

# Altered pancreatic morphology in the offspring of pregnant rats given reduced dietary protein is time and gender specific

Astrid Chamson-Reig<sup>1</sup>, Sandra M Thyssen<sup>1</sup>, Edith Arany<sup>1,2</sup> and David J Hill<sup>1,2,3,4</sup>

<sup>1</sup>Lawson Health Research Institute, St Joseph's Health Care, 268 Grosvenor Street Room H404, London, Ontario, Canada N6A 4V2

Departments of <sup>2</sup>Medicine, <sup>3</sup>Physiology and Pharmacology, <sup>4</sup>Paediatrics, University of Western Ontario, London, Ontario, Canada N6A 4V2

(Requests for offprints should be addressed to D J Hill; Email: dhill@lri.sjhc.london.on.ca)

## Abstract

Restriction of dietary protein during gestation and lactation in the rat results in a reduction in  $\beta$  cell mass, insulin content and release in the offspring, and glucose intolerance when the offspring reach adulthood. The present study was designed to identify if a particular developmental window existed during prenatal development when endocrine pancreatic development was most susceptible to nutritional insult. Pregnant rats received a low-protein (8%, LP), but isocaloric diet from conception to parturition, during the first 2 weeks of gestation (LP (1–2)), the second week only (LP (2)), or the third week (LP (3)). At other times, they received a 20% protein (C) diet, while control animals received this diet continuously. When the offspring were examined at 130 days age, animals that had received LP diet had a significantly impaired glucose tolerance compared with control-fed animals. Pancreatic morphology was examined in the offspring on postnatal days 1 and 21. The LP diet resulted in a significant decrease in the numbers of large (more than 10 000  $\mu\text{m}^2$ ) and medium (between 5000 and 10 000  $\mu\text{m}^2$ ) sized islets present at postnatal day 1 for all LP treatments. Consequently, mean islet area and the mean number of  $\beta$  cells

were reduced. The impact of LP diet was most pronounced in LP (2) for females and in LP (3) for males, and this was greater than for continuous LP exposure. Insulin and Glut-2 mRNA expression were impacted negatively by LP in early and late gestation, but increased following administration in mid-gestation. Total pancreatic insulin content was not altered by LP treatment. Pdx-1, a transcription factor associated with both  $\beta$  cell development and insulin gene transcription, was decreased in female offspring following LP (1–2) and LP (3), but not in males. Pancreatic expression of nestin mRNA, and the abundance of nestin-immunoreactive cells within islets, was decreased by all LP treatments. By postnatal day 21, the mean islet area and number of  $\beta$  cells had largely recovered. However, insulin and Glut-2 mRNAs were elevated in offspring exposed to LP diet, particularly in females. The studies show that LP dietary insult in early, middle, or late gestation, all result in a relative deficiency of  $\beta$  cells following birth, due to a failure to develop larger islets, but that females were particularly susceptible in mid-gestation and males in late gestation.

*Journal of Endocrinology* (2006) **191**, 83–92

## Introduction

Retrospective epidemiological studies have shown that an increased incidence of hypertension, cardiovascular disease, type 2 diabetes, and osteoporosis in adulthood is linked to a low birth weight, and that the susceptibility to particular metabolic diseases is statistically associated with morphometry at birth (Barker 1998). Individuals who had low ponderal index at birth were also thin, tended to be more insulin resistant in adulthood with an impaired glucose tolerance, low muscle lipid content, and reduced rates of glycolysis (Phillips *et al.* 1994, 1996, Taylor *et al.* 1995). The maintenance of birth length in these infants suggests that the fetal insult occurs after 36 weeks when the human fetus accumulates substantial fat and glycogen stores (Bernstein *et al.* 1997). The incidence of adult hypertension was increased in those who were proportionately small at birth,

but these individuals showed no increased risk of adult cardiovascular disease (Barker 1998). Conversely, individuals born disproportionately small had a greater risk of adult cardiovascular events. Proportionate smallness at birth is a reflection of reduced skeletal length, and is likely to represent an earlier onset of the fetal insult than that which occurs in disproportionate growth. A direct linkage between fetal nutrition and adult metabolic disease was shown from follow-up studies of individuals born during the Dutch famine of 1944/1945 (Ravelli *et al.* 1998), where third trimester famine exposure, in particular, led to adult glucose intolerance and type 2 diabetes. In contrast, maternal malnutrition in early gestation, but not in later fetal development, was associated with a higher body mass index and waist circumference in the adults at age 50 with a profound sexual dimorphism, the relative obesity being seen in the females but not in the males (Ravelli *et al.* 1999).

Dietary modifications in early life, such as altered composition, excess or restricted intake, can also predispose to adult metabolic abnormality in rodent models (Alvarez *et al.* 1997, Garofano *et al.* 1997). Protein restriction within an otherwise isocaloric diet during pregnancy and lactation in the rat results in moderate intrauterine growth restriction and altered development of the pancreas and insulin-sensitive tissues (Reusens *et al.* 1995, Hales & Ozanne 2003, Guan *et al.* 2005). These morphological changes to the pancreas are irreversible. While in early adult life, such animals show an improved glucose tolerance compared with control-fed rats with a greater insulin-stimulated glucose uptake by muscle and adipose (Langley *et al.* 1994), by 15 months of age, they show a significantly worse glucose tolerance, particularly in female offspring (Hales *et al.* 1996). A reduction of dietary protein to 8% throughout gestation (low-protein (LP) diet) resulted in a reduced pancreatic weight in the offspring at birth with reduced  $\beta$  cell mass, islet size, and pancreatic vascularity, a diminished rate of  $\beta$  cell replication, but increased developmental apoptosis (Petrik *et al.* 1999, Boujendar *et al.* 2003). When nutritional restriction was stopped at birth, the islet morphology partly recovered, but when extended to weaning, the effects were irreversible. Islets isolated in late fetal life following LP diet showed a lower basal insulin release and a blunted insulin release in response to glucose, arginine, or leucine (Cherif *et al.* 1998). Analysis of  $\beta$  cell cycle kinetics *in situ* suggested that the cycle length was increased by LP, with an extended G<sub>1</sub> phase (Petrik *et al.* 1999). This may reflect a re-programming of cell cycle kinetics that might be imprinted on a precursor stem cell population prior to  $\beta$  cell differentiation. The precise identity of  $\beta$  cell precursors during islet development is unclear, although during pancreatic organogenesis they derive from neurogenin-3-positive cells present within the pancreatic ductal epithelium (Gu *et al.* 2002, Mellitzer *et al.* 2004). Previously, it was suggested that cells immunoreactive for the intermediate filament protein, nestin, which are present in the developing human and rodent pancreas, represented endocrine cell progenitors (Hunzinker & Stein 2000). However, we and others showed that nestin-immunoreactive cells *in vivo* are mesenchymal in origin, and are more closely related to endothelial cell formation (Treutelaar *et al.* 2003, Joannette *et al.* 2004). Nestin-positive cells within the pancreatic ducts and islets, were reduced at birth in the rat following exposure to LP diet, and rescued in parallel with  $\beta$  cell mass by supplementation of the LP diet with specific amino acids, such as taurine (Joannette *et al.* 2004). This suggests that the presence of nestin-immunoreactive cells in the developing pancreas is at least a useful marker of islet formation, and may reflect the development of a supportive vasculature, which is a source of  $\beta$  cell tropic growth factors (Lammert *et al.* 2001, 2003).

We have utilized the LP-fed rat model to study in more detail the critical periods of embryonic and fetal development during which a nutritional insult can invoke altered postnatal metabolism, and also to determine if this phenotype shows

sexual dimorphism as seen in human populations. We have measured endocrine pancreatic morphology and the expression of genes key for insulin release, since these are early indicators of adult glucose intolerance in this model. Our rationale was that an altered morphology of the endocrine pancreas seen following administration of LP diet during early gestation only would reflect primarily an influence on pancreatic embryogenesis and endocrine progenitor cells. The effectiveness of an insult in mid-gestation would be more likely to represent a change in islet formation, which begins around day E12 (Soria 2004), while in late gestation, the LP diet would most likely influence  $\beta$  cell mass and maturation. Islet mass approximately doubles and glucose sensitivity substantially increases in  $\beta$  cells in the last 72 h of fetal life, between 19 and 21 days' gestation.

## Materials and Methods

### Animals

Female Wistar rats were purchased from Charles River (Montreal, PQ, Canada). The animals were housed in the animal facilities at the Lawson Health Research Institute in a temperature-controlled room with a ratio of 12 h light:12 h darkness cycle and were given food and water *ad libitum*. Nulliparous rats weighing 250–300 g were mated on the night of proestrus. The presence of sperm in the vaginal smear was confirmed the next morning, and this was taken as the first day of pregnancy. Pregnant females were immediately allocated to one of the following five experimental conditions. Control animals (control) were fed with a 20% (w/w) protein diet, while a second group received an 8% (w/w) isocaloric protein diet (LP). These two groups were fed with the same diet throughout gestation and lactation. The other three groups were designed to study the effects of the protein restriction during a particular window of gestation. Pregnant rats were fed 8% protein diet on the first 2 weeks of gestation LP (1–2), during the second week LP (2), or the third week LP (3). At remaining times, they received control diet. Diets were purchased from Bioserv (Frenchtown, NJ, USA) and were isocaloric, calories being balanced in the LP diet by the addition of carbohydrates. Protein restriction was achieved by reduction in casein content.

Litter size was reduced to four male and four female pups at birth and litters were killed either on postnatal day 1 by decapitation, or day 21 using CO<sub>2</sub> asphyxiation. Four litters of animals of each age were used. The pancreata were dissected, weighed, and fixed in 10% formalin, or snap-frozen in liquid nitrogen for further analysis. The animals were killed, blood was collected for glucose measurement, and 150  $\mu$ l serum prepared for the measurement of insulin. Glucose was measured with a hand-held glucometer DEX-2 (Bayer). Five animals were subjected to a glucose tolerance test on postnatal day 130. A bolus of i.p. glucose (2 g/kg weight) was given after a 5-h fast and blood sampled from the tail vein for up to 2 h for glucose measurement.

All procedures were performed with the approval of the Animal Ethics Committee of the University of Western Ontario, and in accordance with the guidelines of the Canadian Council on Animal Care.

### Immunohistochemistry

After 24 h fixation in formalin, pancreata were dehydrated, embedded in paraffin, and 5 µm thick sections prepared and mounted on Super frost-plus slides (Fischer Scientific, Toronto, ON, Canada). Immunohistochemistry was performed to localize insulin or nestin by a modified avidin–biotin–peroxidase method (Hsu *et al.* 1981). In brief, slides were incubated overnight at 4 °C in a humidified chamber with horse-anti-mouse insulin antibody (1:10 000 dilution; Sigma Aldrich) or mouse anti-nestin monoclonal antibody (1:200 dilution; Developmental Studies Hybridoma Bank, University of Iowa, USA). The secondary antiserum was biotinylated goat-anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) applied for 2 h at room temperature. Slides were washed and incubated with avidin and biotinylated horseradish peroxidase for 2 h at room temperature. Immunoreactivity was then visualized with Sigmafast diaminobenzidine (Sigma) tablet set. Tissue sections were counter-stained with Carrazi's hematoxylin and mounted under glass coverslip with Eukit (Ruth Wagener Ent, Newmarket, ON, Canada). To establish specificity of the antiserum, controls included substitution of the primary antibody with non-immune serum or omission of the secondary antibody.

### Morphometric analysis

The tissue sections were analyzed using a Carl Zeiss transmitted light microscope at a magnification of  $\times 25$  or  $\times 400$ . Analyses were performed with Northern Eclipse (version 6.0) morphometric analysis software (Empix Imaging, Mississauga, ON, Canada). An islet was defined as containing a cluster of greater than four insulin-immunoreactive cells. For each section of pancreas, the following were determined: the total area of pancreatic tissue, the percent contribution of acinar tissue, the number of islets, the area of the pancreatic tissue represented by endocrine cells, the area of each islet, and the area occupied by  $\beta$  cells (insulin positive) within each islet. Islets were arbitrarily separated by area into large (more than 10 000 µm<sup>2</sup>), medium (between 5000 and 10 000 µm<sup>2</sup>), and small (less than 5000 µm<sup>2</sup>). Islet number was expressed per squared millimeter total pancreatic area. Tissue represented both the head and tail of the pancreas.

### RIA

The animals were killed and the blood samples were collected from each animal; serum was separated and stored at  $-20$  °C for circulating insulin measurement with a sensitive rat insulin RIA kit (Linco Research, Inc., St. Charles, MO, USA). Total

protein was recovered from the protein phase following RNA extraction using Trizol (Invitrogen Life Technologies) and total insulin content was assayed by rat insulin kit (Linco). The assay sensitivity was 3 pM and the inter- and intraassay coefficients of variation were 4 and 9% respectively.

### Reverse transcription and real-time PCR

Total RNA was extracted from pancreas using Trizol (Invitrogen) and subsequently purified by RNeasy Mini kit (Qiagen Inc., Mississauga, ON, Canada) coupled with on-column DNase digestion with the RNase-free DNase set (Qiagen) according to manufacturer's instructions. Reverse transcription (RT) was performed using Omniscript RT Kit (Qiagen) following the manufacturer's instructions. Total RNA, 5 µg, was reverse transcribed in a total volume of 50 µl using oligo(dT) primers from Sigma. For every RT reaction set, one RNA sample was set up without reverse-transcriptase enzyme to provide a negative control. Reactions were incubated at 25 °C for 10 min, 42 °C for 50 min, and 70 °C for 15 min. Following RT, samples were diluted by adding diethylpyrocarbonate-treated distilled water.

Two different methods were used to quantify mRNAs using real-time PCR. Real-time quantitative PCR was performed using Taqman probe technologies in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) to determine the relative abundance of the transcription factor Pdx-1 (Mn00435565\_m1) and nestin (Rn00564394\_m1). Glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as endogenous control (Taqman Rodent GAPDH Control Reagents, P/N: 4308313). Briefly, PCRs were run in triplicate 50 µl reactions that contained 25 µl Taqman Universal PCR Master Mix (P/N 4304437), 2.5 µl of 20 $\times$  Assays-on-Demand Gene Expression Assay Mix (Applied Biosystems), and 150 ng cDNA. Two-step PCR cycling was carried out as follows: 50 °C, 2 min for 1 cycle; 95 °C, 10 min for 1 cycle; and 95 °C, 15 s; 60 °C, 1 min for 45 cycles.

Secondly, SYBR Green I Quantitative real-time PCR was used to study the expression of insulin, Glut-2, and used  $\beta$ -actin as endogenous control. Specific primers shown in Table 1 were designed using Primer express Software (Applied Biosystems) and were purchased from Sigma Genosys (Oakville, ON, Canada). SYBR-Green I Master Mix (Qiagen) was used as recommended (25 µl Master Mix, 2 µl primers 15 µM each, and 400 ng RT product in a total volume of 50 µl in triplicate). Thermal cycling conditions were 15 min at 95 °C, followed by 50 cycles of 15 s at 95 °C, 30 s at 61.5 °C, and 72 °C followed by a standard dissociation curve. Samples were run on a 96-well plate on the ABI PRISM 7900HT Sequence Detection System. The specificity of the SYBR Green 1 assay was verified by performing a melting-curve analysis and sub-sequencing of the PCR products. The comparative threshold (CT) method ( $\Delta\Delta$ CT method) was used and validation experiments were performed to demonstrate that efficiencies of targets and

**Table 1** Details of primers used for quantitative PCR

Primer name	Primer pair sequence	Fragment size (bp)	Reference
Insulin	5'-tcacacctggtggaagctc-3' 5'-acaatgccacgcttctgc-3'	180 (genomic: 679)	Soria (2004)
Glut-2	5'-caagatcaccggaaccttgg-3' 5'-attccgctactgcaagct-3'	310	Hsu <i>et al.</i> (1981)
$\beta$ -actin	5'-gcccctctgaaccctaag-3' 5'-catcaaatgccagtgga-3'	139 (genomic: 603)	Petrik <i>et al.</i> (2001)

reference (house keeping gene) were approximately equal (the plot of log input amount vs  $\Delta$ CT has a slope  $<0.1$ ).

### Statistical analysis

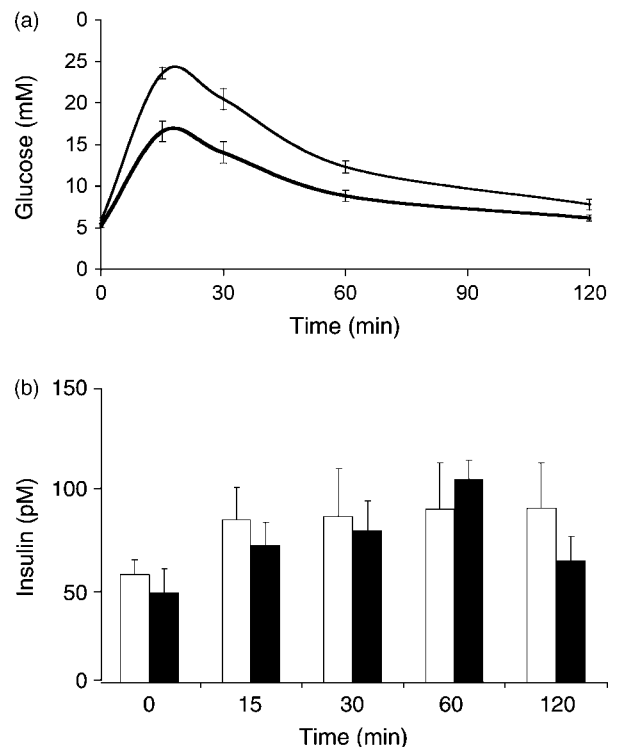
Data are presented as means  $\pm$  S.E.M. For pancreas histomorphometry, the operator was blind to tissue identity. Statistical analyses were performed using STATISTICA v5.5 (StatSoft, Inc., Tulsa, OK, USA). Group means derived from at least five sections from each pancreas, each separated by at least 50  $\mu$ m, were compared by two-way ANOVA. Upon significant interactions, differences between individual group means were analyzed by Duncan's test. Differences were considered statistically significant at  $P < 0.05$ . For quantitative measurement of target gene mRNA using PCR, the different LP treatments were compared with the control and analyzed by two-way ANOVA, followed by a Duncan's *post hoc* test when interaction was present. Significance was set at  $P < 0.05$ . These calculations were performed using STATISTICA v5.5.

## Results

The relative mean weight ( $\pm$  S.E.M.,  $n=3$  litters) of the pancreas did not differ between control-fed and any LP diet group on postnatal days 1 or 21 (day 1, control  $3019 \pm 327$  mg/kg body weight, LP  $3222 \pm 572$  mg/kg; day 21, control  $3999 \pm 778$  mg/kg, and LP  $3921 \pm 264$  mg/kg). The area of pancreas occupied by acinar tissue did not differ between control-fed animals and any LP-fed group at either age. Blood glucose levels in control-fed animals were  $5.8 \pm 0.5$  mM (mean  $\pm$  S.E.M.) on day 1 and  $5.2 \pm 0.3$  mM on day 21, and did not differ in any LP-fed group. Similarly, basal plasma insulin levels did not differ between control- and LP-fed animals on postnatal day 21 ( $52 \pm 7$  nM). An i.v. glucose tolerance test was performed on female offspring at 130 days of age. Glucose tolerance was significantly impaired in animals that had received continuous LP diet compared with controls (Fig. 1a). Basal or glucose-stimulated plasma insulin values did not differ between LP- and control-fed animals throughout the glucose challenge (Fig. 1b).

We analyzed the effects of maternal LP diet administered for various periods within fetal development and on pancreatic morphology in the offspring. The LP diet did

not affect either the percentage area of pancreas occupied by acinar cells, or the total number of islets per squared millimeter of pancreas when administered throughout gestation, or during any developmental window for either females or males, either at postnatal day 1 or 21 (Table 2). When the islets were separated by area into large ( $> 10\,000 \mu\text{m}^2$ ), medium ( $5000\text{--}10\,000 \mu\text{m}^2$ ), or small islets ( $< 5000 \mu\text{m}^2$ ), their proportional presence was approximately 75, 13, and 12% respectively. The numbers of medium and large islets were significantly reduced in each of the LP windows,



**Figure 1** (a) Mean blood glucose levels ( $\pm$  S.E.M.) during an i.v. glucose tolerance test performed on five female rats at 130 days of age. Animals were offspring of mothers that received control diet (lower curve) or LP diet (upper curve) continuously from conception until birth. The area under the curve was significantly greater in offspring of LP-fed animals than for control-fed rats ( $38.8 \pm 1.2\%$  increase,  $P < 0.002$ ). (b) Plasma insulin levels (mean  $\pm$  S.E.M.) in the above animals (control-fed, open bars; LP-fed, closed bars) at each time point. These did not differ between groups.

**Table 2** Number of islets per mm<sup>2</sup> of pancreas

	Control	LP (1–2)	LP (2)	LP (3)	LP
Day 1					
Females	11.61 ± 1.22	9.83 ± 1.15	10.85 ± 0.77	11.59 ± 1.17	11.81 ± 1.59
Small	8.64 ± 1.42	8.43 ± 1.28	10.25 ± 0.71	10.17 ± 1.41	10.33 ± 1.52
Medium	1.53 ± 0.15	0.78 ± 0.19 <sup>†</sup>	0.37 ± 0.09 <sup>‡</sup>	0.74 ± 0.13 <sup>†</sup>	0.88 ± 0.19*
Large	1.44 ± 0.08	0.62 ± 0.18 <sup>†</sup>	0.23 ± 0.05 <sup>‡§</sup>	0.68 ± 0.14 <sup>†</sup>	0.60 ± 0.15*
Males	11.80 ± 0.56	10.50 ± 0.98	10.56 ± 0.92	12.46 ± 0.29	11.90 ± 1.66
Small	9.23 ± 0.65	9.48 ± 0.91	9.17 ± 1.21	11.18 ± 0.46	10.18 ± 1.58
Medium	1.42 ± 0.10	0.59 ± 0.13 <sup>†</sup>	0.58 ± 0.26 <sup>†</sup>	0.83 ± 0.15*	0.86 ± 0.13*
Large	1.15 ± 0.20	0.52 ± 0.16*	0.80 ± 0.04	0.44 ± 0.13*	0.86 ± 0.06
Day 21					
Females	3.88 ± 0.81	3.10 ± 0.46	4.03 ± 0.44	3.51 ± 0.12	4.58 ± 1.05
Small	3.19 ± 0.79	2.75 ± 0.50	3.64 ± 0.56	2.98 ± 0.20	3.99 ± 1.10
Medium	0.34 ± 0.05	0.15 ± 0.03*	0.19 ± 0.06	0.19 ± 0.06	0.20 ± 0.03
Large	0.35 ± 0.04	0.20 ± 0.07	0.19 ± 0.06	0.34 ± 0.06	0.38 ± 0.07
Males	4.31 ± 0.38	3.40 ± 0.01	3.57 ± 0.17	3.35 ± 0.23	3.50 ± 0.28
Small	3.78 ± 0.40	3.03 ± 0.12	3.26 ± 0.16	2.84 ± 0.30	2.96 ± 0.32
Medium	0.28 ± 0.02	0.13 ± 0.04*	0.20 ± 0.07	0.16 ± 0.01	0.29 ± 0.02
Large	0.36 ± 0.07	0.25 ± 0.09	0.18 ± 0.01	0.36 ± 0.06	0.25 ± 0.06

Mean ± S.E.M. for 3–4 separate litters (15–20 animals). \**P* < 0.05, <sup>†</sup>*P* < 0.01, <sup>‡</sup>*P* < 0.001 vs control; <sup>§</sup>*P* < 0.05 vs males.

and following continuous administration, at postnatal day 1 (Table 2). These differences were greater in LP (2) in females and LP (3) in males. By postnatal day 21, the islet size distribution was no longer different between any LP treatment and control, except for a smaller number of medium-sized islets in LP (1–2) for both sexes. Consequent to the reduction in medium and larger islets following LP treatment, mean islet area was reduced in all treatment windows by postnatal day 1 (Table 3). This was most pronounced in females during LP (2), and in males during LP (3). As with islet number, these differences had largely disappeared by day 21.

When total β cell area was calculated as the percentage occupied by insulin-immunoreactive cells per squared millimeter of pancreas at postnatal day 1, this was reduced in all LP windows and with continuous treatment, in both males and females for medium and large islets (Fig. 2). This showed that the reduction in islet size was associated with a significant reduction in the number of β cells also. The percentage of β cells within individual islets did not change between the various LP treatments (data not shown), suggesting that the reduced islet size involved a depletion of other endocrine cell types also. Individual β cell area was also

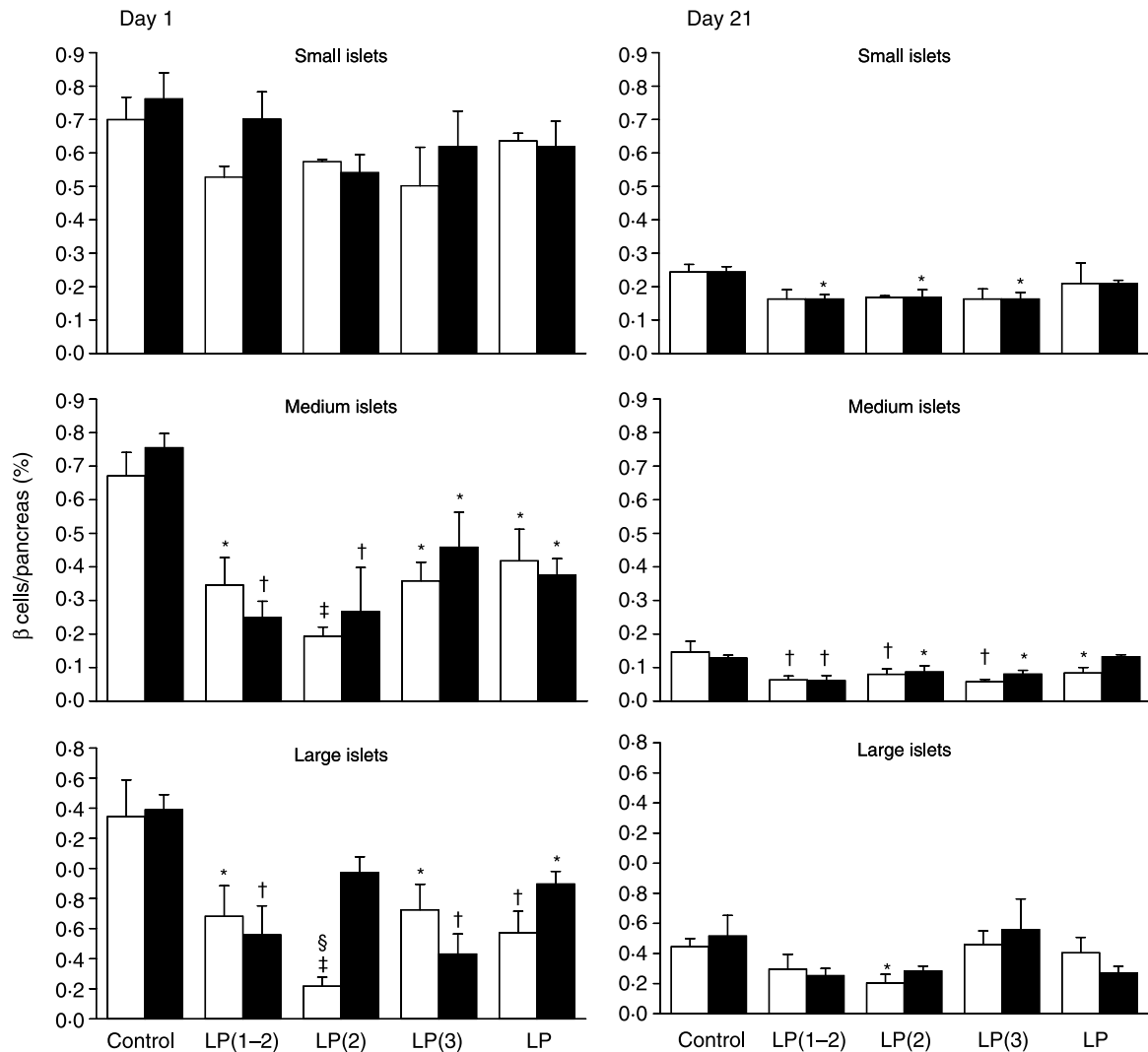
unchanged following LP treatments. The contribution of β cells was most reduced during LP (2) for females and LP (3) in males. The reduced β cell relative area in all LP windows persisted at postnatal day 21, but now affected mainly small- and medium-sized islets.

The decrease in area of pancreas occupied by β cells was not associated with differences in the insulin content in the total pancreas, which was not different between controls and any LP window on day 1 (control 98 ± 13 pg/mg pancreas) or day 21 (control 49 ± 16 pg/mg). However, measurement of insulin mRNA showed profound changes related to both gender and LP treatment window. In rats of both gender, insulin mRNA was significantly higher in LP (2) than in control-fed animals on postnatal day 1 (Fig. 3a), but not for other windows or continuous LP administration. By postnatal day 21, an increased insulin mRNA content was seen for both males and females for LP (1–2), in females for LP (2), and males for LP (3). We extended this analysis to examine the expression of two additional markers of β cell function: the transcription factor Pdx-1, and the glucose transporter Glut-2. Pdx-1 mRNA was significantly reduced in the pancreas of female rats only in LP (1–2) and LP (3) at day 1, but not following continuous

**Table 3** Mean islet area (μm<sup>2</sup>)

	Control	LP (1–2)	LP (2)	LP (3)	LP
Day 1					
Females	4111 ± 119	2787 ± 652	1276 ± 122 <sup>‡§¶</sup>	2176 ± 382*	2703 ± 435*
Males	3743 ± 292	2050 ± 264*	2530 ± 548*	1771 ± 143 <sup>†</sup>	2557 ± 269*
Day 21					
Females	3730 ± 930	2876 ± 861	1782 ± 620	3041 ± 387	2769 ± 745
Males	3163 ± 424	2140 ± 330	1829 ± 315	458 ± 842	2808 ± 441

Mean ± S.E.M. for 3–4 separate litters (15–20 animals). \**P* < 0.05, <sup>†</sup>*P* < 0.01, <sup>‡</sup>*P* < 0.001 vs control; <sup>§</sup>*P* < 0.05 vs LP (1–2) and LP (3); <sup>¶</sup>*P* < 0.05 vs males.

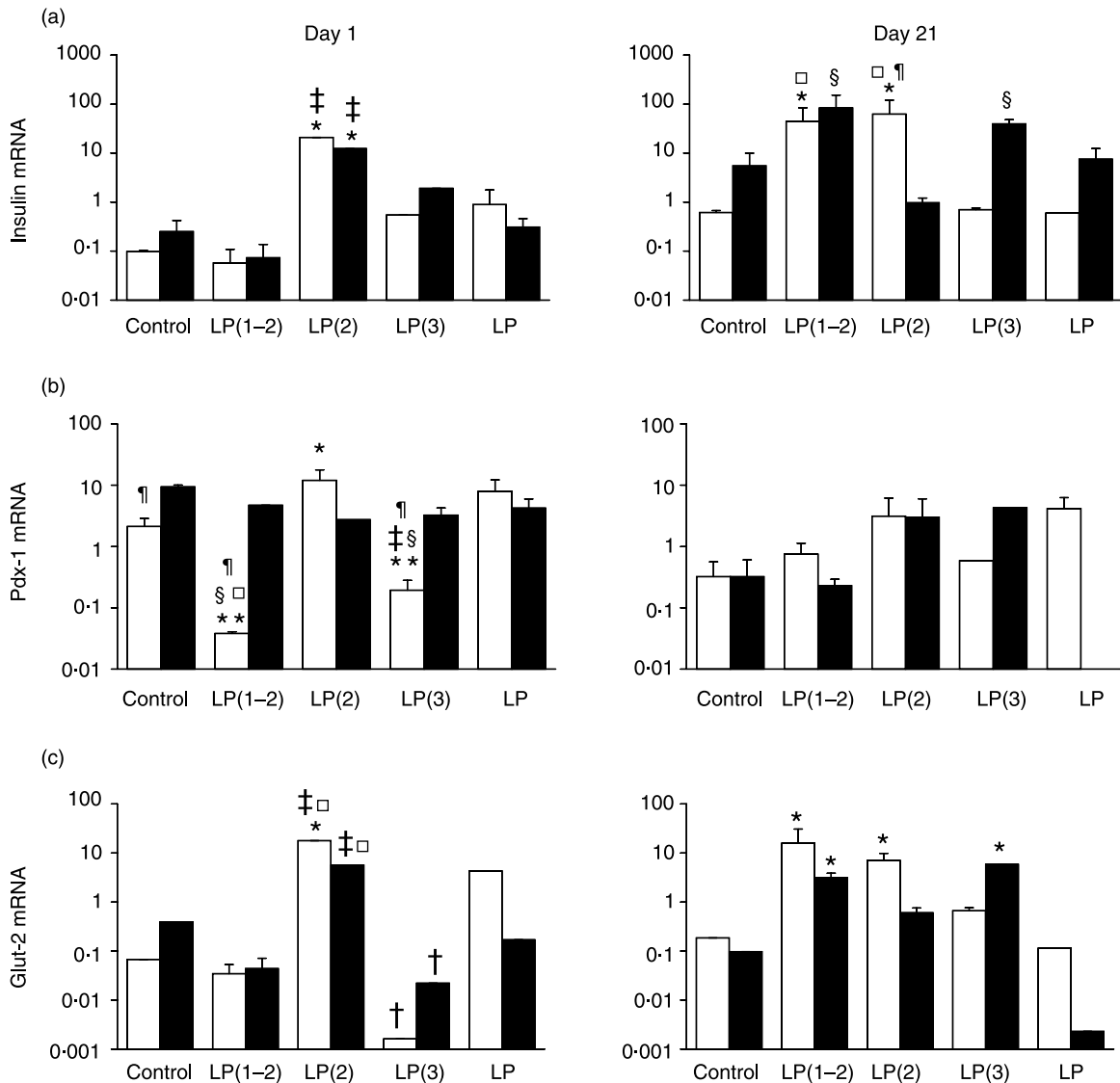


**Figure 2** Area occupied by insulin-immunoreactive  $\beta$  cells per squared millimeter of pancreas at postnatal days 1 and 21 following exposure of mothers to control diet, low-protein diet from conception to birth (LP), during the first 2 weeks of gestation (LP (1–2)), during the second week only (LP (2)), or during the third week (LP (3)). At other times, animals received a control diet. Islets were separated by size into large (more than  $10\,000\ \mu\text{m}^2$ ), medium (between  $5000$  and  $10\,000\ \mu\text{m}^2$ ), and small (less than  $5000\ \mu\text{m}^2$ ). Female (open bars) and male (closed bars) animals were analyzed separately. Figures represent mean values  $\pm$  S.E.M. derived from 3 to 4 litters (15–20 animals). \* $P < 0.05$ , † $P < 0.01$ , ‡ $P < 0.001$  vs gender-specific control; § $P < 0.05$  vs male for same group.

administration (Fig. 3b). These differences were absent by day 21. Glut-2 mRNA expression was significantly increased in both sexes in LP (2) compared with controls on day 1, which was reduced following LP (3) administration, but was unaltered following continuous administration (Fig. 3c). By day 21, Glut-2 mRNA was increased relative to controls for LP (1–2) in both sexes, in LP (2) for females and LP (3) in males. Hence, parallel changes occurred in the expression of mRNAs for insulin and Glut-2 that, unlike many changes in islet morphology, persist at postnatal day 21.

We have previously reported that nestin-immunoreactive stellate cells adjacent to pancreatic ducts, and within islets are closely associated with islet mass and may play a supportive role

in endocrine cell formation (Joanette *et al.* 2004). Quantification of mRNA for nestin in whole pancreas showed a significant reduction following each individual LP treatment window relative to control-fed rats on day 1 (Fig. 4), the reduction being greatest in LP (3) and least with continuous LP treatment. This was similar for both sexes. At postnatal day 21, pancreatic nestin mRNA expression had a tenfold lower expression than at day 1 (not shown). Localization of nestin using immunohistochemistry showed a significant reduction in the number of cells juxtaposed to interlobular ducts on day 1 (Fig. 5a) following continuous LP treatment, but not for individual LP treatment windows. When nestin-positive cells within islets were considered, a reduction was found in the



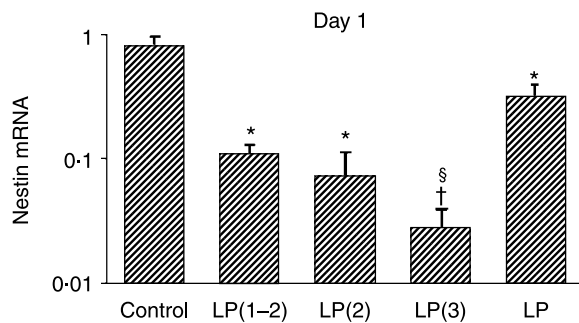
**Figure 3** mRNA expression for insulin (a), Pdx-1 (b), and Glut-2 (c) in intact pancreas was quantified by real-time PCR and expressed relative to the levels of  $\beta$ -actin (a, c) or GAPDH (b). Female (open bars) and male (closed bars) animals were analyzed separately at postnatal days 1 and 21 following exposure of mothers to control diet, low-protein diet from conception to birth (LP), during the first 2 weeks of gestation (LP (1–2)), during the second week only (LP (2)), or during the third week (LP (3)). At other times, animals received a control diet. Figures represent mean values  $\pm$  s.e.m. derived from 3 to 4 litters (15–20 animals) shown on a logarithmic scale. \* $P$ <0.05,  $P$ <0.01, † $P$ <0.001 vs gender-specific control; \* $P$ <0.05 vs LP (1–2); † $P$ <0.05 vs LP (2); ‡ $P$ <0.05 vs LP (3); § $P$ <0.05 vs male for same group.

numbers within smaller islets only for all LP treatments (Fig. 5b). This did not differ between sexes.

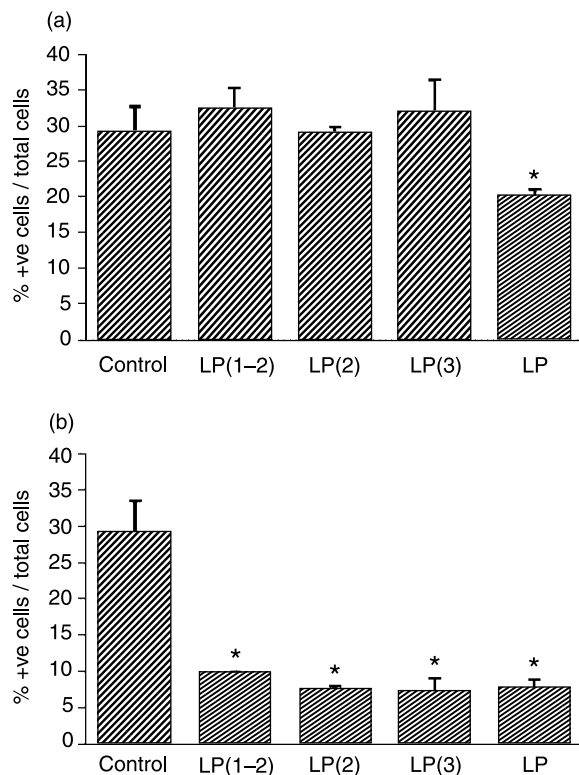
## Discussion

Intrauterine nutritional perturbation has a profound effect on the development of the endocrine pancreas in the offspring, resulting in glucose intolerance, once they reach adulthood (Reusens *et al.* 1995, Hales *et al.* 1996). Using the model of LP

diet from conception until weaning in the rat, we and others have reported that  $\beta$  cell mass is significantly lower in the pancreas of fetuses and neonates due to a lower rate of  $\beta$  cell proliferation, greater developmental apoptosis, and a lower capillary density within the islets (Cherif *et al.* 1998, Petrik *et al.* 1999, Boujendar *et al.* 2003). Such a phenotype could derive from the disruption of a variety of developmental processes including: (a) an altered phenotype and lineage commitment of embryonic stem cells and endoderm tissue post-implantation, (b) altered development of endocrine



**Figure 4** mRNA expression for nestin in intact pancreas was quantified by real-time PCR and expressed relative to the levels of GAPDH. Values did not differ between males and females and are analyzed together at postnatal day 1 following exposure of mothers to control diet, low-protein diet from conception to birth (LP), during the first 2 weeks of gestation (LP (1–2)), during the second week only (LP (2)), or during the third week (LP (3)). At other times animals received a control diet. Figures represent mean values  $\pm$  S.E.M. derived from 3 to 4 litters (15–20 animals) shown on a logarithmic scale. \* $P < 0.05$ , † $P < 0.01$  vs control; § $P < 0.05$  vs LP.



**Figure 5** Percentage of nestin-immunoreactive cells adjacent to ductal epithelium (a) or within smaller islets (less than  $5000 \mu\text{m}^2$ ) (b). Values did not differ between males and females and are analyzed together at postnatal day 1 following exposure of mothers to control diet, low-protein diet from conception to birth (LP), during the first 2 weeks of gestation (LP (1–2)), during the second week only (LP (2)), or during the third week (LP (3)). At other times animals received a control diet. Figures represent mean values  $\pm$  S.E.M. derived from 3 to 4 litters (15–20 animals). \* $P < 0.05$  vs control.

precursor cells during pancreatic embryogenesis, (c) a failure of growth of islets once established, or (d) inadequate islet vascularity and angiogenesis to support the substantial increase in  $\beta$  cell mass that occurs shortly before birth. The results of this study suggest that an LP insult during any of these developmental windows has a deleterious effect on  $\beta$  cell mass at birth. It is possible that following LP administration in LP (1–2) or LP (2), there was a recovery of  $\beta$  cell mass by postnatal day 1, and immediate impact of LP diet at these times has been underestimated. The LP diet used here was made isocaloric with the control diet through the addition of carbohydrate. High carbohydrate alone may impact the development of the endocrine pancreas, although there are no specific data in rat models for its impact during fetal life. However, the administration of a high carbohydrate diet to the neonatal rat resulted in a deficit in pancreatic endocrine tissue, and a relative reduction in the presence of large islets (Petrik *et al.* 2001).

We previously observed that a reduced rate of  $\beta$  cell proliferation in the offspring of LP-fed animals involved a likely elongation of cell cycle time, suggesting a fundamental change in cell cycle kinetics perhaps ‘imprinted’ at the level of the endocrine pancreatic stem cell or precursor (Petrik *et al.* 1999). Such cells have been identified at low frequency within the mouse pancreas that are capable of multi-lineage potential, including all pancreatic endocrine cell types (Seaberg *et al.* 2004). Exposure to LP diet during weeks 1 and 2 of pregnancy substantially decreased mean islet area by postnatal day 1 with a failure to develop larger islets. When maternal rats were given LP diet in the pre-implantation period only, from conception for 4 days, birth weight and postnatal growth rate were reduced (Kwong *et al.* 2000). Isolation and culture of inner cell mass cells from blastocysts, or later embryonic or trophectoderm cells, showed a decreased rate of proliferation *in vitro*. This would support a long-term effect of early nutritional insult on stem and precursor cell phenotype. While the effects of LP diet during weeks 1 and 2 of gestation on  $\beta$  cell presence were similar between male and female offspring, a decrease in the expression of Pdx-1 mRNA, a transcription factor involved in both  $\beta$  cell development and insulin gene transcription, was only reduced in females. Despite the reduced  $\beta$  cell mass, at postnatal day 1 the offspring maintained pancreatic insulin gene expression and protein content. Recently, it has been shown that a differential expression of Pdx-1 within the pancreas occurs following the induction of intrauterine growth restriction by ligation of the uterine vessels, which will impact nutritional sufficiency (Lesage *et al.* 2001). It therefore seems likely that intrauterine nutrition can influence  $\beta$  cell mass in the offspring through an altered organogenesis of the endocrine pancreas involving changes to key lineage determinants such as Pdx-1. Pdx-1 expression is repressed by glucocorticoids during pancreatic organogenesis (Shen *et al.* 2003). However, while corticosterone levels are elevated in rat fetuses following maternal calorie reduction (Lesage *et al.* 2001), maternal and fetal plasma corticosterone levels were not changed following LP diet (Fernandez-Twinn *et al.* 2003).



Administration of LP diet for week 2 of pregnancy only had a greater impact on islet size, and the number of  $\beta$  cells, than did LP for both weeks 1 and 2, or week 3 alone, within female offspring. Conversely, the greatest impact of LP diet on male offspring was in week 3 of pregnancy. This suggests possible sexual dimorphism in critical genes determining  $\beta$  cell development. This did not obviously relate to differences in the pancreatic expression of Pdx-1 or Glut-2 mRNAs. However, a gender-specific programming of insulin secretion and action has been reported in the offspring of LP-fed mothers. Post-feeding insulinemia was reduced in males compared with females, although the acute insulin release was increased in males (Sugden & Holness 2002). Calcium uptake and glucokinase activity was reduced in islets from animals of both sexes (Heywood *et al.* 2004). Though these results are complicated by the demonstrated sexual dimorphism in insulin-dependent metabolism following maternal LP diet (Ozanne *et al.* 1996), they also imply a gender-specific response in insulin secretion in response to protein restriction *in utero*. Low-protein diet reduced the expression of protein kinase A  $\alpha$  within pregnant rat islets, which is implicated in the growth and function of  $\beta$  cells (Milanski *et al.* 2005). It is not known if these changes extend to the islets of the offspring.

Administration of LP diet for the third week of pregnancy only, when  $\beta$  cell mass approximately doubles in the fetus, limited islet size and the pancreatic content of  $\beta$  cells to a similar extent as administration of LP diet throughout gestation. This window of administration resulted in the lowest expression of pancreatic Glut-2 mRNA relative to controls. Expansion of  $\beta$  cell mass in late gestation is dependent on tropic growth factors such as insulin-like growth factor (IGF)-II, which is reduced following LP diet (Petrik *et al.* 1999). This is not specific to pancreas, since hepatocytes from offspring of LP-fed animals showed a reduced rate of proliferation, a lower expression of IGF-I, and an increased synthesis of IGF-binding proteins (El-Kattabi *et al.* 2003). The relevance of impaired growth factor presence or action to the reduction in islet mass has been demonstrated by its reversal following the administration of the glucagon-like polypeptide-1 analog, exendin 4, to growth retarded, neonatal offspring from mothers subjected to uterine vessel occlusion (Stoffers *et al.* 2003).

Offspring of LP-fed rats have reduced islet vascularity (Boujendar *et al.* 2003). Endothelial precursor cells within the developing pancreas contribute to capillary growth, and we have shown that continuous LP administration throughout pregnancy limits the presence of putative endothelial precursor cells expressing nestin, CD34, and c-kit (Joanette *et al.* 2004). The presence of nestin-positive cells adjacent to pancreatic ducts was reduced following continuous LP administration, but not by administration within any individual window. However, administration of LP diet during any window caused a profound reduction in the frequency of nestin-positive cells within islets, while pancreatic nestin mRNA levels, while reduced in all

developmental windows, was significantly lower in LP (3) than with continuous LP administration. This suggests that the rapid pancreatic endocrine development that occurs in the third week of gestation is highly dependent on a parallel increase in vascularity, and this is severely limited by LP diet.

LP diet was discontinued in all animals at birth when the lactating mothers were switched to control diet. By postnatal day 21, almost all differences in mean islet size between LP- and control-fed offspring had disappeared. However, there were still relative deficiencies in the pancreatic content of  $\beta$  cells, and an over-compensation of insulin and Glut-2 mRNA expression. This was apparent in all individual developmental windows, but not after continuous LP administration. Offspring of rats administered LP diet from conception to birth showed relatively impaired glucose tolerance compared with control-fed animals by postnatal day 130, but circulating insulin levels during the glucose challenge were not altered. Since the glucose intolerance resulting from this model has been shown to involve a decrease in insulin sensitivity in target tissues, this may reflect an inability of islets to mount a compensatory increase in insulin secretion.

In summary, LP diet present in early or late gestation impacted endocrine pancreatic morphology at birth. Insulin-secretory potential is likely to be most directly linked to the number of  $\beta$  cells present in the pancreas, represented as relative area occupied within the pancreas. The contribution of  $\beta$  cells was reduced in most LP groups at day 1 and 21, but was most apparent in medium and large size islets. When considered as pancreatic area occupied by  $\beta$  cells, such islets account for the majority of  $\beta$  cells. Despite these deficits, the pancreatic content of insulin did not differ between LP- and control-fed animals, suggesting a compensatory response by the whole pancreas to maintain euglycemia. Islet number, as a measure of the developmental ability to form islet structures, was not changed by LP diet, but the ability of the numerically superior smaller islets to grow medium or large was impaired. This is likely to account for the reduction in total  $\beta$  cells per pancreas at birth. However, following the lifting of LP diet at birth, the number of medium and large size islets could recover by weaning, but still tended to be deficient in  $\beta$  cells. This is likely to contribute to the glucose intolerance observed in adulthood.

## Acknowledgements

We are grateful to Canadian Institutes of Health Research, the Canadian Diabetes Association, the Juvenile Diabetes Research Foundation, the Stem Cell Network Centre of Excellence, and the Ontario Research and Development Challenge Fund for financial support. We thank Ms Brenda Strutt, Ms Catherine Currie, and Becky McGirr for technical support. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

## References

- Alvarez C, Martin MA, Goya L, Bertin E, Portha B & Pascual-Leone AM 1997 Contrasted impact of maternal rat food restriction on fetal endocrine pancreas. *Endocrinology* **138** 2267–2273.
- Barker DJP 1998 *Mothers, Babies and Health in Later Life*. Edinburgh: Harcourt Brace & Co Ltd.
- Bernstein JM, Goran MI, Amini SB & Catalano PM 1997 Differential growth of fetal tissues during the second half of pregnancy. *American Journal of Obstetrics and Gynecology* **176** 28–32.
- Boujendar S, Arany E, Hill D, Remacle C & Reusens B 2003 Taurine supplementation during fetal life reverses the vascular impairment caused to the endocrine pancreas by a low protein diet. *Journal of Nutrition* **133** 2820–2825.
- Cherif H, Reusens B, Ahn MT, Hoet JJ & Remacle C 1998 Effects of taurine on the insulin secretion of fetal rat islets from dams fed a low protein diet. *Journal of Endocrinology* **159** 341–348.
- El-Kattabi I, Gregoire F, Remacle C & Reusens B 2003 Isocaloric maternal low-protein diet alters IGF-I, IGFBPs, and hepatocyte proliferation in the fetal rat. *American Journal of Physiology* **285** E991–E1000.
- Fernandez-Twinn DS, Ozanne SE, Ekizoglou S, Doherty C, Gusterson B & Hales CN 2003 The maternal endocrine environment in the low-protein model of intra-uterine growth restriction. *British Journal of Nutrition* **90** 815–822.
- Garofano A, Czernichow P & Breant B 1997 *In utero* undernutrition impairs rat beta-cell development. *Diabetologia* **40** 1231–1234.
- Gu G, Dubauskaite J & Melton DA 2002 Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* **129** 2447–2457.
- Guan H, Arany E, van Beek JP, Chamson-Reig A, Thyssen S, Hill DJ & Yang K 2005 Adipose tissue gene expression profiling reveals distinct molecular pathways that define visceral adiposity in the offspring of maternal protein restricted rats. *American Journal of Physiology–Endocrinology and Metabolism* **288** E663–E673.
- Hales CN & Ozanne SE 2003 For debate: fetal and early post-natal growth restriction lead to diabetes, the metabolic syndrome and renal failure. *Diabetologia* **46** 1013–1019.
- Hales CN, Desai M, Ozanne SE & Crowther NJ 1996 Fishing in the stream of diabetes: from measuring insulin to the control of fetal organogenesis. *Biochemical Society Transactions* **24** 341–350.
- Heywood WE, Mian N, Milla PJ & Lindley KJ 2004 Programming of defective rat pancreatic beta-cell function in offspring from mothers fed a low-protein diet during gestation and the suckling periods. *Clinical Science* **107** 37–45.
- Hsu SM, Raine L & Fanger H 1981 Use of avidin–biotin peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *Journal of Histochemistry and Cytochemistry* **29** 577–580.
- Hunziker E & Stein M 2000 Nestin-expressing cells in the pancreatic islet of Langerhans. *Biochemical and Biophysical Research Communications* **271** 116–119.
- Joanette EA, Reusens B, Arany E, Thyssen S, Remacle C & Hill DJ 2004 Low protein diet during early life causes a reduction in the frequency of cells immunopositive for Nestin and CD34 in both pancreatic ducts and islets in the rat. *Endocrinology* **145** 3004–3013.
- Kwong WY, Wild AE, Roberts P, Willis AC & Fleming TP 2000 Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development* **127** 4195–4202.
- Lammert E, Cleaver O & Melton D 2001 Induction of pancreatic differentiation by signals from blood vessels. *Science* **294** 564–567.
- Lammert E, Cleaver O & Melton D 2003 Role of endothelial cells in early pancreas and liver development. *Mechanisms of Development* **120** 59–64.
- Langley SC, Browne RF & Jackson AA 1994 Altered glucose tolerance in rats exposed to maternal low protein diets *in utero*. *Comparative Biochemistry and Physiology* **109A** 223–229.
- Lesage J, Blondeau B, Grino M, Breant B & Dupouy JP 2001 Maternal undernutrition during late gestation induces fetal overexposure to glucocorticoids and intrauterine growth retardation, and disturbs the hypothalamo–pituitary adrenal axis in the newborn rat. *Endocrinology* **142** 1692–1702.
- Mellitzer G, Martin M, Sidhoum-Jenny M, Orvain C, Barths J, Seymour PA, Sander M & Gradwohl G 2004 Pancreatic islet progenitor cells in neurogenin 3–yellow fluorescent protein knock-add-on mice. *Molecular Endocrinology* **18** 2765–2776.
- Milanski M, Arantes VC, Ferreira F, de Barros Reis MA, Carneiro EM, Boschero AC, Collares-Buzato CB & Latorraca MQ 2005 Low-protein diets reduce PKA $\alpha$  expression in islets from pregnant rats. *Journal of Nutrition* **135** 1873–1878.
- Ozanne SE, Smith GD, Tikerpa J & Hales CN 1996 Altered regulation of hepatic glucose output in the male offspring of protein-malnourished rat dams. *American Journal of Physiology* **270** E559–E564.
- Petrik J, Reusens B, Arany E, Remacle C, Hoet JJ & Hill DJ 1999 A low protein diet alters the balance of islet cell replication and apoptosis in the fetal and neonatal rat, and is associated with a reduced pancreatic expression of insulin-like growth factor II. *Endocrinology* **140** 4861–4873.
- Petrik J, Srinivasa M, Aalinkeel R, Coukell S, Arany E, Patel MS & Hill DJ 2001 A chronic high carbohydrate diet causes an altered ontogeny of pancreatic islets of Langerhans in the neonatal rat. *Pediatric Research* **49** 84–92.
- Phillips DIW, Barker DJP, Hales CN, Hirst S & Osmond C 1994 Thinness at birth and insulin resistance in adult life. *Diabetologia* **37** 150–154.
- Phillips DIW, Caddy S, Ilic V, Fielding BA, Frayn KN, Borthwick AC & Taylor R 1996 Intramuscular triglyceride and muscle insulin sensitivity: evidence for a causative relationship. *Metabolism* **45** 947–950.
- Ravelli AC, van der Meulen JH & Michels RP 1998 Glucose tolerance in adults after prenatal exposure to the Dutch famine. *Lancet* **351** 173–177.
- Ravelli AC, van der Meulen FH, Osmond C, Barker DJ & Bleker OP 1999 Obesity at the age of 50 y in men and women exposed to famine prenatally. *American Journal of Clinical Nutrition* **70** 811–816.
- Reusens B, Dahri S, Snoeck A, Bennis-Taleb N, Remacle C & Hoett JJ 1995 Long-term consequences of diabetes and its complications may have a fetal origin: experimental and epidemiological evidence. *Diabetes* **35** 187–198.
- Seaberg RM, Smukler SR, Kieffer TJ, Enikolopov G, Asghar Z, Wheeler MB, Korbitt G & van der Kooy D 2004 Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. *Nature Biotechnology* **22** 1115–1124.
- Shen CN, Seckl JR, Slack JM & Tosh D 2003 Glucocorticoids suppress beta-cell development and induce hepatic metaplasia in embryonic pancreas. *Biochemical Journal* **375** 41–50.
- Soria B 2004 *In vitro* differentiation of pancreatic B cells. *Differentiation* **68** 205–219.
- Stoffers DA, Desai BM, DeLeon DD & Simmons RA 2003 Neonatal exendin-4 prevents the development of diabetes in the intrauterine growth retarded rat. *Diabetes* **52** 734–740.
- Sugden MC & Holness MJ 2002 Gender-specific programming of insulin secretion and action. *Journal of Endocrinology* **175** 757–767.
- Taylor DJ, Thompson CH, Kemp GJ, Barnes PR, Sanderson AL, Radda GK & Phillips DI 1995 A relationship between impaired fetal growth and reduced muscle glycolysis revealed by <sup>31</sup>P magnetic resonance spectroscopy. *Diabetologia* **38** 1205–1212.
- Treutelaar MK, Skidmore JM, Dias-Leme CL, Hara M, Zhang L, Simeone D, Martin DM & Burant CF 2003 Nestin lineage cells contribute to the microvasculature but not endocrine cells of the islet. *Diabetes* **52** 2503–2512.

Received in final form 5 July 2006

Accepted 11 July 2006

Made available online as an Accepted Preprint  
21 August 2006