Regulation of postnatal pancreatic *Pdx1* and downstream target genes after gestational exposure to protein restriction in rats

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Abstract

The study carried out in our laboratory demonstrated that protein restriction (low protein, LP) during fetal and neonatal life alters pancreatic development and impairs glucose tolerance later in life. In this study, we examined the role of the transcription factor *Pdx1*, a master regulator of β -cell differentiation and function along with its downstream target genes insulin, *Glut2* and glucokinase (GK). The role(s) of these genes and protein products on the pancreata of male offspring from mothers exposed to LP diets were assessed during gestation, weaning, and adult life. Pregnant rats were allocated to two dietary treatments: control (C) 20% protein diet or LP, 8% protein diet. At birth, offspring were divided into four groups: C received control diet all life, LP1 received LP diet all life, LP2 changed the LP diet to C at weaning, and LP3 switched to C after being exposed to LP during gestation only. Body weights (bw) were significantly ($P < 0.001$) decreased in all LP groups at birth. At weaning, only the LP3 offspring had their body weight restored to control levels. *Pdx1* or any of the *Pdx1*-target genes were similar in all diets at day 21. However, at d130 *Pdx1* mRNA expression and protein abundance were significantly decreased ($P < 0.05$) in all LP groups. In addition, insulin mRNA and protein were decreased in LP1 and LP3 groups compared with C, *Glut2* mRNA and GLUT2 protein levels were decreased in LP3 and GK did not change between groups. Intraperitoneal glucose tolerance test revealed impaired glucose tolerance in LP3 males, concomitant with decreased β -cell mass, islet area, and PDX1 nuclear protein localization. Collectively, this study suggests that restoring proteins in the diet after birth in LP offspring dramatically impairs glucose homeostasis in early adulthood, by altering *Pdx1* expression and downstream-target genes increasing the risk to develop type 2 diabetes.

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Introduction

Epidemiological studies in humans have suggested an association between poor fetal or infant growth (due to adverse intrauterine conditions) and increased risk of developing glucose intolerance (type 2 diabetes mellitus, develop type 2 diabetes (T2DM)) and metabolic syndrome later in life (Hales *et al.* 1991, Lewis *et al.* 2001). In rats, it has been demonstrated that protein restriction to 8% (low protein, LP) to an overall isocaloric diet during gestation resulted in a moderate intrauterine growth restriction (IUGR) and reduced pancreatic weight in offspring at birth (Dahri *et al.* 1995, Hales & Ozanne 2003, Guan *et al.* 2005). In addition, we have shown that this LP diet given during pregnancy at different windows of fetal development results in a relative deficiency of β cells following birth, due to a failure to develop larger islets. These developmental changes in the pancreas affect male and female fetuses differently (Chamson-Reig *et al.* 2006).

Interestingly, if the LP diet was extended into neonatal life, the pancreas showed reduced β -cell mass, islet area and pancreatic vascularity with increased β -cell apoptosis and decreased rate of β -cell duplication and reduction in IGF2 expression (Petrik *et al.* 1999). Moreover, if the LP offspring were given a control diet after weaning until 130 days, they became glucose intolerant with striking sexual dimorphisms in key insulin signaling pathways such as reduced AKT phosphorylation in response to insulin in the adipose and skeletal muscle in males (Chamson-Reig *et al.* 2009).

Therefore, in order to further understand the mechanisms by which glucose impairment occurs after the periods of protein restriction, we examined the master transcription regulator *Pdx1*, known to control the endocrine cell lineage during embryonic development in mice and humans (Ohlsson 1993, Jonsson *et al.* 1994, Offield *et al.* 1996) and to regulate a number of genes involved in the maintenance of β -cell identity and

function, e.g. insulin (Chakrabarti *et al.* 2002, Le Lay *et al.* 2004), *Glut2* (Waeber *et al.* 1996, Lottmann *et al.* 2001), glucokinase (GK) (Watada *et al.* 1996), and also *Pdx1* itself (Gerrish *et al.* 2001). To address these questions, four separate dietary regimes were employed in this perinatal rat model. Only male were examined, because as mentioned previously they were shown to be more prone to develop glucose intolerance later in life LP2. The groups included a normal (20%) protein diet administered throughout life C, a low (8%) protein diet throughout life LP1, until weaning LP2 or gestation only LP3. We have chosen to restore proteins after restriction at specific windows of development given that postnatal accelerated growth of IUGR offspring has been demonstrated to exacerbate the effects of IUGR-related programming and reduce the lifespan of these offspring (Ozanne *et al.* 2004, Chen *et al.* 2009, Tarry-Adkins *et al.* 2009).

Materials and methods

Animal procedures

Virgin female Wistar rats weighing 250–300 g were purchased from Charles River Laboratories (Montreal, QC, Canada) and were bred and housed in our facilities at Lawson Health Research Institute (London, ON, Canada). The animals were maintained in a temperature-controlled room at 22 °C on a 12 h light:12 h darkness cycle with food and water available *ad libitum*. Nulliparous females were cycled and mated with male rats on the night of pro-estrous. Pregnancy was confirmed by the presence of a vaginal plug and/or sperm the next morning and this was noted as day 1 of pregnancy. The dams were fed with a control diet C 20% (w/w) protein or a LP diet, 8% (w/w) protein during gestation. After birth, offspring were divided into four groups. Group 1 C were fed with C diet until day 130, Group 2 LP1 received LP diet until day 130, Group 3 LP2 were fed LP diet during gestation and lactation (day 21), and Group 4 LP3 received LP diet during gestation only. The 130 day time point was chosen based on our previous published work, which demonstrated that male LP2 offspring first exhibit glucose intolerance at this age (Chamson-Reig *et al.* 2006). The offspring at birth were restricted to eight pups to maintain uniform neonatal growth.

All procedures were performed with the approval of the Animal Care Committee of the University of Western Ontario in accordance with guidelines given by the Canadian Council for Animal Care. The diets were purchased from Bio-Serv (Frenchtown, NJ, USA) and control (C=cat# F-4576) and LP diet (LP=cat# F-4575) where isocaloric and the reduction of protein was balanced by the addition of carbohydrates with normal fat content (Snoeck *et al.* 1990, Desai *et al.* 1996). The detailed composition of the diet is given in Table 1.

Each experimental group was comprised eight mothers. The pups were weighed at day 1, 21, and 130. The males were killed by CO₂ asphyxiation at weaning or at 130 days of age, and the pancreata were dissected, weighed, either fixed in 10% formalin or snap-frozen in liquid nitrogen or in RNA*later* and then stored at –80 °C until further analysis.

Table 1 Composition of the diets.

Diet component	Control (C, g/kg) (20% casein)	Low protein (LP, g/kg) (8% casein)
Casein	220	90
Sucrose	213	243
Cellulose fibre	50	50
Cornstarch	80	80
Vitamin mix	2.5	2.5
Mineral mix	47	48
Soya oil	43	43
DL-Methionine	2	0.8
Chlorine chloride	4	40

Intraperitoneal glucose tolerance test

At 130 days of age, three to five male offspring from C or LP3 treatment groups were subjected to an intraperitoneal glucose tolerance test (IGTT). After ~14 h of fasting, the animals were tested for blood glucose by tail vein lancing with a hand held glucometer (Ascensia Breeze Bayer HealthCare, Mishawaka, IN, USA). A single bolus of 2 g/kg glucose was injected into the peritoneal space (i.p.) and blood glucose was monitored at 5, 15, 30, 60, 90, and 120 min.

Quantitative real-time PCR

At sacrifice, at least eight pancreata (1/l) were dissected and stored in RNA*later* (Ambion, Inc., Austin, TX, USA) at –80 °C until RNA extraction. Total RNA was extracted using the Qiagen RNeasy Plus MINI Kit (Qiagen, Inc.) according to the manufacturer's specifications and was stored at –80 °C for further analysis. The integrity of RNA was confirmed on a 2% Agarose TAE gel. The quantity of RNA in the samples was measured by spectrophotometry (Eppendorf, Bio-photometer, Mississauga, ON, Canada). The samples were considered acceptable with an absorbance ratio for the 260/280 value of 1.7–2.

RNA was reverse transcribed into first-strand cDNA using a Superscript III Reverse Transcriptase (Invitrogen). Initially, 4 µg of the RNA sample were measured and DEPC-treated water was added. A master mix containing 10× RT buffer, 25 mM magnesium chloride (MgCl₂), 0.1M dithiothreitol (DTT), 10 mM deoxyribonucleotide triphosphates mix (dNTPs: dATP, dCTP, dGTP, and dTTP) (Invitrogen 10297-018), RNase Out Recombinant RNase Inhibitor, and Superscript III Reverse Transcriptase (200 U/µl) was used. Total master mix was added to each DNase I-treated RNA sample, placed in the thermocycler with the following program: 25 °C for 10 min, 50 °C for 50 min, followed by 85 °C for 5 min to terminate the reaction. cDNA samples were then stored at –20 °C until further use.

Real-time quantitative PCR (qRT-PCR) was performed using SYBR Green Supermix (Bio-Rad Laboratories) in a Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories) sequence-detection system. The primer sequences were designed and purchased from Invitrogen and are given in Table 2. The following cycle conditions were used: step 1, 50 °C for 2 min; step 2, 95 °C for 10 min; step 3, 72 °C for 15 s; and step 4, 60 °C for 1 min. Data were automatically computed using CFX Manager Software (Bio-Rad Laboratories). Relative fold changes were calculated using the comparative cycle times

Table 2 List of primer sequences for qRT-PCR.

Genes	Forward primer (5'–3')	Reverse primer (5'–3')
<i>Pdx1</i>	CCGCGTTCATCTCCC-TTTC	TGCCCCACTGGCTTTTCCA
<i>Insulin</i>	CCCAGGAGAGCGT-GGCATT	CATTGCAGAGGGGTGGGCGG
<i>Glut2</i>	GTCCAGAAAGCCC-CAGATACC	TGCCCCCTAGTCTTTTCAAGCT
β -actin	ACGAGGCCAGAG-CAAGA	TTGGTTACAATGCCGTGTTCA

(Ct) method with β -actin as the reference gene. Δ Ct values for each probe set were standardized to the experimental samples with the lowest transcript abundance (highest Ct value). The relative abundance of each primer set compared with calibrator was determined by the formula: $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ was the standardized Ct value.

Separation of cytoplasmic and nuclear fractions and protein quantification

The different cellular compartments were separated as previously described (Kawamori *et al.* 2003, Hagman *et al.* 2005). Briefly, snap-frozen pancreatic tissue was placed into ice-cold 250 μ l NE1 (10 mM HEPES pH 7.9, 10 mM $MgCl_2$, 5 mM KCl, 1% Triton X, 0.1 mM EDTA, 1 mM DTT), supplemented with a mini-protease inhibitor (Roche Diagnostics). The samples were homogenized for 30 s using a PolyTron PT 2100 (Kinematica AG, Switzerland) and then incubated on ice for 15 min. The homogenates were then centrifuged at 12 000 rpm for 10 min at 4 °C and supernatants were saved as they had the cytoplasm fraction in a fresh-labeled tube placed on ice. The pellets were then re-suspended in ice-cold 1000 μ l NE1 and vortexed briefly, then centrifuged at 5000 rpm for 5 min at 4 °C. The supernatant were aspirated off while adding 250 μ l of NE2 (25% glycerol, 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 500 mM NaCl, 0.2 mM EDTA), supplemented with a mini-protease inhibitor tablet (Roche Diagnostics) to pellet. The re-suspended pellet was incubated on ice for 1 h and vortexed every 5 min then spun at 13 000 rpm for 20 min. The supernatants were removed to a new tube and then quantified and stored at –80 °C until later use.

Protein concentration was determined using a MICRO BCA Protein Assay Reagent Kit involving the same steps as above. Gel electrophoresis and immunoblotting were conducted as described, using rabbit anti-histone H3 antibody (1:1000, Millipore 06-755, Billerica, MA, USA) for the nuclear fraction as internal loading control and a mouse anti β -tubulin MAB (1:1000, Millipore 05-661) was used as an internal loading control for cytoplasmic fraction.

Western blotting analysis of Pdx1, insulin, Glut2, and Gk

Fifty milligram samples of frozen pancreata were placed in 1 ml lysis buffer (50 mM Tris–HCl (tri-hydroxymethyl aminomethane) pH 7.4, 150 mM sodium chloride, 1 mM phenylmethanesulfonyl fluoride, 0.1% Igepal (MP Biomedical, Solo, OH, USA), 0.1% Trasyolol, Complete Mini tablet (Roche Diagnostics). The samples

were homogenized (IKA, T10 Basic S1 Disperser) then spun at 13 000 g at 4 °C for 30 min. The supernatants were removed to new tubes and then quantified and stored at –80 °C until later use. Protein concentration was determined using a MICRO BCA Protein Assay Reagent Kit (Thermo Scientific Pierce Protein Research Products 23235, Rockford, IL, USA).

Twelve percent polyacrylamide gels were prepared and samples from three to five animals per treatment (C, LP1, LP2, LP3) and age (d21 and d130) were loaded (50 μ g protein in 20 μ l). The samples were separated by running in Laemmli electrophoresis buffer (0.03% Tris–Base (EMD Chemicals, Mississauga, ON, Canada), 0.14% glycine, 0.01% SDS) for ~45 min to 1.5 h at 130 V. The gel was then removed and stacked in an Invitrogen iBlot machine (Invitrogen) for a 7-min semi-dry transfer onto nitrocellulose membrane (Invitrogen).

After transferring, the membranes were stained with Ponceau Red S solution (0.1% Ponceau S, 5% acetic acid) for 1–2 min to visualize protein quality and ensure proper transfer. Ponceau S was washed off and a blocking step followed by incubating the membrane in 5% non-fat dry milk in Tris-buffered saline Tween 20 (TBS-T), 0.024% Tris–Base (EMD Chemicals), 0.08% sodium chloride, and 0.05% Tween 20 (Sigma–Aldrich P1379) for 60 min at room temperature. The membranes were initially probed for β -actin (the internal loading control) using primary antibody against rat β -actin (Abcam, Cambridge, MA, USA) in a 1:5000 dilution and incubated overnight at 4 °C in a rocking platform. The membranes were then washed with TBS-T and a secondary antibody α -rabbit-IgG-HRP (Santa Cruz Biotechnology sc-2004) in a 1:10 000 dilution was added and incubated for 60 min at room temperature on a rocking platform. The membranes were washed in TBS-T followed by TBS and a chemiluminescence reaction was then performed by the incubation with picoLUCENTPLUS (G-Biosciences 786-02, A Geno Technology, Inc., Maryland Heights, MO, USA) for 5 min for visualization of the proteins with a Syngene ChemiGenius² Bioimaging System (Syngene, Frederick, MD, USA) with digital CCD camera (Hamamatsu, Photonics Systems C4742-80-12AG, Bridgewater, NJ, USA). The images were taken in a series of 30 s, 1 min, 3 min, and 5 min exposure times and then edited with GeneSnap (Syngene) software and bands were analyzed by densitometry using a Gene tools software (Syngene) and normalized to β -actin. The nitrocellulose membranes were stripped by incubating for 30 min in stripping buffer (100 mM β -ME, 2% SDS, 62.5 mM Tris–HCl, pH 6.7 in water) at 55 °C, washed in TBS-T, and probed with a rabbit anti-PDX1 antibody (1:1000) (generous gift from Dr Chris Wright, Vanderbilt University, Nashville, TN, USA) followed by α -rabbit-IgG-HRP secondary antibody (1:1000) (Santa Cruz Biotechnology sc-2004) and visualized, as described previously. The same steps were used for the other proteins with some modification. For the detection of *Glut2*, goat anti-GLUT2 antibody (1:200) Santa Cruz Biotechnology, sc-7580) was used with secondary anti-goat IgG-HRP (Santa Cruz Biotechnology sc-2020). To detect GK, a rabbit anti-GK antibody (1:200) (Santa Cruz Biotechnology sc-2020) was used with secondary goat anti-rabbit IgG-HRP (1:10 000) (Santa Cruz Biotechnology, sc-2004).

For the detection of insulin, 16% polyacrylamide separating gels was used. The membranes were probed with a rabbit

anti-insulin antibody (1:200) (Santa Cruz Biotechnology, sc-9168) followed by a goat anti rabbit secondary antibody (1:10 000) (Santa Cruz Biotechnology, sc-2004). Visualization of the protein bands was performed by chemiluminescence and was analyzed as described previously.

Immunohistochemistry and immunofluorescence

Pancreatic tissue was fixed in 10% neutral buffered formalin for 24–36 h and embedded in paraffin. Tissue sections of 5 μ m were cut from the head region of the pancreas and mounted on SuperFrost Plus glass slides (Fisher Scientific, Ottawa, ON, Canada) and incubated at 50 °C for 24–36 h. The tissues were rehydrated through a series of graded alcohols.

Dual staining was performed on pancreas tissue sections to localize the presence of α -cells and β -cells within the islets of Langerhans by a modified avidin biotin-peroxidase method (Hsu *et al.* 1981). All antisera were diluted in Dako-Cytomation antibody diluent (DakoCytomation, Mississauga, ON, Canada). The tissue sections were blocked with 5% horse serum (Cedarlane Laboratories, Hornby, ON, Canada) before application of anti-glucagon (Sigma; 1:2000 dilution). Biotinylated horse anti-mouse (Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody (1:30 dilution). The slides were incubated with ExtrAvidin peroxidase (Sigma), and color was developed in fresh diaminobenzidine (BioGenex, San Ramon, CA, USA). Goat serum (5%, Cedarlane) was then applied followed by incubation with an rabbit anti-insulin antiserum (Santa Cruz; 1:200 dilution). Biotinylated goat anti-rabbit (Vector Laboratories) was used as a secondary antibody (1:30 dilution). Vectastatin ABC-AP Kit and then Vector Red Alkaline Phosphatase Substrate Kit I (Vector) were used to visualize insulin-positive β -cells. The tissue sections were counterstained with Carazzi's hematoxylin, and the slides were dehydrated in a series of graded alcohols. They were counterstained with Carrazi's hematoxylin and mounted under glass coverslip with Eukit (Ruth Wagoner Ent, Newmarket, ON, Canada).

To identify the localization of PDX1 in the islets two methods were used: a modified avidin biotin-peroxidase method (Hsu *et al.* 1981) as described previously, using a goat anti-PDX1 (a gift from Chris Wright, Vanderbilt University; 1:10 000 dilution) as a primary antibody and biotinylated rabbit antigoat (vector BA-500; 1:100) as a secondary antibody. To show co-localization of PDX1 within β -cells, we performed immunofluorescence (IF) using a similar protocol as the one described previously adding an antigen retrieval step. Briefly, the tissues were incubated at 95 °C for 25 min with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) and after cooling at room temperature, we proceeded with the addition of a rabbit anti-insulin antiserum (Santa Cruz; 1:200 dilution) and goat anti-PDX1 (1:10 000 dilution) as primary antibodies. Donkey anti-rabbit (Alexafluor 488; 1:500) and donkey anti-goat (AlexaFluor 555; 1:500) were used respectively as secondary antibodies and incubated for 2 h. Nuclear counterstain DAPI (Sigma-Aldrich, 1:500) was applied before the addition of coverslip with anti-fade mounting solution (Life Technologies). To establish the specificity of the antibodies, controls included substitution of the primary antibody with non-immune serum or omission of the secondary antibody.

Morphometric analysis

Analysis of pancreatic sections was performed using a Carl Zeiss Axioskop transmitted light microscope (Carl Zeiss, Inc., New York, NY, USA) with QImaging MicroPublisher 3.3 Real Time Viewing camera (QImaging, Burnaby, BC, Canada). Digital images were captured at 40 $\times$ or 20 $\times$ objective lens, depending on the size of islet. The image analysis was performed using Northern Eclipse Version 7.0 morphometric analysis software (Empix Imaging, Inc., Mississauga, ON, Canada). For morphometric analysis, three to four animals per group were selected. Subsequently, each pancreas was completely sectioned at 5 μ m and three random sections (separated at least for 30 μ m) from each pancreas were then analyzed. Multiple fields of view were assessed upon the entire pancreas, to ensure all islets were analyzed. For each pancreatic section, the following measurements were determined: total pancreatic area islet area and total area occupied by β -cells and β -cells mass (mg).

Statistical analyses

Data were presented as mean $\pm$ S.E.M. Unpaired two-tailed Student's *t*-test was used to compare control (C) vs low protein LP groups. One-way ANOVA followed by Student–Newman–Keuls procedure was used to compare more than two groups. The differences were considered statistically significant at $P < 0.05$. Statistical analysis was performed using Graph Pad Prism Version 4 (Graph Pad, Inc., San Diego, CA, USA).

Results

Effect of the LP diets on body weight at birth, weaning, and at 130 days old in male rats

To understand the effect of the different diets, body weight measurements were performed on day 1 (birth), day 21 (weaning), and day 130 (d130) of postnatal life on offspring of control and the different LP treatments. At birth, offspring from mothers exposed to the LP diet were pooled, as at that point there was no differences between the LP groups, although their body weight were significantly decreased (4.756 ± 0.118) compared with the control group (6.94 ± 0.249 , $P < 0.001$, Table 3).

At day 21, both LP1 and LP2 (grouped together at this age) offspring exhibited significantly decreased body weight (28.20 ± 0.20) ($P < 0.001$) compared with C group (49.5 ± 0.25). However, no changes were seen in the LP3 (51.04 ± 0.30).

By d130, the body weight was still significantly reduced in LP1 (410 ± 0.14) $P < 0.001$ and LP2 (475.2 ± 0.25) ($P < 0.05$) groups compared with C group (535.6 ± 0.35). In contrast, LP3 offspring (610 ± 0.14) were significantly ($P < 0.05$) increased compared with the control group (Table 3). Collectively, these results suggest that males exposed to LP diets exhibited a reduced body weight at all ages with the exception of the LP3 group, which at d130 were higher to the controls.

Table 3 Body weight at day1, 21, and d130 in male rats.

Body weight	Control	LP1	LP2	LP3
d1	6.94 ± 0.24	4.75 ± 0.25 [†]		
d21	49.5 ± 0.25	28.20 ± 0.20 [†]		51.04 ± 0.32
d130	530 ± 0.25	410 ± 0.3 [†]	470 ± 0.25*	610.02 ± 0.4*

At d1, all LP group were pooled as they were exposed to the same conditions. At d21 (weaning), as both LP1 and LP2 groups received the same diet during pregnancy and lactation, data of the two groups were pooled into one group: LP1/2. Eight to 20 animals were tested per group and measurements were expressed as mean ± S.E.M. in grams and significance was determined using Student's *t*-test. (**P* < 0.05, [†]*P* < 0.001) vs control.

Effect of the LP diet on *PDX1* and *PDX1*-target gene expression on offspring pancreata at d21

By quantitative real-time PCR (qRT-PCR) and western blotting analysis, no significant changes were found in *Pdx1* gene expression and protein abundance in LP groups compared with control at d21 (Fig. 1A and B). In addition, protein abundance of the *PDX1*-target genes, insulin, *Glut2*, and *Gk* was also unaltered at this age (Fig. 2A, B, and C). These results indicate that at weaning, the LP diets did not affect the expression and translation of the genes involved in β -cell differentiation and function.

Effect of the LP diets on *Pdx1* gene expression and cellular localization in offspring pancreata at d130

PDX1 cellular localization in β -cells is very important, as nuclear *PDX1* represents the active and functional fraction of the protein. By postnatal day 130, a significant (*P* < 0.05) decrease in *Pdx1* mRNA and *PDX1* protein abundance was observed in males in LP1 (1.48 ± 0.18), LP2 (1.98 ± 0.182), and LP3 (0.987 ± 0.17) groups compared with C group (4.09 ± 1.2) (Fig. 3A and B). This was associated with a significant decrease in the nuclear localization of *PDX1* protein in LP3 group (*P* < 0.05) compared with C group; however, nuclear localization of *PDX1* in LP1 and LP2 did not show any significant changes as shown in Fig. 4E and G. In contrast, LP3 groups showed a significant increase in the cytoplasmic localization of *PDX1* protein opposed to LP1 and LP2 groups where the cytoplasmic *PDX1* was significantly decreased as compared with C group (Fig. 4F and H). In addition, ICC and IF revealed that *PDX1* nuclear co-localization within β -cells was also decreased in LP3 offspring. These data suggest that restoring protein after birth such as in the LP3 group dramatically affects β -cells function by reducing *Pdx1* activity (Fig. 4B, and C).

Effect of the LP diets on the expression of *PDX1* downstream-target genes in offspring pancreata at d130

By postnatal day 130, insulin gene expression was examined using qRT-PCR and results indicated significant decreases in insulin gene expression in the LP1 (368 ± 18 , *n* = 8) and LP3 groups (213.5 ± 20 , *n* = 7) compared with C group (670 ± 80 , *n* = 9) (*P* < 0.05),

while no significant changes were observed in the LP2 group (632 ± 15 , *n* = 7; Fig. 5A). Insulin protein abundance showed a significant decrease in LP1 (1.55 ± 0.598 , *n* = 3) and LP3 (1.043 ± 0.112 , *n* = 4) groups as compared with C group (2.43 ± 0.31 , *n* = 4) (*P* < 0.05),

Figure 1 (A) *Pdx1* mRNA levels in samples from control (C, white bar), LP1/2 (grey bar), and LP3 (black bar) groups of offspring at d21. One-way ANOVA followed by Newman–Keuls was performed (*n* = 6–9). (B) Representative western blot for *PDX1* protein samples from control (C), LP1/2, and LP3 groups. Graphs represent results four separate western blottings. Protein abundance was calculated relative to the internal control (β -actin). One-way ANOVAs followed by Newman–Keuls was performed (*n* = 4–5).

Figure 2 (A) Representative western blottings for insulin and the internal control, β -actin, from pancreatic tissue of d21 male groups: control (C), LP1/2, and LP3. (B) Representative western blottings of GLUT2 protein levels. (C) Representative western blottings of GK protein expression levels. The graphs summarize the results of four separate membranes. One-way ANOVA followed by Newman–Keuls was performed. Data were expressed as mean $\pm$ S.E.M. relative to the internal control, β -actin ($n=3-4$).

while no significant changes were found in LP2 group (2.68 ± 0.33 , $n=3$) compared with C group (Fig. 5B).

Quantitative RT-PCR of *Glut2* mRNA showed a significant decrease in *Glut2* gene expression in LP3 groups as compared with C group ($P<0.01$), while no change in LP1 and LP2 groups compared with C group was observed (Fig. 6A). Levels of GLUT2 protein abundance showed no significant change ($P>0.05$) in LP1 and LP2 groups compared with C group. However, GLUT2 protein abundance in LP3 group was decreased as compared with C groups ($P<0.05$) (Fig. 6B). No significant changes were observed in GK protein abundance in LP1, LP2, or LP3 groups compared with C groups (Fig. 7). Collectively, this suggests that both LP1 and LP3 offspring were affected by the dietary treatments, as insulin was significantly reduced in both groups. Moreover, LP3 was the group that was further affected at this age as it also had reduced GLUT2 expression.

Effect of LP3 diet on IGT in offspring at d130 of postnatal life

As PDX1 protein nuclear localization exclusively in LP3 group was significantly decreased compared with control groups, we conducted glucose tolerance test for this group to determine any disturbance in glucose homeostasis, which could be related to possible changes in insulin synthesis and secretion. Fasting blood glucose was high in LP3 group (7.9 ± 0.95 mmol/l) and continued to be high after 2 h of glucose load (Fig. 8A). The area under curve was calculated and analyzed using unpaired *t*-test and showed a significant increase ($P<0.01$) in LP3 group as compared with C group (Fig. 8B). These data confirm that the LP3 had the major impairment in glucose tolerance likely due to the reduction in *Pdx1*, insulin, and *Glut2* that increased the failure to respond to a glucose overload.

Morphological changes in LP3 group pancreata compared with C group

To determine whether the impairment of glucose tolerance was attributed to β -cell dysfunction, histological analysis of β -cell area and mass was assessed. Morphometric quantitative analysis demonstrated that the percentage of total β -cell area/total islet area was significantly decreased in LP3 group (46.37 ± 2.656) compared with C group (69.14 ± 6.475) ($P<0.05$) (Fig. 9D and E). The LP3 group also showed a significant ($P<0.05$) decrease in β -cell mass compared with C group (Fig. 9C).

Discussion

Over the past two decades, it has been proposed that nutritional regimens during critical periods of development may have a long-lasting impact on organ structure

Figure 3 (A) *Pdx1* mRNA levels in samples from control (C, white bar), LP1, LP2 (grey bar), and LP3 (black bar) groups of offspring at d130. One-way ANOVAs followed by Newman–Keuls was performed ($n=6-9$) (* $P<0.05$). (B) Representative western blotting for PDX1 protein samples from control (C), LP1, LP2, and LP3 groups. Graphs represent results of four separate western blotting. Protein abundance was calculated relative to the internal control, β -actin. One-way ANOVAs followed by Newman–Keuls was performed ($n=4-5$) (* $P<0.05$).

and function and will affect the pattern of adult disease (Barker *et al.* 1989). Administration of a LP diet during pregnancy in rats has been studied extensively and leads to low birth weight in offspring with an important impairment in pancreatic development within the endocrine compartment (Snoeck *et al.* 1990, Petrik *et al.* 1999, Holemans *et al.* 2003, Heywood *et al.* 2004, Chamson-Reig *et al.* 2006).

In addition, the effect of protein restriction depended on the timing or the critical period when the food insult was given during pregnancy. Restriction in early-,

mid-, or late-gestation had different effects on the placenta and fetal weight (Villar & Belizan 1982, Painter *et al.* 2005). Moreover, increasing the amount of protein in the diet at birth/or weaning in these LP offspring led to impaired glucose homeostasis and beta cell dysfunction at d130 in a gender-specific manner (Chamson-Reig *et al.* 2009, Tang *et al.* 2013). Sexual differences were also observed in humans, as males previously exposed to protein restriction in utero develop obesity (Anguita *et al.* 1993), hypercholesterolemia, and triacylglycerolemia (Desai *et al.* 1996).

In this study, we examined the effects of a LP diet given during pregnancy, lactation, and all life in male offspring to determine the critical window that affected pancreatic and β -cell development leading to impaired glucose

Figure 4 (A and B) Nuclear localization of PDX1 in groups on control (C) and low-protein (LP) diets at d130. (B) Nuclear co-localization of PDX1 in control d130 male rats, by immunohistochemistry (IHC) and IF. (C) Cytoplasmic localization of PDX1 in the islets of LP3 d130 rats. (D) PDX1 co-localization with β -cells in LP3 at d130. Arrows show nuclear and cytoplasmic localization. Magnification bar = 100 μ m. Nuclear and cytoplasmic fractions of pancreatic samples were separated and subjected to western blotting analysis using antibodies against PDX1, Histone H3 and tubulin. The two latter proteins were used as internal control markers of the nuclear and cytoplasmic fractions respectively. (E and F) Representative western blotting analysis of nuclear and cytoplasmic fractions. (G and H) Graphs represent data from four separate membranes. One-way ANOVAs followed by Newman–Keuls was performed. Scale bars are mean $\pm$ S.E.M. ($n=4-5$) (* $P<0.05$).

Figure 5 (A) Gene expression and (B) protein abundance of insulin from male groups at d130 of offspring age. One-way ANOVAs followed by Newman–Keuls was performed. Bars are mean $\pm$ S.E.M. ($n=3-5$) (* $P<0.05$).

homeostasis later in life. We showed that all offspring from mothers fed with the different combinations of LP diets had a significantly lower body weight at birth ($P<0.001$) than the control groups. Moreover, offspring from LP1 and LP2 groups (fed LP throughout lactation) also had reduced body weight at weaning. This lower body weight was maintained in the LP1 group until d130. Recently, we showed that the LP1 diet improved β -cell function in males (Tang *et al.* 2013) at d130. A previous study demonstrated that 20-week-old male offspring with the same diet LP1 were more insulin sensitive than offspring kept on a normal diet following birth (Sugden & Holness 2002), although no differences in insulin secretion and action were reported with females at the same age (Sugden & Holness 2002). In contrast, we now indicate that the LP3 offspring had a higher body weight than the C and the other LP groups at 130 days of age. This increase in body weight seen in the LP3 group is known as catch-up growth and has been directly related to obesity and metabolic syndrome in adult life (Eriksson *et al.* 2002, Barker *et al.* 2005,

Ozanne & Hales 2005, Bieswal *et al.* 2006, Ong 2006, Pinheiro *et al.* 2009). Prevention of postnatal catch-up growth normalizes both glucose tolerance and obesity (Jimenez-Chillaron *et al.* 2006). Moreover, postnatal catch-up growth results in an accelerated cellular aging by islet telomere shortening (Tarry-Adkins *et al.* 2009).

It is known that, during normal fetal development, *Pdx1* is involved in both pancreas formation and β -cell differentiation. Furthermore, in mature β -cells, *Pdx1* transactivates insulin and other β -cell-related genes such as *Glut2* and *Gk*. In this study, we demonstrated that at d21 there were no significant changes in the expression of *Pdx1*, insulin, *Glut2* or *Gk* genes in any of the treatment groups. However, at 130 days *Pdx1* gene expression and PDX1 protein abundance were reduced in all LP groups. This reduction of *Pdx1* expression either genetically or acquired has been suggested to cause type 2 diabetes and β -cell dysfunction (Stoffers *et al.* 1997, Kulkarni *et al.* 2004, Brissova *et al.* 2005). PDX1 cellular localization in β -cells is very important, as nuclear PDX1 represents the active and functional fraction of the protein and PDX1 nuclear translocation

Figure 6 Gene expression and protein abundance of GLUT2 in male groups at d130 of age. (A) Gene expression $n=8-9$ per group (** $P<0.01$). (B) Representative western blot assessing GLUT2 expression. Quantification of band intensities were examined and analyzed. One-way ANOVA followed by Student–Newman–Keuls was performed. Scale bars are mean $\pm$ S.E.M. (* $P<0.05$).

Figure 7 Western blotting analysis for GK protein from male offspring at d130. Graph represents results obtained from four different western blots. Quantification of bands and one-way ANOVA test were performed. Scale bars are mean $\pm$ S.E.M. ($n=3-4$).

from the cytosol enhances insulin gene transcription (Elrick & Docherty 2001) while PDX1 shuttling to the cytosol impairs insulin secretion (Poitout *et al.* 2006). In this study we found that PDX1 cellular localization was altered with the diets. Whereas *Pdx1* gene expression was decreased in all LP groups, we demonstrated that PDX1 nuclear localization was significantly reduced in the LP3 group compared with the other groups.

Moreover, as stated above, PDX1 regulates genes involved in β -cell identity, function, and downstream target genes related in glucose homeostasis (i.e., insulin, *Gk*, and *Glut2*). It is well established that insulin expression and its transcriptional regulation involve *Pdx1* (Le Lay *et al.* 2004) as it interacts with other proteins at the insulin promoter such as $\beta 2$ /Neuro D1, *E47/Pan1*, p300 co-activator and Bridge-1 (Thomas *et al.* 1999, Ohneda *et al.* 2000, Qiu *et al.* 2002) and *Glut2* promoter (Waeber *et al.* 1996, Lottmann *et al.* 2001). Inactivation of *Pdx1* in mice leads to loss of islet cell phenotype and a reduction in insulin and *Glut2* expression that leads to diabetes (Ahlgren *et al.* 1998). In our present study, insulin gene expression and protein abundance was reduced in LP1 and LP3 at 130 days. In addition to insulin, *Glut2* was only reduced in LP3 at 130 days of age that concomitantly showed glucose intolerance by IGTT. This is of great importance considering that a reduction in *Glut2* expression in β -cell has been shown to strongly impair glucose signaling of insulin secretion and synthesis (Guillam *et al.* 2000). In this study, our data suggest that the reduction in *Glut2* may alter glucose transport into the β -cell, leading to less phosphorylation of PDX1 and less translocation of PDX1 to the nucleus affecting PDX1 activation and consequently insulin synthesis and

secretion. On the other hand, the decreased activation of PDX1 (nuclear localization) reduces *Glut2* and other gene transactivation. However, the question remains whether decreased expression of *Glut2* is due to decreased PDX1 nuclear localization or that PDX1 nuclear localization occurs as a result of decreased *Glut2* expression. Moreover, the attenuation of PDX1 expression and its effects in the other downstream genes may partially explain the mechanism of β -cell dysfunction. *Gk* (glucose sensor in β -cells), another target gene for PDX1, was not altered in any of our LP-treated groups at 130 days. Finally, the decreased *Pdx1* gene expression in LP3 resulted in changes in pancreas morphometry with reduced islet cell area, β -cell mass, β -cell area, and glucose intolerance. Given that *Pdx1* expression is tightly correlated with beta cell number (Stoffers 2011), a loss of PDX1 expression in our LP3 offspring would suggest that the decrease in islet size observed is likely due to a decrease in β -cell number.

In summary, we presented and confirmed evidence that the postnatal period plays a major role in determination of the consequences of LP diet on fetal growth and on the expression of transcription factors involved in the

Figure 8 Intraperitoneal glucose tolerance test. (A) Intraperitoneal glucose tolerance test (IGTT) was performed on male offspring of LP3 (closed circles) and control (C) (open circles) groups. (B) Area under curve (AUC) was calculated and analyzed using unpaired *t*-test. Data are presented as mean $\pm$ S.E.M. ($n=3$). A significant increase (** $P<0.01$) in area under curve in LP3 group (black bar) compared with control (C) group (white bar).

Figure 9 Morphological analysis from pancreas exposed to the four dietary treatments. β -cell mass, islet area and total % of β -cell area, in LP diet-treated group were analyzed. (* $P < 0.05$). Control (A) and LP3 (B) micrographs of pancreatic islets are illustrated, where brown represents glucagon-producing cells (arrow, alpha cells in periphery) and pink/red indicates insulin-producing cells (arrow, beta cells in core). Morphological analysis also showed a significant statistical difference between LP3 (black bars) and control (white bars) groups in β -cell mass and islet cell area. Data are presented as means $\pm$ S.E.M. as a percentage of endocrine area, derived from three or four animals per group. Magnification bar = 100 μ m. β -cell area mass, islet cell area, and β -cell area were significantly decreased (* $P < 0.05$) in LP3 group compared with the control (C) group (C, D, and E respectively).

maintenance and function of beta cells (*Pdx1* and its downstream genes). Protein restriction throughout life LP1 resulted in offspring with lower body weight and reduced PDX1 and targets genes with unaltered β -cell function (Tang *et al.* 2013). Nevertheless, when protein was reduced during gestation LP3 only, offspring had a higher body weight compared with the C group but presented a significant reduction of *Pdx1* and downstream genes with dramatic consequences in glucose homeostasis in early adulthood. These studies shed some light on the further understanding of the etiology and progression of T2DM due to dietary deficiencies in pregnancy and may serve for the future development of strategies for prevention and treatment of this disease.

Declaration of interest

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

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