Developmental programming of neonatal pancreatic β-cells by a maternal low-protein diet in rats involves a switch from proliferation to differentiation

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Rodríguez-Trejo A, Ortiz-López MG, Zambrano E, Granados-Silvestre MA, Méndez C, Blondeau B, Bréant B, Nathanielsz PW, Menjivar M. Developmental programming of neonatal pancreatic β-cells by a maternal low-protein diet in rats involves a switch from proliferation to differentiation. Am J Physiol Endocrinol Metab 302: E1431–E1439, 2012. First published March 20, 2012; doi:10.1152/ajpendo.00619.2011.—Maternal low-protein diets (LP) impair pancreatic β-cell development, resulting in later-life failure and susceptibility to type 2 diabetes (T2D). We hypothesized that intrauterine and/or postnatal developmental programming seen in this situation involve altered β-cell structure and relative time course of expression of genes critical to β-cell differentiation and growth. Pregnant Wistar rats were fed either control (C) 20% or restricted (R) 6% protein diets during pregnancy (1st letter) and/or lactation (2nd letter) in four groups: CC, RR, RC, and CR. At postnatal days 7 and 21, we measured male offspring β-cell mass, proliferation, aggregate number, and size as well as mRNA level for 13 key genes regulating β-cell development and function in isolated islets. Compared with CC, pre- and postnatal LP (RR) decreased β-cell fraction, mass, proliferation, aggregate size, and number and increased Hnf1α, Hnf4α, Pdx1, Isl1, Rfx6, and Slc2a2 mRNA levels. LP only in pregnancy (RC) also decreased β-cell fraction, mass, proliferation, aggregate size, and number and increased Hnf1α, Hnf4α, Pdx1, Rfx6, and Ins mRNA levels. Postnatal LP offspring (CR) showed decreased β-cell mass but increased β-cell fraction, aggregate number, and Hnf1α, Hnf4α, Rfx6, and Slc2a2 mRNA levels. We conclude that LP in pregnancy sets the trajectory of postnatal β-cell growth and differentiation, whereas LP in lactation has smaller effects. We propose that LP promotes differentiation through upregulation of transcription factors that stimulate differentiation at the expense of proliferation. This results in a decreased β-cell reserve, which can contribute to later-life predisposition to T2D.

postnatal β-cell growth; nutrition; transcription factors

IMPAIRED INSULIN SYNTHESIS AND SECRETION in type 2 diabetes (T2D) results from β-cell dysfunction and/or reduced β-cell mass (22, 28). Environmental challenges during intrauterine and early postnatal life affect development and can result in persistent, altered, programmed β-cell structure and function. As a result, offspring are predisposed to later-life dysfunction of glucose and insulin metabolism and a greater T2D risk (26, 49). Compelling data for developmental programming come from human epidemiological (49) and controlled animal studies using challenges such as exposure to low global maternal nutrition (13), maternal protein restriction (37), and fetal exposure to glucocorticoid levels higher than appropriate for the current stage of differentiation and development (4).

Growth-restricted offspring of rat mothers fed isocaloric low-protein diets (LP) through gestation and lactation show impaired glucose-stimulated insulin secretion (GSIS) (7, 19), reduced β-cell mass (10, 35, 41), and age-dependent development of glucose intolerance, insulin resistance, and diabetes (18, 33, 36). Maternal LP limited to gestation or lactation also induces alterations in growth, β-cell function, and glucose homeostasis, although effects on β-cell structure are less well defined. If prenatal LP is followed by adequate postnatal nutrition after birth, growth recuperates and overall GSIS is restored during early life (2, 19, 50), although as adults offspring become glucose intolerant and insulin resistant when challenged with a hypercaloric diet (5). In contrast, offspring of mothers fed a LP only in lactation show growth restriction (50), GSIS impairment, and glucose intolerance in young adult life (16, 30, 31). These observations indicate that both the prenatal and early postnatal periods are separate critical windows of susceptibility for programmed glucose and insulin metabolism by dietary interventions and that distinct outcomes in insulin secretion may be due to alterations in different developmental stage-specific mechanisms of β-cell function and mass.

β-Cell programming resulting from maternal LP is likely to reflect altered expression of a cassette of key genes controlling various stages of pancreatic development and function of mature β-cells, such as forkhead box A2 (Foxa2) (12), hepatocyte nuclear factor (HNF)1 homeobox B (Hnf1b) (47), HNF1 homeobox A (Hnf1a) (27), HNF4α (Hnf4a) (11, 44), pancreatic and duodenal homeobox 1 (Pdx1) (48), regulatory factor X6 (Rfx6) (43), neurogenic differentiation 1 (Neurod1) (17), Isl1 LIM homeobox 1 (Isl1) (9), NK6 homeobox 1 (Nkx6-1) (38), and paired box 4 (Pax4) (42), that encode transcription factors. During development, these genes play central roles in endodermal specification of derived pancreatic cells (Foxa2, Hnf1b, Hnf1a, and Hnf4a), pancreas formation, and endocrine cell differentiation (Pdx1, Rfx6, and Neurod1) as well as in
endocrine lineage divergence and growth (Isl1, Pax4, and Nkx6-1). Their role and position in the hierarchy of transcription factor expression in the developing pancreas has been revealed by studies of gene-specific inactivation in mice. Abolition of these genes has demonstrated that they are also implicated in regulating β-cell mass, insulin production, and secretion. Furthermore, gene sequence variations of these transcription factors can cause diabetes in humans (6, 21, 40). Therefore, it is plausible that alterations in their expression contribute to developmental β-cell programming by LP given that they are crucial for adequate development and function of β-cells.

One general hypothesis relating to developmental programming is that adverse conditions during development promote progenitor cell differentiation at the expense of proliferation, resulting in organ maturation and survival but reduced organ growth. As a result the organ, although mature, may have reduced functional capacity. β-Cells undergo major structural (growth of β-cell mass) and functional changes during early postnatal life (1, 8, 15, 23, 29). Interestingly, recent evidence suggests that the marked neonatal β-cell mass increase is achieved by both proliferation of preexisting β-cells and differentiation or neogenesis from progenitor cells (32). These precursor cells may be located within the islets since it has been suggested that β-cells do not arise from ductal or acinar cells after birth (24). We hypothesized that timing of maternal protein restriction (gestation and/or lactation) results in specific and developmental stage-dependent postnatal alterations in expression of genes important for β-cell function, such as Ins (insulin), Slc2a2 [solute carrier family 2 (facilitated glucose transporter), member 2; Glut2], and Kcnj11 (potassium inwardly reflecting channel, subfamily J, member 11; Kir6.2), and in structural features of β-cells. If this is the case, it would influence separate parameters, i.e., the secretory capacity of individual β-cells and/or the functional capacity of all the β-cells. Structural alterations of β-cells may be a consequence of maternal LP affecting timing of gene expression of these transcription factors that play a central role in regulating β-cell differentiation and proliferation. Such changes may promote differentiation but also affect β-cell secretory capacity because they are also implicated in both insulin synthesis and secretion. Therefore, we assessed effects of prenatal and/or postnatal exposure to a maternal LP on β-cell structure and proliferation as well as on islet gene expression of Foxa2, Hnf1b, Hnf6a, Hnf4a, Pdx1, Rfx6, Neurod1, Isl1, Pax4, Nkx6–1, Ins, Slc2a2, and Kcnj11 in male offspring during lactation.

MATERIALS AND METHODS

Animals. All procedures followed Mexican regulations for the use and care of laboratory animals (NO-062-ZOO-1999) and were approved by the Animal Ethics Committee, Faculty of Chemistry, Universidad Nacional Autónoma de México. Male and nulliparous female Wistar rats (Harlan Laboratories, Mexico City, Mexico) were housed at 22–24°C on a 12:12-h light-dark cycle with free access to laboratory chow and water. Females weighing 225–250 g (90–110 days of age) were mated. The day on which pregnancy was confirmed by sperm in vaginal smears was considered as gestation day 1. Mothers were housed individually and fed ad libitum either a control diet (“C” diet; 20% protein, Harlan TD.91352; Harlan, Madison, WI) or isocaloric LP (“R” diet; 6% protein, Harlan TD.90016) throughout gestation and/or lactation to establish four experimental groups [1st letter, pregnancy diet (imposed on gestation day 1); 2nd letter, lactation diet (imposed on the day of delivery)]; CC, control (12 mothers); RR, pre- and postnatal LP (11 mothers); RC, prenatal LP/postnatal control diet (11 mothers); and CR, postnatal LP (10 mothers). Maternal body weight and food intake were measured three times weekly at 7 AM from gestational day 1 until lactation day 21. The day after delivery, litter size was adjusted to 10 pups/litter (5 males and 5 females when possible) and male offspring body weight recorded. Separate litters were studied at postnatal day (PND) 7 and PND 21. Three male pups per litter (5–6 litters/group at PND 7 and 5–6 litters/group at PND 21) were randomly selected, removed from their mothers, weighed, fasted for 4 h, and anesthetized with pentobarbital sodium (25 μg/g ip) for blood collection by cardiac puncture and pancreatic dissection (1 male pup/litter) or islet isolation (2 male pups/litter).

Serum glucose and insulin. Glucose was measured by the glucose oxidase method (Randox Laboratories, County Antrim, UK) and insulin by rat insulin ELISA kit (Mercodia, Uppsala, Sweden). Intra-assay coefficients of variation were <2 and <4%.

Immunohistochemistry and immunofluorescence. Pancreata were removed, weighed, and dissected. The pancreatic tail was fixed (4% paraformaldehyde-PBS) and paraffin embedded, sectioned completely (5 μm), and mounted. Insulin immunohistochemical staining was performed to assess β-cell structure (13). Eight sections (240 μm apart) per pancreas were incubated with blocking buffer (3% BSA-0.1% Tween 20 in Tris-buffered saline) for 30 min, followed by guinea pig anti-insulin antibody (Dako, Carpinteria, CA) for 1 h and incubation with horseradish peroxidase-conjugated anti-guinea pig IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. Immunoreactivity was visualized with diaminobenzidine (Dako), and sections were counterstained with hematoxylin. To determine β-cell proliferation, double immunofluorescence staining for Ki-67 and insulin was carried out on three to six sections (240 μm apart) per pancreas. Sections were microwaved for 3 min in 10 mmol/l citrate buffer for antigen retrieval, permeabilized with 0.3% Triton X-100 in Tris-buffered saline for 20 min, and incubated with blocking buffer for 30 min. After overnight incubation at 4°C with guinea pig anti-insulin (Dako) and mouse anti-Ki-67 (BD Pharmingen, San Diego, CA) antibodies, detection was performed using FITC-labeled anti-guinea pig IgG and Texas Red-labeled anti-mouse IgG secondary antibodies (Jackson ImmunoResearch Laboratories).

β-Cell morphometric and proliferation analyses. Area of insulin-positive cells and pancreatic tissue surface was quantified by computer-assisted measurements with a Leica DMRB microscope provided with a camera coupled to a Quantimet 500 MC computer (screen magnification ×24) using Leica Qwin software (Wetzlar, Germany) (13). β-Cell fraction (%β-cells in the pancreas) was calculated as the ratio of insulin-positive area to total pancreatic tissue area on the entire section. β-Cell mass was obtained by multiplying β-cell fraction by the corresponding pancreatic weight and β-cell mass/body weight determined. Aggregates composed of more than three immunoreactive insulin cells (diameter >25 μm) were counted and expressed as number of β-cell aggregates per area of pancreatic tissue (cm²). The mean area of β-cell aggregates was also measured. The rate of proliferating β-cells was calculated as the ratio of ≥50 cells colocalizing for insulin and Ki-67 to the total number of insulin-positive cells visualized with a Reichert Polyscan microscope (Vienna, Austria).

Islet isolation. Islets were isolated by collagenase digestion (25). The pancreas was perfused via its duct with 1 mg/ml collagenase XI (Sigma-Aldrich, St. Louis, MO) in HBSS (Gibco-Invitrogen, Grand Island, NY). Pancreatic digestion was carried out at 37°C for 20 min, after which cold HBSS was added. The suspension was centrifuged at 290 g for 30 s at 4°C, washed twice with HBSS, resuspended in HBSS-10% FBS (Gibco-Invitrogen), and poured onto a 70-μm cell strainer (BD Falcon; BD Biosciences, San Diego, CA). Islets were rinsed, hand-picked, and washed twice with PBS.
Gene expression analysis. RNA was extracted from freshly isolated islets using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), following the manufacturer’s instructions. RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE), and cDNA was synthesized by reverse transcription of RNA with the SuperScript II kit (Invitrogen), using random hexamers. Relative gene expression of Foxa2, Hnf1β, Hnf1α, Hnf4α, Pdx1, Rfx6, Neurod1, Isl1, Pax4, Nkx6–1, Ins, Slc2a2, and Kcnj11 was assessed by quantitative PCR with a Light Cycler 2.0 thermal cycler (Roche Applied Science, Hague Road, IN), using the Light Cycler Taqman Master kit, probes from the Universal ProbeLibrary, and specific primers (Table 1) generated with the ProbeFinder design assay software (http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP03000). Data of each gene were normalized to the housekeeping gene Hprt1 (hipoxantine phosphoribosyltransferase 1).

Statistical analysis. Only male offspring were studied. Data are expressed as means ± SE; n refers to the number of either mothers or litters for each measurement. Body weights at conception and maternal food intake were analyzed using Student’s t-test. Maternal body weight at the end of gestation adjusted to litter size was assessed by a univariate analysis of variance and litter size and offspring PND1 body weight with a Mann-Whitney test. One-way ANOVA, followed by Games-Howell post hoc testing, was performed to analyze body weight and food intake of mothers in lactation and all offspring data on PNDs 7 and 21. Two-way ANOVA test was used to assess gene expression. Statistical analyses were carried out with SPSS 15.0 software (SPSS, Chicago, IL), and P values <0.05 were considered statistically significant.

RESULTS

Effect of LP on maternal body weight, daily food intake, and litter size. Mothers fed control (C) and LP (R) diets throughout pregnancy had similar body weights at conception (Table 2). However, body weight gain was reduced by 7% in R mothers by the end of gestation, although average daily food intake and litter size were unchanged. Maintaining LP in lactation up to PND 21 led to a 15–20% body weight reduction in RR mothers compared with controls (C). Although food intake rate was similar between groups at PND 7, it was decreased by 43% at PND 21 in RR mothers. Dietary change after delivery (RC and RR and 2.7-fold lower than RC in the presence of LP did not decrease pancreatic weight in CR pups at PND 7, although in CR and RR pups the pancreas weighed ∼40% of the weight in CC and RC by PND 21. All groups had an equivalent pancreatic tissue mass per unit of body weight at PND 7 and PND 21.

Table 1. Primer sequences used for quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Product Size, bp</th>
<th>Probe No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxa2</td>
<td>CCAGAACACCGAGATCTCTT</td>
<td>ACGGCCCTGCAGCACTATTT</td>
<td>109</td>
<td>95</td>
</tr>
<tr>
<td>Hnf1β</td>
<td>AGCGCGGACGGTGCAAGCAG</td>
<td>GGAGGGCCCTGCTTTTATGT</td>
<td>94</td>
<td>107</td>
</tr>
<tr>
<td>Hnf1α</td>
<td>AGCCAGAGTCTGCTGCTAGAG</td>
<td>CGCCACAGACAGCAAGCAAG</td>
<td>135</td>
<td>68</td>
</tr>
<tr>
<td>Hnf4α</td>
<td>AATTCTGCTTGATTTGTTCT</td>
<td>ATGAGACCTGCTGCTGTTT</td>
<td>100</td>
<td>83</td>
</tr>
<tr>
<td>Pdx1</td>
<td>TGGGCGCTTTGTTGATTTT</td>
<td>AGGGTCGTGCTGCTAGCTG</td>
<td>110</td>
<td>68</td>
</tr>
<tr>
<td>Rfx6</td>
<td>TGTCGCTCTGGTAATTTGATT</td>
<td>GGCGGACCTGCTGCTGCTG</td>
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<td>68</td>
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<tr>
<td>Neurod1</td>
<td>AGCCAGAAGCCAGAGTG</td>
<td>TTTGTCATGCTGCTGCTGC</td>
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<td>1</td>
</tr>
<tr>
<td>Isl1</td>
<td>AGGACCGAACGCAACAAACT</td>
<td>TGTCATGCTGCTGCTGCTG</td>
<td>79</td>
<td>83</td>
</tr>
<tr>
<td>Pax4</td>
<td>GAGCCAGCGAGCTGCTTCC</td>
<td>GAGGTCGTGCTGCTGCTG</td>
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<td>130</td>
</tr>
<tr>
<td>Nkx6–1</td>
<td>GAGCCAGATGCTGCTGGAGACAA</td>
<td>CGAAATATATTGAATTTG</td>
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<td>126</td>
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<tr>
<td>Ins</td>
<td>GTCAGACTGCTGCTGCTGCTG</td>
<td>CGAAATATATTGAATTTG</td>
<td>83</td>
<td>29</td>
</tr>
<tr>
<td>Slc2a2</td>
<td>TTTTCTGCTGCTGCTGCTG</td>
<td>CGAAATATATTGAATTTG</td>
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<tr>
<td>Kcnj11</td>
<td>CTGCTGAGTAGCTGCTGCTG</td>
<td>CGAAATATATTGAATTTG</td>
<td>93</td>
<td>107</td>
</tr>
<tr>
<td>Hprt1</td>
<td>GTCTGCTGCTGCTGCTGCTG</td>
<td>CGAAATATATTGAATTTG</td>
<td>126</td>
<td>22</td>
</tr>
</tbody>
</table>

Foxa2, forkhead box A2; Hnf1β, hepatocyte nuclear factor (HNF1) homeobox B; Hnf1α, HNF1 homeobox A; Hnf4α, hepatocyte nuclear factor 4α; Pdx1, pancreatic and duodenal homeobox 1; Rfx6, regulatory factor X6; Neurod1, neurogenic differentiation 1; Isl1, ISL LIM homeobox 1; Pax4, paired box 4; Nkx6–1, NK6 homeobox 1; Ins, insulin; Slc2a2, solute carrier family 2 (facilitated glucose transporter), member 2; Kcnj11, potassium inwardly rectifying channel, subfamily J, member 11; Hprt1, hipoxantine phosphoribosyltransferase 1; bp, base pairs.

Effect of maternal LP on male offspring body and pancreatic weight. On PND 1, male R offspring body weight was decreased by 15% (Table 2). Continued exposure to LP limited the growth of RR pups, which were 46% smaller on PND 7 and nearly one-third the weight of CC pups by PND 21. In contrast, dietary normalization of RC mothers with the diet in lactation led to similar body weight gain to CC and greater gain than RR offspring at PNDs 7 and 21. Maternal LP after birth reduced growth in CR pups; body weight decreased by 30% compared with CC at PND 7 and by 50% at PND 21.

Effect of maternal LP on offspring serum glucose and insulin concentration. Serum glucose concentration at both postnatal ages and insulin on PND 7 did not differ by treatment (Table 2). However, on PND 21, insulin levels of RC pups were 65 and 37% higher than in CC and RR, respectively, indicating potentially early development of insulin resistance in RC pups. In CR offspring, serum insulin was 45% lower than CC and RR and 2.7-fold lower than RC in the presence of normal glucose, suggesting early increased insulin sensitivity and/or impaired insulin secretion.

Effect of maternal LP on β-cell structure and proliferation compared with the control diet. In CC offspring (Fig. 1), β-cell mass increased from PNDs 7 to 21, whereas other morphological measurements decreased, except for β-cell aggregate size, which remained unchanged. For each LP group, data were normalized to CC for comparison with CC at PND 7 or 21 as well as the other two LP groups (Fig. 1, upper middle, lower middle, and bottom). In RR offspring (Fig. 1) compared with CC, β-cell fraction decreased by 45 and 25% at PND 7 and PND 21, respectively, whereas β-cell mass was reduced by 72% at PND 21 compared with continued dietary LP (RR). LP in lactation reduced body weight and food intake by 20 and 40%, respectively, in CR mothers compared with CC and RC mothers at PND 21.
70% at both ages. The proliferation rate decreased ~50% at both ages. The mean number of β-cell aggregates was reduced at PND 7, and the size of β-cell aggregates decreased by 25% at both ages. Compared with CC, β-cell fraction and β-cell mass at both ages were decreased in RC offspring (Fig. 1), and the proliferation rate was reduced 25% and 30% at PND 7 and PND 21, respectively. The number of β-cell aggregates was reduced by 25% in RC offspring at both ages, whereas β-cell aggregate size was reduced only at PND 21. Interestingly, β-cell mass, β-cell proliferation at both ages, and β-cell fraction and β-cell aggregate size at PND 7 were increased compared with RR offspring, showing the effect of restoring the normal maternal diet (Fig. 1, lower middle). In CR offspring compared with CC (Fig. 1), β-cell mass decreased 45% at PND 21 and β-cell proliferation 10% at PND 7. When normalized for body weight, β-cell mass per unit did not differ between groups (data not shown).

**Effect of maternal LP on male offspring expression of transcription factor and β-cell genes.** There were no dietary group or age-related changes in six genes: Hnf1b, Foxa2, Nkx6-1, Neurod1, Pax4, and Kcnj11. Therefore, we report changes in seven genes: Hnf1a, Hnf4a, Pdx1, Isl1, Rfx6, Ins, and Slc2a2 (Fig. 2). In the CC group, in the first six of these there was a consistent pattern of decreased expression between PNDs 7 and 21. Slc2a2 mRNA was unchanged (Fig. 2, top). For the three experimental groups, mRNA expression calculated relative to CC at the same age is shown in Fig. 2, upper middle, lower middle, and bottom. Compared with CC, all significant changes with age in the three experimental groups were in the direction of increased expression (Fig. 2, upper middle, lower middle, and bottom). At PND 7 compared with CC, RR offspring showed increased mRNA for three genes: Hnf1a, Hnf4a, and Rfx6. At PND 21 the same three genes remained elevated as well as Pdx1, Isl1, and Slc2a2 (Fig. 2, upper middle).

A comparison of CC and RC (indicating R effects in pregnancy) showed increased Hnf1a, Pdx1, Rfx6, and Ins at PND 7, but only Hnf4a was elevated at PND 21. Effects of R in lactation are shown by comparing CC and CR. Hnf1a, Hnf4a, Rfx6, and Slc2a2 were increased in both PND 7 and PND 21.

**DISCUSSION**

Compelling evidence from controlled animal studies indicates that structural and functional alterations in β-cells caused by a suboptimal nutritional environment during early life can contribute to development of glucose intolerance and increased susceptibility to later-life T2D (45, 46). However, the underlying mechanisms remain poorly defined. We used the well-established model of feeding prenatal and early postnatal LP to pregnant rats, which induces diabetes in the offspring (36), to determine early postnatal life changes in β-cell growth and expression of a cassette of genes involved in pancreatic development and β-cell function in relation to their potential role in β-cell programming.

Defective insulin secretion in offspring exposed to prenatal and early postnatal LP (2, 7, 19) may potentially be due to altered structure of β-cells. Growth-restricted preweanling and weanling pups with normal fasting glucose and insulin concentrations showed a marked reduction in pancreatic weight, β-cell fraction, and mass and decreased mean size of β-cell aggregates. By PND 21, β-cell mass was reduced in all three

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**Table 2. Body weight and food intake of mothers, litter size, and body and pancreas weight, serum glucose, and insulin concentrations of the offspring**

<table>
<thead>
<tr>
<th>Day</th>
<th>C</th>
<th>R</th>
<th>CC</th>
<th>RR</th>
<th>RC</th>
<th>CR</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mothers</td>
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<tr>
<td>Gestation</td>
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<tr>
<td>Body weight, g</td>
<td>G1 246.8 ± 2.5</td>
<td>248.6 ± 2.3</td>
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<tr>
<td>Food intake, g/day</td>
<td>18.0 ± 0.3</td>
<td>18.8 ± 0.4</td>
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<tr>
<td>Litter size, pups/litter</td>
<td>11.8 ± 0.2</td>
<td>11.6 ± 0.3</td>
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<tr>
<td>Lactation</td>
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<tr>
<td>Body weight, g</td>
<td>L7 268.5 ± 5.0</td>
<td>228.5 ± 7.2*</td>
<td>266.7 ± 7.2†</td>
<td>261.4 ± 5.0†</td>
<td></td>
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<tr>
<td>Food intake, g/day</td>
<td>23.4 ± 1.0</td>
<td>18.1 ± 1.9</td>
<td>25.8 ± 2.4</td>
<td>23.3 ± 1.3</td>
<td></td>
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</tr>
<tr>
<td>Pancreas weight, mg</td>
<td>L7 5.0 228.5</td>
<td>13.1 ± 0.8*</td>
<td>13.1 ± 0.8</td>
<td>7.9 ± 1.0*</td>
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<tr>
<td>Glucose, mmol/l</td>
<td>L7 4.8 16.8</td>
<td>40.8 ± 3.7*</td>
<td>122.4 ± 8.6‡</td>
<td>39.5 ± 3.2*</td>
<td></td>
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<tr>
<td>Insulin, pmol/l</td>
<td>L7 0.5 6.2</td>
<td>5.3 ± 0.5</td>
<td>6.2 ± 0.8</td>
<td>4.8 ± 0.5</td>
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<tr>
<td>Data are expressed as means ± SE; n = 22/group. C, control group; R, low-protein diet group in gestation period; CC, control group; RR, prenatal and postnatal low-protein diet group; RC, prenatal low-protein diet/postnatal control diet group; CR, postnatal low-protein diet group in lactation period; G1, gestation day 1; G21, gestation day 21; P1, postnatal day 1; L7, lactation day 7; L21, lactation day 21; P7, postnatal day 7; P21, postnatal day 21. L7/P7 CC, n = 6; P7 RR, R, and CR, n = 5; L21/P21 CC, RR, and RC, n = 6; P21 CR, n = 5. *P &lt; 0.05 vs. C or CC; †P &lt; 0.05 vs. RR; ‡P &lt; 0.05 vs. RC from the same age.</td>
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</table>
experimental groups but was most impaired when restriction occurred prenatally and was not completely recovered when mothers returned to the control diet at birth. Importantly, pancreatic weight and β-cell mass were proportional to body weight in all groups. However, β-cell mass may not increase in later postnatal life in RR pups, as occurs in the model of maternal caloric restriction in gestation and lactation (14), and thus, adult R offspring would have a decreased reserve of β-cells. The altered pancreatic phenotype demonstrated by LP offspring predisposes them to later-life T2D (36). Offspring weaned onto normal protein diets eventually show decreased insulin responses to glucose, even when normal glucose toler-
Fig. 2. Effect of exposure to maternal low-protein diet on pancreatic islet gene expression of Hnf1a, Hnf4a, Pdx1, Isl1, Rfx6, Ins, and Slc2a2 in offspring during lactation. Relative mRNA levels by RT-PCR on PND 7 (open bars) and PND 21 (filled bars). CC, RR, RC, and CR groups are indicated (1st letter, diet in pregnancy; 2nd letter, diet in lactation) and show data normalized to control values (denoted by dotted line) on PND 7 or PND 21 (5 or 6 litters/group). Data are expressed as means ± SE. Dotted line denotes the control value. #Significant differences (P < 0.05) between PND 7 and PND 21; *significant differences (P < 0.05) vs. control (CC) values. Different letters denote significant differences (P < 0.05) between RR, RC, and CR groups.

Protein restriction accelerates β-cell development. 

Host is maintained early in life due to improved insulin sensitivity (3, 34). The decrease in β-cell proliferation and mass will clearly make these pups vulnerable, especially to obesogenic diets (49).

Matching of the trajectory of differentiation and cellular proliferation during development is key to achieving optimal structure and functional capability of all organs, including the pancreas. Impairment of pancreatic insulin secretion could result from altered expression of genes implicated in pancreatic islet growth, cell differentiation, and insulin secretory capacity.

Apart from Isl1 at PND 21 and Ins at PND 7, each of the genes investigated was increased in at least one group of pups at least at one age.

The various gene changes observed may either be a direct response to the nutritional challenge or represent compensation for initial changes. In addition, although the islet is comprised of greater than 90% of β-cells, these changes may take place partially or completely in other cell types. Offspring islet expression of Hnf1a, Hnf4a, Pdx1, Isl1, Rfx6, and Slc2a2 was affected by prenatal and early postnatal exposure to LP, indi-
cating that their expression is regulated potentially by decreased amino acid availability. Gene overexpression of all of these transcription factors suggests premature differentiation.

Hnf1α and Hnf4α are implicated in pancreatic development and control insulin secretion partly by regulating glucose transporter Slc2a2 transcription (5, 39, 44). Their overexpression in RR offspring might occur as a compensatory mechanism to maintain glucose homeostasis during early life since insulin secretion is impaired (2, 7, 19). However, upregulation of Hnf1α and Hnf4α expression may have detrimental effects on β-cell structure, because previous studies demonstrate that Hnf1α and Hnf4α overexpression decrease β-cell proliferation and induce apoptosis, leading to a reduction in β-cell mass (11, 27).

Rfx6 regulates pancreatic islet cell differentiation (43) and expression of genes encoding factors involved in Ins transcription (40). Its overexpression in RR offspring might represent a compensatory mechanism to promote transcription of its target genes to sustain insulin production. Message for Rfx6 was the most affected gene, being increased in four of the six age-by-treatment groups. Since this gene is necessary for specification of several islet cell types (43), caution must be expressed in conclusions solely regarding its role in driving β-cell growth and differentiation.

Pdx1 is crucial for pancreatic development, endocrine cell differentiation, and adult Ins expression and β-cell neogenesis (20). A study in a pancreatic β-cell line showed that its overexpression promotes insulin production without insulin gene expression being affected (48). Hence, upregulation of Pdx1 expression in RR offspring likely represents accelerated pancreatic maturation that would result in better maintenance of adequate insulin levels, at least initially. Since LP impairs β-cell proliferation, it may lead to premature differentiation, by upregulating Pdx1 expression, to form β-cells at the expense of proper β-cell expansion. Thus, LP restricts growth of β-cells, so protein can be used for the maintenance of existing β-cells. Indeed, an increased number of differentiated β-cells and fewer precursor cells would support our suggestion of accelerated differentiation of β-cells that have reduced proliferation capability. Further studies will be necessary to elucidate this.

Isl1 is required for the differentiation, proliferation, and maintenance of endocrine cells during intrauterine pancreatic development and in early postnatal life (9). Accordingly, its overexpression in RR offspring may influence premature islet cell formation, including that of β-cells.

It is known that most of the β-cell mass of adult rats forms after birth and that maturation of insulin secretion occurs in early postnatal life (1, 8). Therefore, any environmental factor in this critical window of development could have long-term consequences on pancreatic function. Offspring exposed to LP in gestation followed by a normal maternal diet during lactation (RC) underwent catchup growth and showed increased serum insulin by weaning, maintaining relatively normal glucose homeostasis since glucose levels were normal. Increased plasma insulin was accompanied in the RC group by increased Ins mRNA expression. Increased insulin secretion in the presence of normal glucose suggests that insulin resistance is already present in these neonates by PND 21, and later-life challenges to pancreatic function may well precipitate overt diabetes. Previous studies with animals experiencing this developmental history indicate that increased insulin concentrations are still observed in adulthood and that nutritional challenges lead to glucose intolerance and insulin resistance (3, 50). All the morphometric markers of β-cell development were decreased in RR offspring compared with CC controls. Nutritional recovery in RC only partially restored all variables compared with RR pups, indicating that the underlying mechanisms of β-cell morphological alterations probably originate in fetal life. Hnf1α, Hnf4α, Pdx1, and Rfx6 expression were upregulated in RC offspring and might also represent a similar mechanism to that seen in weaning offspring with prenatal and postnatal protein restriction. Nevertheless, except for Hnf4α, their expression was transiently increased on PND 7, suggesting that these mechanisms set in place in utero are no longer necessary in postnatal life since the offspring recuperates from the effect of prenatal protein restriction. Ins expression was also induced at PND 7 in RC, consistent with early development of insulin resistance.

In sharp contrast, offspring of mothers fed a normal diet in pregnancy but LP during lactation (CR) showed growth restriction, decreased insulin concentration (suggesting defective insulin secretion and/or improved insulin sensitivity), and reduced β-cell mass. Decreased β-cell mass in CR pups may reflect only the severe reduction in pancreatic weight rather than a negative effect on β-cell structure since β-cell fraction and aggregate number were slightly increased and β-cell aggregate size was unchanged. Moreover, β-cell proliferation in CR offspring was decreased on PND 7, but it was similar to that of CC by PND 21. Conversely, prenatal LP (RR and RC groups) reduced β-cell fraction, mass, aggregate size, and number partly as a result of decreased β-cell proliferation. Increased β-cell apoptosis could also contribute to reduce β-cell mass in all LP groups. These data suggest that postnatal LP (CR) affects mainly pancreatic weight, whereas prenatal LP, regardless of the postnatal diet (RR and RC), has detrimental effects on β-cell development. Hnf1α, Hnf4α, and Slc2a2 expression were upregulated in CR offspring. It is also possible that Hnf1α and Hnf4α overexpression reduce β-cell mass, although proliferation was not significantly affected. Rfx6 expression was upregulated as a further potential compensatory mechanism to sustain insulin production.

Taken together, these results suggest that RR and CR pups showing increased gene expression, perhaps as a compensation mechanism for GSIS impairment (2, 7, 16, 19, 30, 31) and decreased β-cell mass, may be predisposed to β-cell exhaustion from later-life attempts to compensate, resulting in glucose intolerance and predisposition to T2D. Conversely, postnatal recuperation of nutrition (RC) restores overall GSIS (2, 19), and because β-cell mass is partially recovered, gene expression is not altered at weaning, and fasting insulin levels are increased; it is likely that glucose intolerance in adult life results mainly from the insulin resistance that appears to develop in early life in the RC offspring.

In conclusion, maternal LP induces gene expression of key transcription factors and β-cell genes, which affect β-cell structure and function in a manner that is dependent on the window of nutrient challenge. Some of these changes may be compensatory mechanisms to maintain survival and glucose homeostasis in early life. The changes we observed would result in the earlier appearance of fewer mature islets with a smaller functional capacity and which, if factors such as overnutrition and associated adiposity occur later in life to-
gather or separately, could lead to later-life diabetes. This study indicates potential mechanisms underlying β-cell programming that are targets for future investigation and highlights the relative and specific importance of dietary interventions during pregnancy and lactation to prevent detrimental structural and functional outcomes in β-cells.

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DISCLOSURES

The authors have no conflicts of interest, financial or otherwise, regarding this article.

AUTHOR CONTRIBUTIONS


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