Novel lean type 2 diabetic rat model using gestational low-protein programming

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OBSTETRICS

Novel lean type 2 diabetic rat model using gestational low-protein programming

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BACKGROUND: Type 2 diabetes (T2D) in lean individuals is not well studied and up to 26% of diabetes occurs in these individuals. Although the cause is not well understood, it has been primarily attributed to nutritional issues during early development.

OBJECTIVE: Our objective was to develop a lean T2D model using gestational low-protein (LP) programming.

STUDY DESIGN: Pregnant rats were fed control (20% protein) or isocaloric LP (6%) diet from gestational day 4 until delivery. Standard diet was given to dams after delivery and to pups after weaning. Glucose tolerance test was done at 2, 4, and 6 months of age. Magnetic resonance imaging of body fat for females was done at 4 months. Rats were sacrificed at 4 and 8 months of age and their perigonadal, perirenal, inguinal, and brown fat were weighed and expressed relative to their body weight. Euglycemic-hyperinsulinemic clamp was done around 6 months of age.

RESULTS: Male and female offspring exposed to a LP diet during gestation developed glucose intolerance and insulin resistance (IR). Further, glucose intolerance progressed with increasing age and occurred earlier and was more severe in females when compared to males. Euglycemic-hyperinsulinemic clamp showed whole body IR in both sexes, with females demonstrating increased IR compared to males. LP females showed a 4.5-fold increase in IR while males showed a 2.5-fold increase when compared to their respective controls. Data from magnetic resonance imaging on female offspring showed no difference in the subcutaneous, inguinal, and visceral fat content. We were able to validate this observation by sacrificing the rats at 4 and 8 months and measuring total body fat content. This showed no differences in body fat content between control and LP offspring in either males or females. Additionally, diabetic rats had a similar body mass index to that of the controls.

CONCLUSION: LP gestational programming produces a progressively worsening T2D model in rats with a lean phenotype without obesity.

Key words: gestational programming, glucose intolerance, insulin resistance, lean diabetes, type 2 diabetes

Introduction

Diabetes has reached epidemic proportions with 1 in 9 affected in the United States, with projected estimates as high as 1 in 3 by 2050 (Centers for Disease Control and Prevention).[1,2] Type 2 diabetes (T2D) has been historically attributed to lifestyle and genetics, however recent studies indicate that an adverse uterine environment is associated with the development of T2D later in life.[3] Although various aspects of T2D are well studied, the pathogenesis, progression, and mechanisms of the developmental origins of T2D are poorly understood.

A recent study on minority US populations showed that 13% of diabetic patients are of normal weight[4,5] with a 5-fold higher incidence in people of Asian origin.[4] Studies from India[5] and the Caribbean islands[6,7] also report the presence of a lean diabetic population, with 1.6-26% and 5% prevalence, respectively. This has been primarily attributed to possible in utero nutritional issues.[8] As obesity is not required for the development of T2D,[5,9,10] in utero nutritional issues could be a causative factor for developing insulin resistance (IR) and glucose intolerance that predispose to T2D. T2D in these lean individuals is not well understood.

Various genetic, diet, and chemically induced diabetic rodent models are utilized to study T2D.[11-13] However, these models are accompanied by obesity and do not accurately mimic the lean T2D phenotype. Therefore, there is a need for a lean diabetic animal model to investigate various aspects of lean phenotype and to study the possible mechanisms of origin of lean T2D. Previous work has demonstrated that a gestational low-protein (LP) diet increases the susceptibility of offspring to the development of metabolic diseases during adulthood.[14,15] Our objective was to characterize a gestational protein-restricted rat model that results in IR and glucose intolerance in offspring during adulthood, but that is not accompanied by obesity.

Materials and Methods

Animals

Timed pregnant (day 4) Wistar rats (~200 g) were procured from Harlan Laboratories (Indianapolis, IN) and were housed in a temperature-controlled room (~23°C) with a 14:10-hour light/dark cycle with unlimited access to food and water. Pregnant rats were fed control (20% protein Teklad diet; Harlan Laboratories) or isocaloric LP (6%) diet from gestational day 4 until delivery. Normal diet was given to mothers after delivery and to pups after weaning until the end of the study. The number of pups in the control and LP litters were culled to 8 pups per mother (pups with weights at extremes were euthanized) to ensure equal nutrient access for all offspring. Body weights and length of pups were recorded on a regular basis to calculate

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body mass index (BMI). Glucose tolerance test (GTT) and euglycemic-hyperinsulinemic clamp were performed in females at diestrus phase to minimize the influence of estrogens. Rats were sacrificed at 4 and 8 months to collect gonadal, perirenal, and inguinal fat pads and their weights were recorded. All experimental procedures were performed with approval by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Glucose tolerance test

GTT was performed on male and female offspring at 2, 4, and 6 months of age to identify the progression of glucose intolerance. Rats were fasted for 6 hours and were administered glucose (1 g/kg body weight intraperitoneally). Blood glucose levels were measured using ACCU-CHEK Nano (Roche, Indianapolis, IN) at 0, 15, 30, 60, 120, and 180 minutes via saphenous puncture. Blood samples were collected in heparin-coated tubes for measuring fasting plasma insulin levels.

Insulin levels

Plasma insulin levels were measured using a rat insulin enzyme-linked immunosorbent assay kit (Mercodia), following the manufacturer's instruction as reported earlier.[16]

Homeostatic model assessment

Homeostatic model assessment (HOMA)-IR and HOMA-insulin sensitivity (IS) were calculated to assess IR and IS of control and LP rats using the following equations[17]:

$$\text{HOMA-IR} = [\text{fasting glucose (mg/dL)} \times \text{fasting insulin (mU/L)}] \div 405$$

$$\text{HOMA-IS} = 10,000 \div [\text{fasting glucose (mg/dL)} \times \text{fasting insulin (mU/L)}]$$

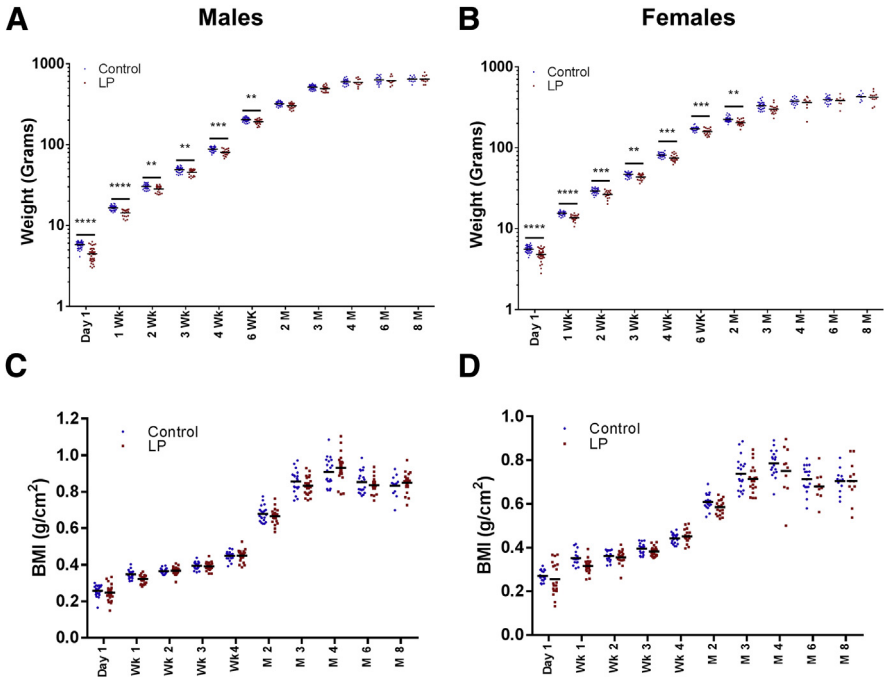
Magnetic resonance imaging

Magnetic resonance imaging (MRI) of body fat for females was done at 4 months of age. Rats were anesthetized, placed in the animal holder (BioSpin; Bruker, Billerica, MA), and imaged using 9.4-T Avance BioSpec Spectrometer/AVIII (Bruker) with a 21-cm horizontal bore and a 72-mm resonator. To delineate the distribution of adipose tissue in the rats, a Dixon fat imaging sequence was used: repetition time = 800 milliseconds; echo time = 12 milliseconds; slice thickness = 2 mm; number of slices = 24; field of view = 7 cm; and 256 × 256 matrix. Saturation slabs were also incorporated. For consistency between animals, the first slice was aligned directly below the kidneys. After acquisition, in house MatLab code was utilized to separate the water and fat images, and the fat images were then quantified. Images (256 × 256 × 24 voxels, 70 × 70 × 48 mm) that were scanned for fat were imported into an imaging program (ROIeditor; www.mristudio.org). All fat was segmented out by setting a threshold over background, which was about 10% of the maximum signal. Visceral, inguinal, and subcutaneous fat was successively segmented out manually, slice by slice, by first inclosing all visceral fat and then segmenting between inguinal and subcutaneous fat. The volumes of separate fat were then generated by subtraction. Total fat volume was computed from the total number of voxels above the threshold in each type. We were constrained by the size of the bore in our MRI device, and therefore unable to scan the males at 4 months of age due to their larger size relative to females.

Euglycemic-hyperinsulinemic clamp

Euglycemic-hyperinsulinemic clamp was done at 6 and 7 months of age for females and males, respectively. Rats were fasted for 6 hours, and were restrained in an appropriately sized restrainer (Kent Scientific Corp, Torrington, CT). Tail vein catheter was inserted using PE-10 tubing at the proximal end of the tail. The catheter

FIGURE 1
Offspring weight and their body mass index (BMI)



Body weights of control and low-protein (LP)-programmed **A**, male and **B**, female offspring and their respective **C** and **D**, BMI from birth to 8 months of age. $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$ (n = 12-33 in males and 12-31 in females for weights and n = 12-20 in males and 10-20 in females for BMI).

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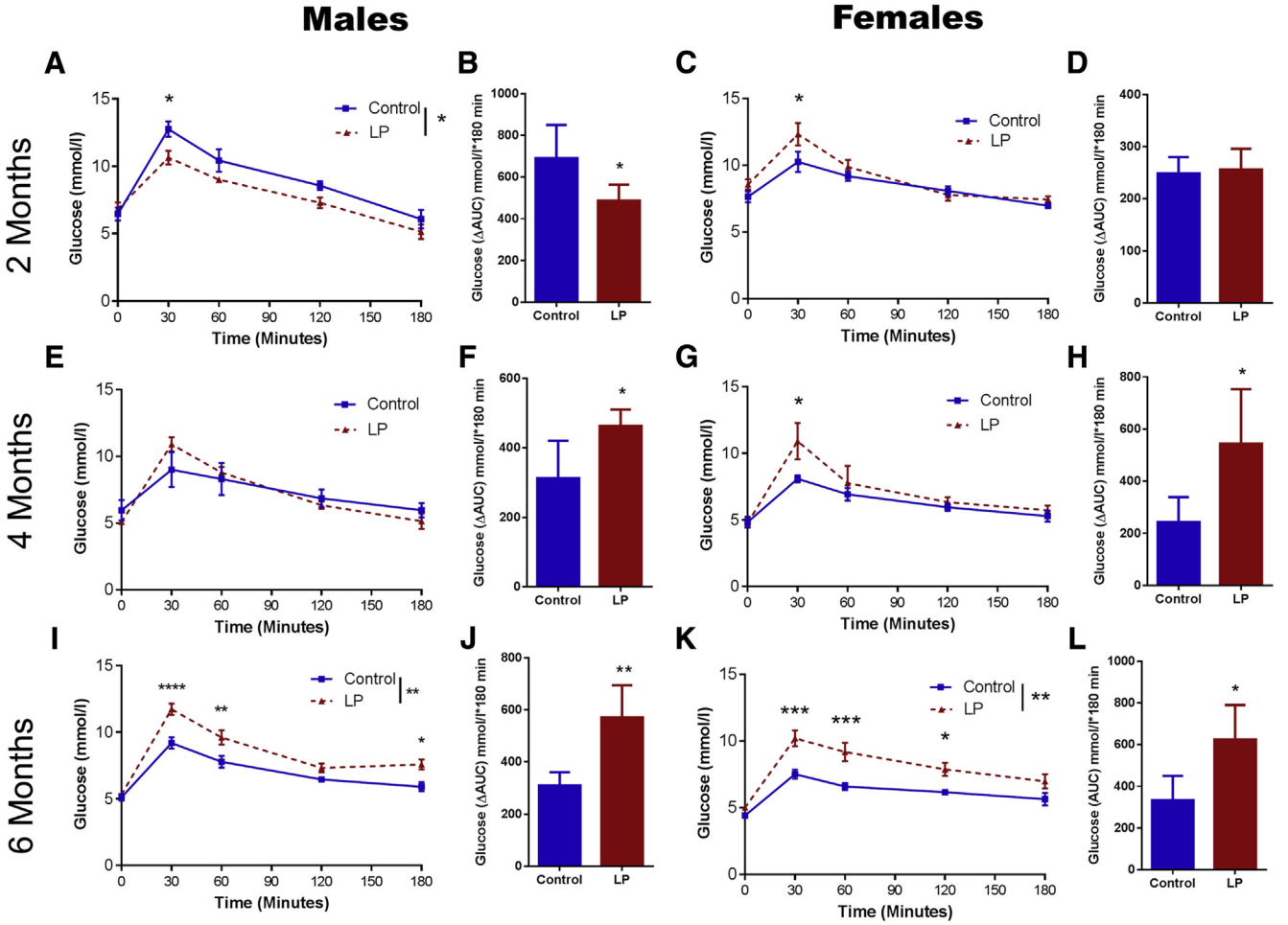
FIGURE 2
Glucose tolerance of males and females at 2, 4, and 6 months



A, **C**, **E**, **G**, **I**, and **K**, Data from glucose tolerance test along with its corresponding Δ area under curve (AUC) of **B**, **F**, and **J**, male and **D**, **H**, and **L**, female low-protein (LP) offspring at 2, 4, and 6 months. *$P < .05$, **$P < .01$, ***$P < .001$, and ****$P < .0001$ (n = 5-6).

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was connected to a "Y" connector that was connected to syringes filled with 50% glucose solution and insulin (HumulinR; Eli Lilly and Co). The syringes were mounted onto a syringe pump (Harvard Apparatus, Holliston, MA). Insulin was constantly infused at a rate of 4 mU/(kg*min) (flow rate of 200 and 150 μL/h for males and females, respectively). Blood samples for measuring glucose were obtained every 10-15 minutes from the tail tip. Glucose infusion rates were adjusted by trial and error until a steady state of blood glucose concentration was reached. Three consecutive readings within a range of ~1 mmol/L blood glucose concentrations were considered to have reached a steady state. Glucose levels were clamped between 5-6 mmol/L.

Statistical analyses

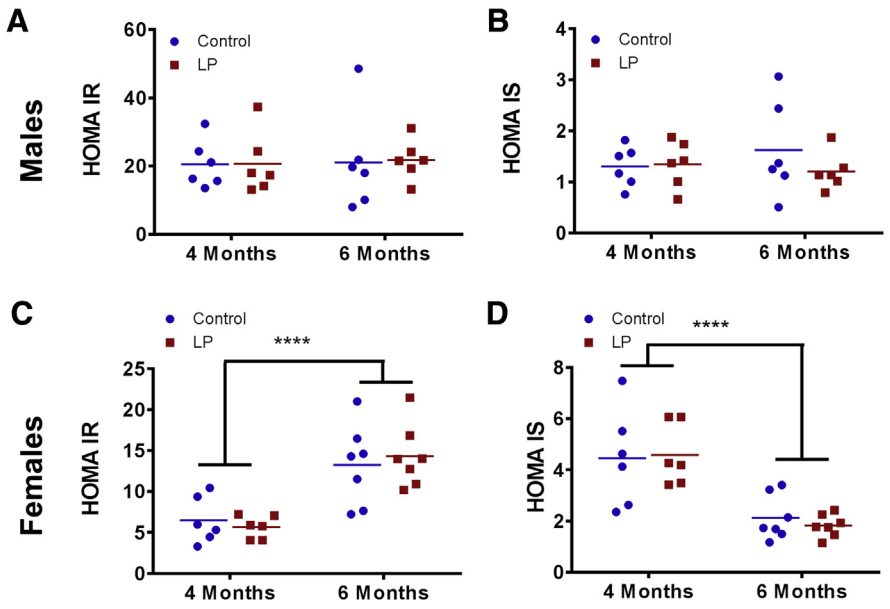
Statistical analyses were performed using GraphPad Prism (La Jolla, CA). Data are presented as scatter plot with mean or mean ± SEM. Comparison between 2 groups was performed using unpaired Student *t* test. Comparisons between multiple groups were done with 2-way analysis of variance followed by Bonferroni test. Differences were considered significant when $P < .05$.

Results

LP offspring exhibited catch-up growth but had similar BMI

Gestational LP programed offspring were smaller at birth when compared to the controls. Male LP pups weighed 4.5 ± 0.15 g and were smaller ($P < .0001$, n = 12-33) when compared to the male control pups (5.8 ± 0.08 g). Female control pups were 5.7 ± 0.07 g whereas females LP pups were significantly smaller ($P < .0001$, n = 10-31) and weighed 4.9 ± 0.14 g. However, both male and female offspring in LP group showed catch-up growth and their weights were similar to the control group

FIGURE 3
Homeostatic model assessment (HOMA) insulin resistance (IR) and insulin sensitivity (IS)



HOMA-IR and HOMA-IS of **A** and **B**, male and **C** and **D**, female offspring at 4 and 6 months of age. ****$P < .0001$ (n = 5-7).

LP, low protein.

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by around 3 months (Figure 1, A and B). Interestingly, both controls and LP-programmed offspring showed similar BMI throughout the study period (n = 12-20 in males and 10-20 in females) (Figure 1, C and D).

LP offspring show progressively worsening glucose intolerance

GTTs were performed on control and LP-programmed offspring at 2, 4, and 6 months to identify the progression of glucose intolerance (n = 5-6 in each group). The blood glucose levels peaked at 30 minutes after bolus intraperitoneal glucose administration and returned to basal levels by 180 minutes. Total glucose response was calculated as the Δ glycemia area under the curve (AUC) using the trapezoidal method as reported earlier.[16] In male offspring, the peak glucose levels at 30 minutes were lower in LP (LP 10.6 ± 0.6 mmol/L vs control 12.8 ± 0.5 mmol/L, $P < .05$) at 2 months (Figure 2, A), similar (LP 10.9 ± 0.5 mmol/L vs control 9.0 ± 1.3 mmol/L) at 4 months (Figure 2, E), and significantly higher (LP 11.7 ± 0.4 mmol/L vs control 9.2 ± 0.4 mmol/L) at 6 months (Figure 2, I) when compared to their corresponding controls. The Δ AUC shows that male LP offspring at 2 months were significantly ($P = .0328$) more glucose tolerant (Δ AUC glycemia, 486.5 ± 35, mmol/L *180 minutes) when compared to their controls (Δ AUC glycemia, 690.6 ± 71, mmol/L *180 minutes) (Figure 2, B). However by 4 months these LP males were more glucose intolerant ($P = .0441$, glycemia, 462.7 ± 24, mmol/L *180 minutes) than controls (Δ AUC glycemia, 312.2 ± 54 mmol/L *180 minutes) (Figure 2, F), and this glucose intolerance further worsened at 6 months ($P = .0056$, Δ AUC glycemia, 571.3 ± 55 mmol/L *180 minutes in LP and 309.1 ± 26 mmol/L *180 minutes in controls) (Figure 2, J). In females, peak glucose levels at 30 minutes after bolus administration were significantly higher in LP at 2 months (LP 12.3 ± 0.8 mmol/L vs control 10.3 ± 0.8 mmol/L, $P < .05$) (Figure 2, C), 4 months (LP 10.9 ± 1.4 mmol/L vs control 8.1 ± 0.3 mmol/L, $P < .05$) (Figure 2, G), and 6 months (LP 10.2 ± 0.6 mmol/L vs control 7.5 ± 0.3 mmol/L, $P < .001$) (Figure 2, K) when compared to their corresponding controls. However, Δ AUC glycemia values showed no differences in their glucose tolerance at 2 months ($P = .7792$, 256.1 ± 17.96, mmol/L *180 minutes in LP and 248.9 ± 16 mmol/L *180 minutes in controls) (Figure 2, D), but showed glucose intolerance by 4 months ($P = .0408$, Δ AUC glycemia, 543.0 ± 105 mmol/L *180 minutes in LP and 243.7 ± 48 mmol/L *180 minutes in controls) (Figure 2, H) and this condition was significantly worsened by 6 months ($P = .0174$, Δ AUC glycemia, 624.4 ± 83 mmol/L *180 minutes in LP and 332.7 ± 53 mmol/L *180 minutes in controls) (Figure 2, L). LP-programmed females appear to have an earlier onset and faster progression of glucose intolerance when compared to the males.

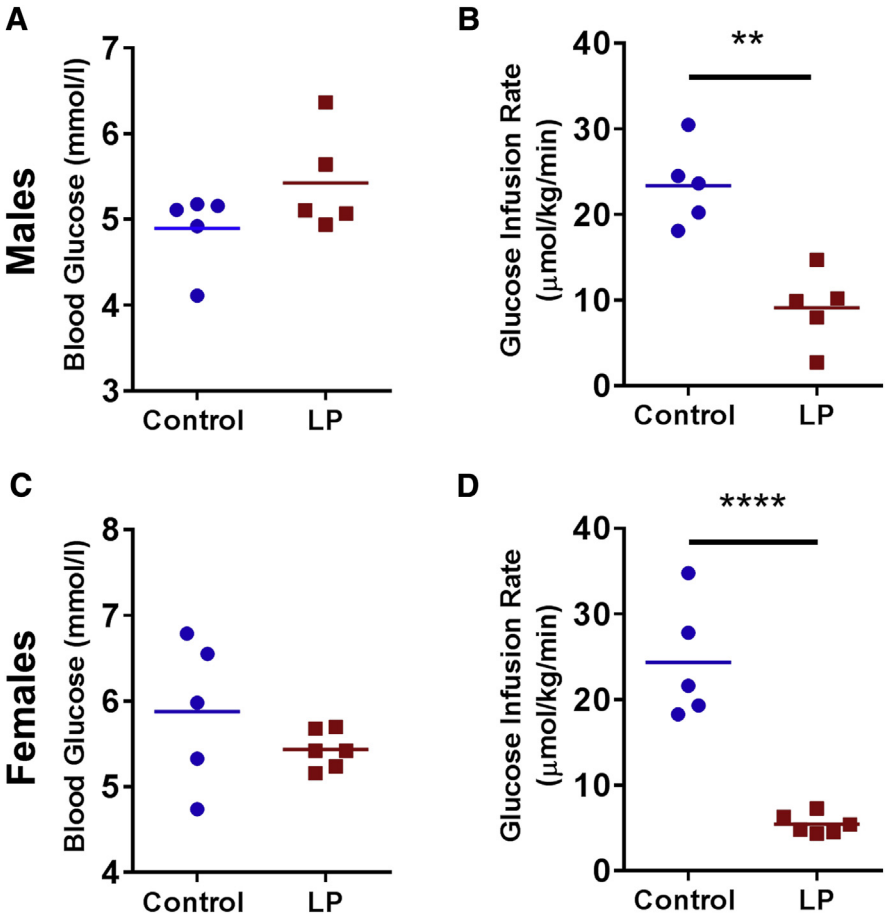
Fasting insulin concentrations did not show any difference between LP and control offspring

Fasting insulin concentrations in plasma were measured in LP and control offspring in both sexes at 4 and 6 months. Insulin levels did not differ at 4 months (control, 479 ± 95 pmol/L; LP, 415 ± 65 pmol/L) or 6 months (control, 468 ± 99 pmol/L; LP, 552 ± 56 pmol/L) in males. Fasting insulin levels in females also did not differ between controls and LP at 4 months (control, 187 ± 35 pmol/L; LP, 163 ± 18 pmol/L) and 6 months (control, 393 ± 55 pmol/L; LP, 383 ± 35 pmol/L). However, insulin levels in the female offspring were increased in both controls and LP at 6 months when compared to 4 months ($P < .0001$).

Homeostatic model assessment

HOMA IR and IS values for both males (Figure 3, A and B) and females (Figure 3, C and D) were similar between the control and LP offspring at 4 and 6 months of age (n = 5-7 in each group). Interestingly, 6-month-old females of both control and LP groups showed more IR ($P < .0001$) and less IS ($P < .0001$) when compared to 4-month-old

FIGURE 4
Euglycemic-hyperinsulinemic clamp for male and female offspring



Clamped blood glucose concentrations along with respective glucose infusion rates to maintain homeostasis for **A** and **B**, male and **C** and **D**, female low-protein (LP)-programmed offspring in comparison with their respective controls. $^{**}P < .01$, $^{****}P < .0001$ (n = 5-6).

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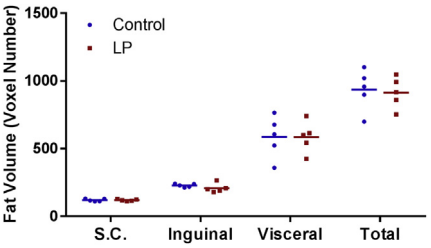
FIGURE 5
Magnetic resonance imaging (MRI) of body fat in female offspring



MRI analysis showing no changes in subcutaneous (S.C.), inguinal, visceral, and total fat content of control and low-protein (LP) offspring in 4-month-old females (n = 5).

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females indicating females show a dramatic increase in IR with age.

LP offspring were IR in euglycemic-hyperinsulinemic clamp

Euglycemic-hyperinsulinemic clamp is the gold standard for measuring IR. Glucose concentrations during the clamp were maintained between 4.9 ± 0.2 and 5.9 ± 0.4 mmol/L during the steady state (Figure 4, A and C). The glucose infusion rate was nearly 2.5-fold lower in LP males when compared to their controls (9.1 µmol/[kg*min] in LP vs 23.4 µmol/[kg*min] in control; $P < .01$, n = 5) (Figure 4, B). In LP females, the glucose infusion rate was 4.5-fold lower than their controls (5.4 µmol/[kg*min] in LP vs 24.4 µmol/[kg*min] in control; $P < .0001$, n = 5-6) (Figure 4, D). Thus, our data show that both male and female LP offspring have significantly greater IR when compared to their respective controls (Figure 4).

MRI shows no change in the fat distribution between LP and control female offspring

MRI was performed to identify if there were differences in the fat content and distribution between control and LP offspring (n = 5). Three-dimensional MRI analyses showed no overall differences between controls and LP offspring and no significant changes in subcutaneous, visceral, and inguinal fat distributions (Figure 5). Representative video images of the MRI scan and 3-dimensional analyses are shown in supplementary Video Files 1-3.

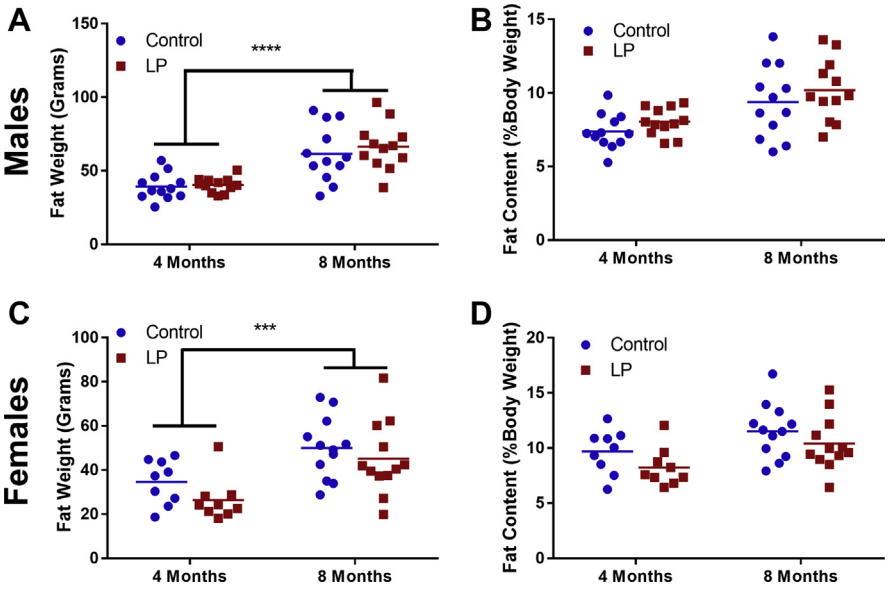
Fat depot weights showed no differences

LP-programmed and control rats from both sexes were sacrificed at 4 and 8 months of age and their fat depots (perigonadal, perirenal, inguinal, and brown adipose tissue) were weighed (n = 12 in males and n = 10-12 in females). No differences were observed in any of the fat depots between control and LP offspring in either males or females (data not shown). Total fat depot weights were similar among control and LP offspring at 4 and 8 months in both sexes (Figure 6, A and C) but they showed a significant ($P < .0001$ in males and $P < .001$ in females) increase in the fat depot weights at 8 months when compared to 4 months. Normalization of the fat depot weights with their respective body weights also showed no differences in the percent fat contents (Figure 6, B and D) between controls and LP. These data indicate that LP offspring of both sexes were not obese and had adipose deposition comparable to their respective controls.

Comment

Diabetes is a metabolic disease caused by defects in insulin secretion, insulin

FIGURE 6
Weights of fat pads from - and 8-month-old male and female offspring



Weights and % body weights of fat pads collected from 4- and 8-month-old **A** and **B**, male and **C** and **D**, female low-protein (LP)-programmed offspring along with their respective controls. ***$P < .001$, ****$P < .0001$ (n = 10-12).

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action, or a combination of both resulting in hyperglycemia.[18] T2D is often associated with obesity and most research investigating T2D is performed using obese animal models. However, various clinical observations have shown the presence of T2D in lean or normal BMI individuals.[9,19-24] This atypical diabetic phenotype does not fit into the traditional classification of diabetes and is known by various names such as Jamaica type diabetes, metabolically obese normal-weight diabetes, malnutrition-related diabetes mellitus, phasic insulin-dependent diabetes, tropical diabetes, mixed-onset type diabetes, J-type diabetes, Z-type diabetes, M-type or type-3 diabetes, and ketosis-resistant growth-onset type diabetes.[6,7,9,25-29] Although the existence of lean diabetes has been observed for decades, the etiology and pathophysiology of disease in this lean population is poorly understood.

One of the common factors that connects various types of atypical lean diabetic phenotypes is the role of early nutrition.[14] Recent studies show that an adverse in utero environment is often associated with the development of T2D in offspring.[3] A gestational LP diet programs offspring to become susceptible to the development of metabolic diseases during adulthood.[14,15] It is important to develop a rodent model that reflects such an adverse gestational environment with fetal nutritional deficiency to investigate the etiology and mechanisms underlying lean T2D. Published literature describing the lean T2D phenotype is often reported in developing nations among people with poor socioeconomic status, strongly suggesting the possibility of developmental programming during early development.[7,14]

We have shown for the first time that gestational LP programming produces a progressively worsening model of T2D in rats that is characterized by a lean phenotype and is without altered adipose tissue amount and distribution compared to controls. Our model shows that both males and females develop glucose intolerance and IR without obesity. Our earlier studies in this model show that males and females have distinct mechanisms leading to glucose intolerance and IR.[30] Our recent study in gastrocnemius muscles shows that male LP offspring are glucose intolerant and IR with increased basal expression and activation of insulin receptor along with compromised Glut4 membrane transport due to defective phosphorylations of IRS-1 and AS160.[16] Defective Glut4 localization in the gastrocnemius muscles has also been reported in lean T2D patients.[31]

LP-programmed females are glucose intolerant with intact Glut4 translocation mechanism. However, they have impaired phosphorylations in GSK3 pathway indicating sex differences in the mechanisms underlying the disease.[30] Fasting insulin levels did not show any changes and consequently HOMA-IR and IS also did not show any changes between the controls and LP-programmed offspring. However, GTT and euglycemic-hyperinsulinemic clamp showed glucose intolerance and IR in both sexes with females displaying a more aggressive phenotype when compared to males. Clinical studies have shown the presence of lean diabetes in both males and females[5,32] with around twice the rate of incidence in females in one cohort.[32] However, large-scale multicentric clinical studies need to be undertaken to validate these findings.

The only other reported lean T2D model is called Goto-Kakizaki rats.[33,34] This model was developed by the repetitive breeding of Wistar rats with the poorest glucose tolerance[33,35] and is characterized by glucose intolerance and flawed glucose-induced insulin secretion with defective glucose metabolism.[34] Although this model develops T2D, it is a reflection of the genetic makeup of the rats[34] rather than developmental programming. Various existing obese rodent T2D models have contributed greatly to our understanding of the disease, however, they are inadequate to study the lean and pre-obese phenotypes associated with T2D.

We have characterized a novel lean T2D rat model that reflects various aspects of lean T2D. Lean T2D is poorly understood partly due the absence of a suitable animal model. This animal model opens up new vistas for investigators to study lean T2D

and the role of nutrition during early development and its metabolic implications in later life. We believe that our unique lean gestational LP programming T2D model will be a useful tool to further understand the etiology, progression, and severity of lean T2D and to investigate the underlying mechanisms, which may help us to devise appropriate treatment strategies. ■

Acknowledgment

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