2% and 5.3%, and inter-assay CV were 7.0% and 19.6% for QC samples containing 9.9 and 35.9 mU.L-1 insulin respectively (n = 10 assays). Plasma glucose concentrations were measured by colorimetric enzymatic analysis on a Hitachi 912 automated metabolic analyser using Roche/Hitachi Glucose/HK kits (Roche Diagnostics GmbH, Mannheim, Germany).

Post-mortem

Lambs were euthanized by overdose of sodium pentobarbitone at d 16. Organs (liver, kidneys, lungs, heart), muscles (semitendinosus, gastrocnemius, soleus, tibialis, extensor digitorum longus, biceps femoris, vastus lateralis, biceps), and dissectable fat depots (left and right perirenal fat, left and right retroperitoneal fat and omental fat) were dissected and weighed for each lamb. Dissected muscle and visceral fat weights were calculated as the sum of weights of these muscles and fat depots, respectively.

Pancreas and islet isolation and immunostaining and morphometric analysis

Each pancreas was rapidly dissected and weighed. Representative mixed aliquots were fixed for 48 h in 4% paraformaldehyde before embedding in paraffin wax. One section per block was immunostained to detect insulin-positive cells, and morphometric analysis of β-cells was performed as described previously, in 20 fields of view per sheep selected by random-systematic sampling [12]. Measures of *in vivo* β-cell function were calculated by dividing total, 1st phase and 2nd phase glucose-stimulated insulin secretion and basal and maximal IDI by β-cell mass. Pancreatic islets were obtained by collagenase digestion of pancreas at 35°C for 40 min, washing and handpicking of islets >100 μm in diameter, with

purity confirmed by immunostaining of aliquots as previously described [36]. Islet aliquots were cultured overnight at 37°C in 95% O_2/5% CO_2 in RPMI 1640 media (Sigma Aldrich, Sydney, Australia).

In vitro β-cell secretion and responses

Static islet incubation and experiments were performed as previously described [36]. Briefly, for each animal and incubation condition, triplicate preparations of 10 islets were handpicked into 1.5 mL tubes. Static incubations were performed at 37°C for 1 hr in KRB/BSA/Forskolin media containing 0, 1.1, 11.1 mM glucose, or 15 mM KCl, or 11.1 glucose plus 5 mM Lysine, 11.1 glucose plus 5 mM Arginine, 1.1 glucose plus 10 mM Leucine, 11.1 glucose plus 10μM Epinephrine, or at 0°C for 1 hr in KRB/BSA/Forskolin media containing 11.1 glucose. Islets were then centrifuged, supernatant collected for insulin analysis and DNA was ethanol-extracted from pellets and quantified by PicoGreen dsDNA Quantification kit (Invitrogen, Melbourne, Australia). In vitro insulin secretion for each replicate was calculated as insulin concentration divided by DNA concentration. In vitro data for an animal was included in analyses provided that insulin secretion in incubations with KCl (test of maximal release) was greater than those obtained from incubations with epinephrine or at 0°C (inhibitory quality controls). Due to technical difficulties with some preparations, in vitro insulin secretion data was obtained successfully for 5 CON, 5 IUGR+Veh and 6 IUGR+Ex-4 lambs.

Statistical analysis

Data for non-repeated measures on each animal were analysed by the mixed models procedure of SPSS for effects of treatment (fixed effect) and including dam as a random (block) effect in the model to account for common maternal environment in twins. Where treatment effects or trends were apparent (P<0.1), we then compared means by the LSD method, based on a priori questions to determine: 1. effects of IUGR (CON cf. IUGR+Veh groups), 2. effects of exendin-4 in IUGR lambs (IUGR+Veh cf. IUGR+Ex-4 groups), and 3. to assess whether exendin-4 restored values to those of controls (CON cf. IUGR+Ex-4 groups). We also confirmed these comparisons between IUGR+Veh and IU-GR+Ex-4 groups using a paired t-test to compare twin siblings, and the significance of this test was consistent with that for LSD comparisons for all measures (data not shown). Neonatal growth patterns and glucose, insulin and insulin:glucose ratios overall and during 1st phase (0–30 min) and 2nd phase (30–210 min) of insulin secretion during the IVGTT were analysed by repeated measures for effects of treatment (between factor), time (within factor) and interactions, and including dam as a random (block) effect in the model to account for common maternal environment in twins. Glucose-stimulated in vitro insulin secretion was analysed by repeated measures for effects of treatment (between factor), glucose concentration (within factor) and interactions. Stimulation and inhibition of in vitro insulin secretion were analysed using repeated measures models for effects of treatment (between factor), stimulation (within factor, 11.1 mM glucose or KCl) or inhibition (within factor, 11.1 mM glucose or epinephrine) and interactions,

Table 1. Effect of IUGR and neonatal exendin-4 on size at birth, postnatal growth and body composition in lambs.

	CON	IUGR+Veh	IUGR+Ex-4	Significance (treatment effect)
Number of animals	7	8	8	
Size at birth				
Birth weight (kg)	6.01±0.21	4.82±0.17*	4.84±0.15*	<0.001
Crown rump length (cm)	56.3±1.4	54.6±1.1	55.1±1.1	NS
Shoulder height (cm)	44.0±0.7	40.1±0.8*	40.9±0.7*	0.008
Abdominal circumference (cm)	40.1±0.4	35.1±0.9*	36.1±0.6*	<0.001
Body mass index (kg.m^{-2})	19.2±1.1	16.3±0.8*	16.0±0.7*	0.040
Neonatal Growth				
AGR$_{weight}$ (g.day^{-1})	309±29	327±14	211±17^	0.001
FGR$_{weight}$ (%.day^{-1})	5.17±0.48	6.86±0.39	4.35±0.32^	0.001
AGR$_{shoulder\ height}$ (cm.day^{-1})	0.390±0.027	0.507±0.037*	0.403±0.038^	0.030
FGR$_{shoulder\ height}$ (%.day^{-1})	0.89±0.06	1.17±0.19	1.00±0.11	NS
AGR$_{abdominal\ circumference}$ (cm.day^{-1})	0.473±0.075	0.782±0.042*	0.544±0.048^	0.002
FGR$_{abdominal\ circumference}$ (%.day^{-1})	1.18±0.19	2.25±0.17*	1.52±0.15^	0.001
Postmortem (d 16)				
Body weight (kg)	11.0±0.5	10.1±0.3	8.33±0.25^*	<0.001
Total liver weight (g)	296±19	285±17	214±7^*	0.002
Total liver weight (% of body weight)	2.70±0.10	2.82±0.14	2.57±0.07^	NS
Summed muscle mass (g)	265±13	228±8*	183±9^*	<0.001
Summed muscle mass (% of body weight)	2.42±0.07	2.26±0.04	2.19±0.08*	0.055
Visceral fat (g)	132±19	118±11	41.7±6.3^*	<0.001
Visceral fat (% of body weight)	1.19±0.17	1.16±0.09	0.495±0.062^*	<0.001

Neonatal growth rates are from d 0 to 16. NS: P>0.1, * different from CON (P<0.05), ^ different from IUGR+Veh (P<0.05).
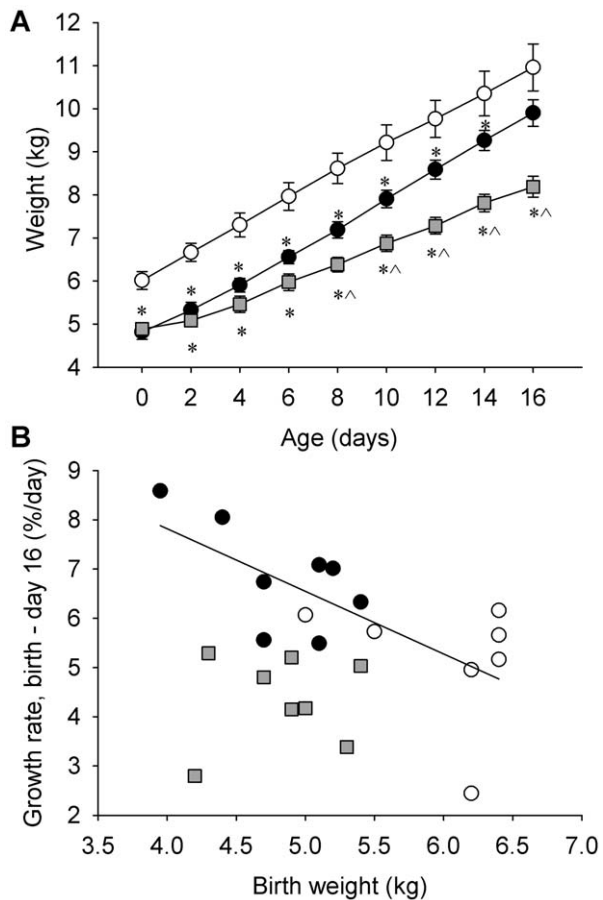doi:10.1371/journal.pone.0056553.t001

Figure 1. Effect of IUGR and neonatal exendin-4 treatment on neonatal growth. Neonatal exendin-4 treatment reduced weight of twin IUGR lambs from 8 days of age (A), and abolished the negative relationship between birth weight and neonatal fractional growth rate (B). CON (white circle) and IUGR+Veh (black circle) lambs were treated once daily with vehicle (0.5% methanol in saline s.c.) and IUGR+Ex-4 (gray square) lambs were treated once daily with exendin-4 (1 nmol.kg^{-1} s.c.). Data in Figure 1A are means ± SEM, and data in Figure 1B are individual animal outcomes. * different from CON (P<0.05),^ different from IUGR+Veh (P<0.05).
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and by mixed model as described above for incubations with individual secretagogues.

Results

Size at birth, neonatal growth and body composition

Lamb weight, abdominal circumference and body mass index at birth were reduced in twin lambs (all IUGR groups) compared to singleton lambs (each P<0.001, Table 1). Absolute and fractional growth rates for weight and abdominal circumference, and absolute but not fractional growth rate for shoulder height, differed with treatment (Table 1). IUGR+Veh lambs had higher FGR for weight and abdominal circumference than CON lambs (P=0.022 and P=0.001 respectively), and by d 16, there was no difference in weight between these two groups (Figure 1A). In control and IUGR+Veh lambs, FGR for weight increased as birth weight decreased (combined: R=−0.700, P=0.002, n=15; Figure 1B), whereas in IUGR+Ex4 lambs, neonatal FGR was not related to birth weight (P>0.3; Figure 1B). Neonatal exendin-4 treatment reduced neonatal growth rates (Table 1) including

for weight (AGR$_{weight}$, −35%, P<0.001), linear growth (AGR$_{shoulder\ height}$, −20%, P=0.031), and organ growth (AGR$_{abdominal\ circumference}$, −30%, P=0.007), and this group were lighter than CON and IUGR + Vehlambs at d16 (Figure 1). Neonatal exendin-4 reduced body weight (−18%, P=0.016) and relative visceral fat mass (−57%, P<0.001) at post-mortem compared to IUGR+Veh lambs (Table 1). IUGR+Ex-4 lambs had lower absolute liver weights than CON (−28%, P=0.001) or IUGR+Veh (−25%, P=0.009) lambs, and lower relative liver weights (as a proportion of body weight) than IUGR+Veh lambs (−9%, P=0.021). Absolute summed muscle mass was lower in IUGR+Veh lambs (−3.7%, P=0.017) relative to CON, and was decreased by exendin-4 treatment relative to CON (−27%, P<0.001) and IUGR+Veh (−25%, P =0.004) groups. Relative summed muscle weight also tended to be lower in IUGR+Veh (−7.6%, P=0.093) and was lower in IUGR+Ex-4 (−9.5%, P=0.019) compared to CON lambs (Table 1).

Insulin secretion, sensitivity and action

Fasting glucose and insulin levels, glucose tolerance and overall, 1st phase and 2nd phase insulin secretion in vivo were similar in IUGR+Veh and CON lambs (each P>0.1, Table 2). Fasting plasma glucose (d 14) was reduced in IUGR+Ex-4 lambs compared to CON (−10%, P=0.022) and IUGR+Veh lambs (−9%, P=0.019, Table 2). Conversely, glucose tolerance was impaired (increased glucose AUC) in IUGR+Ex-4 lambs overall (+132%, +156% respectively), during first phase insulin secretion (+41%, +57%), and during second phase insulin secretion compared to CON and IUGR+Veh lambs (each P≤0.02, Table 2). Across the whole of the IVGTT, and within the 1st phase of insulin secretion, plasma glucose (Figure 2) changed with time (each P<0.001). Fasting plasma glucose in fasting samples was lower in IUGR+Ex4 than in IUGR+Veh lambs (P<0.001), and tended to be lower in IUGR+Ex4 than in CON lambs (P=0.091). Conversely, plasma glucose during the 1st phase of insulin secretion was higher in IUGR+Ex4 than in IUGR+Veh lambs (P<0.001), and plasma glucose during the 2nd phase of insulin secretion did not differ between groups (P>0.3). The pattern of change in plasma glucose with time differed between groups overall (P<0.001) and during the 1st phase of insulin secretion (P=0.003). Fasting plasma insulin in absolute terms and relative to glucose, and insulin secretion (assessed relative to the glucose stimulus as AUC insulin/AUC glucose) did not differ between the groups (Table 2 and Figure 2). Plasma insulin (Figure 2) changed with time throughout the IVGTT (P<0.001), and within 1st (P<0.001) and 2nd phase (P=0.008) of insulin response. The ratio of plasma insulin to glucose (Figure 2), an index of insulin secretion, similarly changed with time throughout the IVGTT (P<0.001), and within 1st (P=0.015) and 2nd phase (P=0.005) of insulin response. Plasma insulin concentrations and the ratio of plasma insulin to glucose ratios during the IVGTT (Figure 2) were higher in IUGR+Ex4 than in IUGR+Veh lambs overall (each P<0.001) and during the 2nd phase of insulin secretion (each P<0.001), and did not differ between other treatment groups. IUGR+Ex-4 lambs had lower insulin sensitivity compared to CON (−44%, P=0.004) and IUGR+Veh lambs (−46%, P=0.002, Table 2). Basal and maximal insulin disposition indices did not differ between groups (Table 2).

Pancreas morphology and β-cell function

Absolute and relative pancreas weights, and numbers of β-cells per islet, β-cell volume density and absolute β-cell mass did not differ with treatment (Table 3). β-cell mass relative to body weight was greater in IUGR+Ex-4 lambs than CON lambs (+36%,

Table 2. Effect of IUGR and neonatal exendin-4 on insulin action in lambs.

	CON	IUGR+Veh	IUGR+Ex-4	Significance (treatment effect)
Number of animals	7	8	8	
Fasting				
Plasma glucose (mmol.L^{-1})	6.47±0.26	6.40±0.11	5.81±0.12*	0.008
Plasma insulin (mU.L^{-1})	20.4±6.0	15.4±2.2	16.4±2.2	NS
Plasma insulin:glucose (mU.mmol^{-1})	3.30±1.11	2.40±0.35	2.83±0.38	NS
AUC glucose (mmol.min.L^{-1})				
Total	62±6	56±3	143±28*	0.003
1st phase	60.9±5.4	54.8±2.9	86.2±5.7*	<0.001
2nd phase	1±1	1±1	57±24*	0.017
AUC insulin (mU.min.L^{-1})				
Total	587±184	590±181	863±178	NS
1st phase	499±133	579±180	650±128	NS
2nd phase	88±61	12±6	213±119	NS
AUC insulin:AUC glucose (mU.mmol^{-1})				
Total	10.8±4.3	10.9±3.6	7.6±1.9	NS
1st phase	8.9±2.8	10.7±3.5	7.8±1.6	NS
2nd phase	26.5±25.9	0.3±0.3	9.6±2.8	NS
Insulin sensitivity (mg.L^{-1}.mU^{-1}.kg^{-1}.min^{-1})	0.097±0.010	0.100±0.011	0.047±0.009*	0.003
Basal IDI (mg.mL.kg^{-2}.min^{-2})	69.7±31.2	39.5±5.5	28.4±10.0	NS
Maximal IDI (mg.mL.kg^{-2}.min^{-2})	138±28	119±27	97±37	NS

Glucose and insulin AUC were measured during an IVGTT (0.25 g glucose.kg^{-1}) at d 14. 1st and 2nd phase values for insulin and glucose were measured from 0–30 and from 30–210 minutes after glucose administration, respectively. Insulin sensitivity was measured during a hyperinsulinemic euglycemic clamp (2 mU insulin.kg^{-1}.min^{-1}) at d 12. NS: P>0.1, * different from CON (P<0.05), ∧ different from IUGR+Veh (P<0.05).
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P = 0.039, Table 3). IUGR+Ex-4 lambs also tended to have higher relative β-cell mass than IUGR+Veh lambs (+28%, P = 0.083, Table 3). Measures of β-cell function did not differ between treatments (Table 3).

In vitro β-cell secretory function

Islet insulin secretion (Figure 3) increased with increasing glucose concentration between 0 and 11.1 mM overall (P = 0.006). Glucose-stimulated insulin secretion tended to be higher overall in IUGR+Veh compared to CON lambs (+420%, P = 0.081), did not differ between IUGR+Ex4 lambs and CON lambs (P = 0.9) and tended to be higher in IUGR+Veh lambs than in IUGR+Ex4 lambs (+20%, P = 0.087). At the highest glucose concentration (11.1 mM), IUGR +Vehlambs had higher insulin secretion than CON lambs (+66%, P = 0.046) and tended to have higher insulin secretion than IUGR+Ex-4 lambs (+58%, P = 0.066 respectively, Figure 3). Within each group of lambs, in vitro insulin secretion at 11.1 mM glucose was between 1.6 and 2-fold higher than that at 0 mM glucose (Figure 3). In vitro insulin secretion was similar from islets incubated with 15 mM KCl or 11.1 mM glucose (P>0.5), and the response to KCl was similar between treatments (P>0.8). In vitro insulin secretion was suppressed by epinephrine treatment compared to glucose-stimulated insulin secretion (−62%, P = 0.001). Suppression of glucose-stimulated insulin secretion by epinephrine was greater in IUGR+Veh than CON lambs in absolute terms (−173 cf. −47 µU insulin/µgDNA, P = 0.044), but not as a proportion of insulin secretion in the absence of epinephrine (−28.8% cf. −7.8%, P = 0.274). Epinephrine suppression of glucose-stimulated insulin secretion was similar in islets from IUGR-Ex4 to that in other groups (P>0.1 for each).

Lysine-, arginine- and leucine-stimulated in vitro islet insulin secretion did not differ between treatment groups (each P>0.3, data not shown).

Discussion

In the present study, twin IUGR lambs caught up in weight by 16 d of age, and had normal in vivo insulin action in their second week of life, with similar β-cell mass to singleton control lambs. Glucose-stimulated in vitro insulin secretion was increased in the IUGR twin lamb relative to controls, suggesting up-regulated β-cell function at this age. Daily exendin-4 treatment of twin IUGR lambs during neonatal life prevented catch-up growth and fat accumulation, and normalised in vitro insulin secretion from their islets, relative to untreated IUGR twins, which may retain adaptive capacity for later life. Glucose tolerance of IUGR lambs was impaired during exendin-4 treatment however, reflecting decreased insulin sensitivity and occurred despite greater in vivo insulin secretion. This may be due to central actions of exendin-4 to inhibit food intake and insulin sensitivity [37,38,39]. Nevertheless, the reduction in fat accumulation and normalised β-cell action in vitro of IUGR lambs suggest that neonatal exendin-4 might have beneficial effects on insulin-regulated glucose homeostasis in later life. These outcomes also demonstrate the biological activity of exendin-4 for the first time in the sheep, at least in the context of individuals who had undergone growth-restriction before birth.

We found similar growth and metabolic responses to IUGR induced by twinning in this study to those seen previously after IUGR induced by restriction of placental growth and function (PR) in sheep. Like the PR lamb, the twin IUGR lambs in the
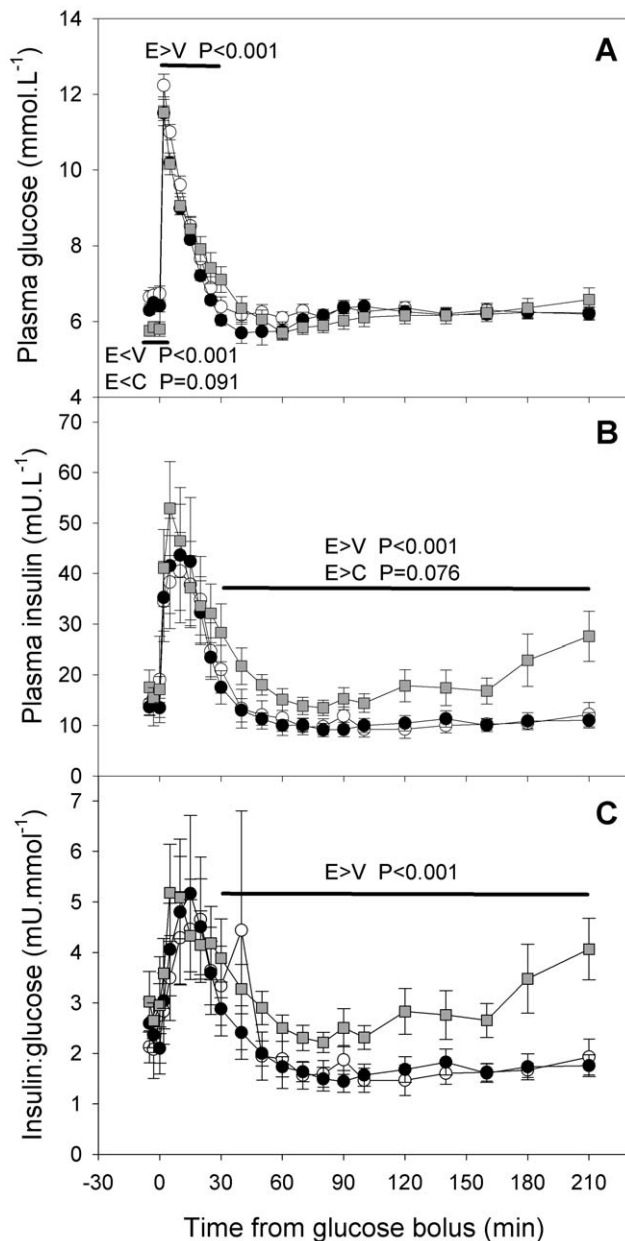
Figure 2. Effect of IUGR and neonatal exendin-4 treatment on in vivo metabolism in young lambs. Glucose tolerance (A), glucose-stimulated insulin secretion (B) and relative glucose-stimulated insulin secretion (C) were measured during an intravenous glucose tolerance test 14 days of age. CON (white circle, n = 7), IUGR+Veh (black circle, n = 8) and IUGR+Ex-4 (gray square, n = 8). Data are means ± SEM. * different from CON (P<0.05).
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present study experienced accelerated neonatal catch-up growth, achieving a normal body weight by 16 d of age in this study and by 30 d of age in our studies in PR lambs [40]. Accelerated fat deposition occurs during accelerated neonatal growth, and in humans catch-up growth is a risk factor for later obesity [41]. PR lambs have fat stores proportionate to their reduced body weight in late gestation [42], and similar to our twin lambs at 16 days in the present study, fat mass relative to body weight is similar in PR and CON lambs at 21 days despite their catch up growth [43]. By 43 days of age, however, the accelerated fat deposition results in

greater visceral fat in PR lambs than their control counterparts [40]. Small size at birth in humans consistently induces insulin resistance in adults and adolescents [44], but this is preceded by enhanced insulin sensitivity in neonates, which reverses to resistance in association with catch-up growth in the first few years of life [45]. There is similar evidence of a reversal from insulin sensitivity to insulin resistance in the lamb following IUGR induced by restriction of placental growth and function (PR). The young PR lamb at 21 days of age has increased expression of insulin receptors and insulin signalling molecules in skeletal muscle [46], although *in vivo* insulin action was not measured. At 30 days, glucose tolerance of PR lambs is normal, despite decreased insulin action caused by falls in both *in vivo* insulin secretion and insulin sensitivity [40,47]. The latter reflects decreased expression of insulin-signalling pathways in skeletal muscle [47]. Impaired glucose tolerance and elevated fasting glucose emerge by 1 year of age in IUGR sheep [48]. The normal insulin sensitivity and glucose tolerance seen here in the twin IUGR lamb may therefore reflect the beginnings of the reversal from insulin sensitivity to insulin resistance occurring during the neonatal catch-up growth they are experiencing at this age.

Neither IUGR nor neonatal exendin-4 treatment in IUGR lambs altered relative β-cell mass at 16 days in the present study, consistent with the lack of effect of PR and neonatal exendin-4 treatment on β-cell mass in young postnatal rats at 2 weeks of age [49]. In the rat, reduced β-cell mass after IUGR emerges by 3 months of age in young adults, and neonatal exendin-4 treatment normalises adult β-cell mass at this age in this model [49]. We hypothesise that these beneficial effects of exendin-4 treatment after IUGR might also emerge with ageing in the sheep. This lack of an immediate response may also reflect the collection of pancreas soon after completion of exendin-4 treatment here and in PR rats. Previous rodent studies have reported increased β-cell replication after similar exendin-4 treatment durations, but differences in β-cell mass are sometimes not apparent until several weeks later [50,51]. Many of the actions of exendin-4 and GLP-1 on insulin secretion are mediated via stimulation of *Pdx-1* expression, a transcription factor important for regulation of β-cell mass as well as function, and which is required for plasticity of β-cell mass and function to increase insulin secretion in response to demand. In the PR rat, prevention of later diabetes following neonatal exendin-4 treatment reflects reversal of epigenetic changes induced by PR in the *Pdx-1* promoter by late gestation, that normally worsen with age and lead to decreased *Pdx-1* expression, loss of β-cell function and subsequent loss of β-cell mass postnatally [15,16,19]. Intriguingly, although neonatal exendin-4 induces epigenetic changes such as increased acetylation and lysine 4 trimethylation at histone H3 in control as well as PR rat juveniles, it only increases *Pdx-1* expression and β-cell mass and improves glucose tolerance in the PR progeny [15,16,19]. Indeed, the *Pdx*-1 promotor becomes methylated and hence partially silenced by adulthood in untreated PR rat progeny, but not in control progeny regardless of exendin-4 treatment, which implies that the levels of histone 3 acetylation and lysine 4 trimethylation in untreated control progeny is already sufficient to prevent later promoter methylation [16]. We do not yet know whether neonatal exendin-4 treatment will affect outcomes in control sheep progeny, as the aim of the present study was to evaluate its efficacy only in the context of IUGR. Whether neonatal exendin-4 acts similarly in the IUGR lamb as in the PR rat, by reversing epigenetic changes in the *Pdx-1* promotor and improves adult β-cell mass and function to delay or prevent the subsequent loss of insulin secretory capacity observed after IUGR in young adult male sheep [11] remains to be determined, and will require separate animal

Table 3. Effect of IUGR and neonatal exendin-4 on pancreas morphology and β-cell function.

	CON (7)	IUGR+Veh (8)	IUGR+Ex-4 (8)	Significance (treatment effect)
Pancreas morphology				
Pancreas weight (g)	10.8±1.5	8.53±0.93	7.90±0.55	NS
Pancreas (% of body weight)	0.103±0.019	0.085±0.009	0.096±0.007	NS
β-cell volume density	0.033±0.005	0.040±0.003	0.049±0.007	NS
β-cell mass (g)	0.326±0.038	0.345±0.054	0.387±0.055	NS
β-cell mass (% of body weight)	0.0030±0.0004	0.0034±0.0005	0.0047±0.0006*	0.070
Islet density (no.mm^{-2})	66.3±9.7	76.9±10.3	91.6±10.5	NS
β-cells/islets	10.9±1.4	10.5±1.3	12.9±1.3	NS
% of islets with <5β-cells	27.7±6.3	23.8±6.4	31.3±6.7	NS
β-cell function				
Insulin secretion (AUC ins) per β-cell mass (mU.min.L^{-1}.g^{-1})	1682±413	1944±588	2190±286	NS
Basal IDI per β-cell mass (mg.mL.kg^{-2}.min^{-2}.g^{-1})	187±60	129±25	85.4±26.5	NS
Max IDI per β-cell mass (mg.mL.kg^{-2}.min^{-2}.g^{-1})	441±85	389±89	269±94	NS

NS: $P>0.1$, * different from CON ($P<0.05$).
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cohorts with long-term follow-up of functional and molecular outcomes

In this study, the decrease in fasting plasma glucose (~9%) and more sustained insulin secretion during exendin-4 treatment in the IUGR neonatal lamb compared to untreated IUGR siblings were generally consistent with responses to exendin-4 in rodents and humans. Medium- to long-term exendin-4 treatment in human T2DM patients (daily 5–10 μg injections for 30 and 82 weeks) [52,53,54], in the obese diabetic *db/db* mouse (1 nmol.kg^{-1}.d^{-1} as daily injections for 14 days) [55], and in the obese *ob/ob* mouse (20 μg.kg^{-1}.d^{-1}, ~ 5 nmol.kg^{-1}.d^{-1} as twice daily injections for 60 days) [56] reduces fasting blood glucose as well as HbA1c, a marker of chronic hyperglycemia. Earlier studies in humans also demonstrated acute decreases in fasting and post-prandial glucose concentrations after a single exendin-4 dose and after 5 days of

twice daily injections with 5 μg exendin-4 [57]. Infusions with GLP-1 and chronic exendin-4 treatment enhance post-prandial and glucose-stimulated insulin secretion in human patients with T2DM, including restoration of 1st phase insulin secretion response to glucose, and sustained elevation of 2nd phase insulin secretion in T2DM patients [57,58,59]. In the diabetic rat, four weeks of twice-daily exendin-4 injections (105 pmol.kg^{-1}) increased 1st and 2nd phase insulin secretion during a hyperglycemic clamp [60]. Whilst we similarly observed increases in second phase insulin secretion in IUGR lambs during exendin-4 treatment, their first phase insulin secretion was unchanged. This apparent difference may be because first phase insulin secretion is normal in the IUGR lamb at this age, whereas previous reports of increased first phase insulin responses after exendin-4 or GLP-1 treatment have all been in the context of diabetes, when first phase
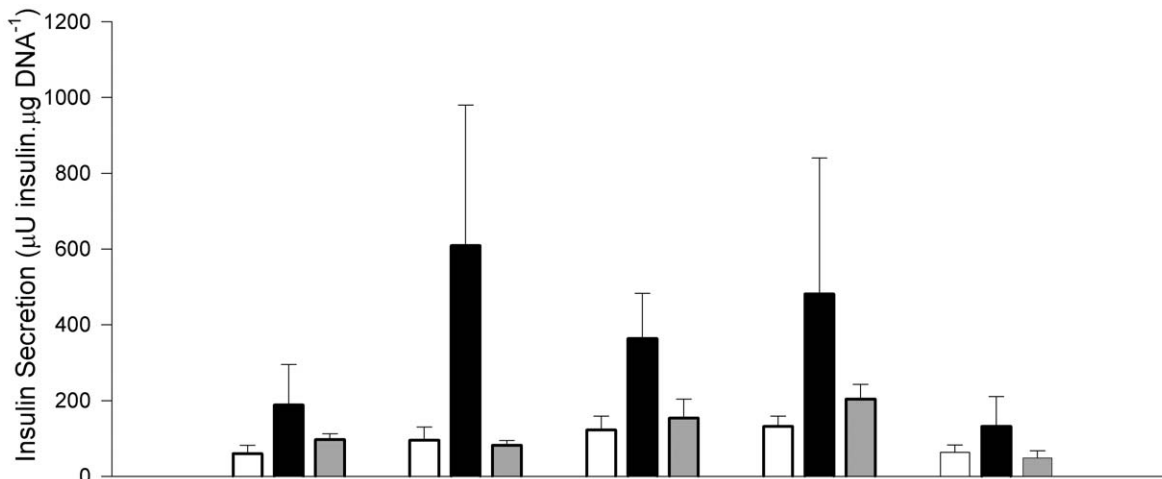

Figure 3. Effect of IUGR and neonatal exendin-4 treatment on in vitro insulin secretion from isolated islets in response to glucose and potassium chloride. CON (white bar, n = 5), IUGR+Veh (black bar, n = 5) and IUGR+Ex-4 (gray bar, n = 6). Data are means ± SEM. Specific contrasts: * $P<0.05$, # $P<0.10$.
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secretion is impaired. The effects of exendin-4 on insulin secretion in IUGR sheep AFTER cessation of treatment remain to be investigated.

We performed *in vitro* testing to measure intrinsic β-cell function independent of systemic input from endocrine and nervous systems [36]. In this study, IUGR neonatal lambs had enhanced *in vitro* glucose-stimulated insulin secretion, or β-cell hypersecretion relative to control lambs, which occurs in obese individuals, as well as early in the pathogenesis of type 2 diabetes [61,62,63]. Interestingly, exendin-4 treatment of IUGR lambs abolished this *in vitro* insulin hypersecretion from isolated islets, suggesting some normalisation of intrinsic β-cell function and its determinants. Together with increased β-cell mass, this suggests that neonatal exendin-4 may improve insulin secretory capacity after IUGR.

In contrast with the improved insulin sensitivity seen after chronic GLP-1 or exendin-4 treatment in human patients with extreme obesity [64] or T2DM [65], insulin sensitivity was profoundly decreased on the 11th day of exendin-4 treatment in neonatal IUGR lambs, relative to their untreated IUGR littermates. In studies of exendin-4 action in rodents, direct measures and calculated indices of insulin sensitivity have either been increased [56,60,66], or not altered [55], immediately following or during chronic (2 – 9 weeks) exendin-4 treatment. We propose that the differential effects of exendin-4 on insulin sensitivity may depend on whether the latter is assessed during treatment or after, whether the subjects are obese and on their developmental stage and growth rate. Exendin-4 reduced weight gain in the IUGR lambs in the present study as well as in PR rat neonates [15], consistent with its actions including decreased food and caloric intake, reduced gastric emptying and induced weight loss or slowed weight gain in mice and rats [67,68] and in adolescent and adult humans [52,53,54,64,69,70,71]. It appears that restricted nutrition reduces insulin sensitivity in growing animals, possibly partly due to reduced mass of insulin-responsive tissues, whereas in older or obese animals the net effect of restricted feeding and consequently reduced fatness is to increase insulin sensitivity. Thus, feed restriction *increases* insulin-stimulated glucose metabolism and insulin sensitivity in adult sheep [72], but decreases insulin-stimulated glucose uptake in muscle of young growing pigs [73]. In mice, exendin-4 can cross the blood-brain barrier [74], and acts centrally to suppress femoral blood flow and whole body insulin sensitivity, via the GLP-1 receptor and activation of PKC-δ signalling pathways in the hypothalamus [37,38], suggesting an additional mechanism for decreased peripheral insulin sensitivity during exendin-4 treatment. As a consequence of their reduced insulin sensitivity, and despite the increased 2nd phase insulin secretion that maintained insulin disposition, glucose tolerance was impaired in IUGR+Ex-4 lambs compared to IUGR+Veh and CON lambs. This contrasts with improved glucose tolerance observed 24 h after completion of medium- to long-term exendin-4 treatment in mature rats [56,59,60,66], during continued long-term exendin-4 treatment in β-cell depleted rats [75], and acutely in T2DM human patients [76]. In some of these studies, the improved glucose tolerance during or after exendin-4 treatment reflects marked improvement

of deficient insulin secretion due to stimulation of β-cell regeneration [75] or up-regulation of β-cell function in T2DM patients [76]. Long-term exendin-4 treatment increases insulin sensitivity in obese humans, genetically-obese rodents and diabetic humans and rodents, measured either during or 16–24 h after completion of treatment [56,64,77,78]. Improved whole-body insulin sensitivity is probably also due to improvements in hepatic insulin sensitivity, with lower post-prandial endogenous glucose production after or during exendin-4 treatment [79]. To our knowledge, this is the first study of the effects of exendin-4 on insulin action treatment in young growing animals. Further studies are needed to define the underlying mechanisms for their reduced insulin sensitivity during treatment.

The profound reduction in visceral fat deposition after IUGR in response to exendin-4 is also of particular potential importance for later glucose homeostasis, given that obesity and particularly visceral fat deposition are strong risk factors for impaired glucose tolerance and T2DM [80,81]. In the PR rat, neonatal exendin-4 reduces weight gain in conjunction with prevention of later diabetes, and this may particularly reduce the risk of T2DM in IUGR subjects [15], since catch-up growth after IUGR is a risk factor for T2DM and for adult obesity [82,83]. Intriguingly, neonatal exendin-4 treatment abolished the negative relationship between birth weight and fractional growth rate in IUGR lambs in the current study. In contrast to its metabolic effects, exendin-4 reduced neonatal growth and adult size in both control and PR rat progeny [15]. This suggests that exendin-4 may act in part, but not only, via the pathway/s responsible for catch-up after IUGR, which include neonatal hyperphagia, elevated insulin sensitivity and increased abundance of thyroid hormones in IUGR lambs [40,84,85]. Longer-term evaluations of growth and composition after cessation of exendin-4 are needed to determine whether this decrease in central adiposity persists in the IUGR sheep.

In conclusion, neonatal exendin-4 treatment increased 2nd phase insulin secretion *in vivo*, normalised *in vitro* insulin secretion and decreased visceral fat at the end of treatment in the IUGR lamb. Neonatal exendin-4 treatment also improves insulin secretion and glucose tolerance in adolescent and adult rat progeny following IUGR, preventing development of diabetes in these animals [15], although the effects during treatment were not measured in the latter study. Investigation of the long-term effects of neonatal exendin-4 on glucose homeostasis and insulin action in the IUGR lamb into adulthood should be a priority for the future.

Acknowledgments

Author Contributions

Conceived and designed the experiments: KLG JAO. Performed the experiments: KLG SAS SNBM MJD MLH. Critically revised manuscript for intellectual content: KLG MJD MLH RAS JAO. Analyzed the data: KLG SAS JAO. Wrote the paper: KLG SAS JAO.

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