

Exposure of the Pregnant Rat to Low Protein Diet Causes Impaired Glucose Homeostasis in the Young Adult Offspring by Different Mechanisms in Males and Females

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The understanding of the mechanisms by which gender dimorphisms are involved in the modulation of insulin sensitivity and glucose tolerance can be crucial to unravel the development of type 2 diabetes. Rats treated with a low protein diet (LP, 8% protein content) during pregnancy and lactation have a reduced β -cell mass at birth and a reduced insulin secretion at weaning. In this study we examined the effect of LP diet on glucose homeostasis from birth to adulthood when offspring previously exposed to LP were subsequently switched to control diet (C, 20% protein content) at weaning. The LP group had a reduced body weight after weaning compared to the C-fed rats, although their food intake was not significantly different. Furthermore, LP males had a significant increase in visceral adiposity relative to their body weight ($P < 0.05$). Intraperitoneal glucose tolerance test (IGTT) showed that glucose clearance was unchanged until 130 days of age when LP-fed females showed elevated blood glucose compared to C, despite similar plasma insulin levels. Females also demonstrated a significant reduction in mean pancreatic islet number, individual islet size and beta cell mass. However, no differences in IGTT or islet morphometry were observed in LP males, although basal insulin levels were two-fold higher. Akt phosphorylation in response to insulin was reduced in adipose and skeletal muscle of adult rats following

exposure to LP diet in early life when compared to control-fed animals, but this was only apparent in males. Plasma testosterone levels were also reduced in males at 130 days age. These data suggest that the development of impaired glucose homeostasis in offspring of LP-fed rats is likely to occur by different mechanisms in males and females. *Exp Biol Med* 234:1425–1436, 2009

Key words: insulin signaling; glucose homeostasis; gender differences; nutrition

Introduction

Epidemiological studies in humans have shown that dietary restriction during pregnancy retards birth weight (1), leading to permanent changes in organ development (2) and, consequently, playing an important role in the predisposition to type 2 diabetes (3, 4). The risk of developing diabetes was also related to gender but the mechanisms underlying this association are unclear. Men were shown to be more insulin resistant than women (5, 7). Others observed significant sex differences for the associations between endogenous testosterone and risk of type 2 diabetes with a high testosterone level being associated with higher risk of type 2 diabetes among women, but with decreased risk of type 2 diabetes among men (8). We have previously demonstrated, using an established rat model of dietary protein restriction (2, 9, 10) administered during pregnancy and lactation, that dietary insufficiency in early life alters normal pancreatic development which may ultimately contribute to impaired glucose homeostasis in adulthood (31). The goals of this study were to extend our previous findings and evaluate the precise time point when gender-related differences of metabolic determinants of insulin sensitivity, and the susceptibility to develop diabetes occur.

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Previous studies have shown that maternal low protein diet (LP) alters growth and glucose metabolism of male (11) and female offspring (9) between 20 to 21 months of age, respectively, by reducing insulin action and protein expression, and causing relative hyperinsulinemia. Others have reported that although the young offspring receiving LP diet during gestation and lactation had better glucose tolerance from 6 weeks to 3 months of age than those receiving a normal diet (12, 13), their ability to maintain glucose tolerance deteriorated with age and became significantly worse by 15 months in the adult male offspring (14, 15). Impaired glucose control was associated with a reduction of insulin-stimulated glucose uptake in both muscle (11) and adipose tissue (16), as well as a reduction in both GLUT 4 and PKC- ζ in muscle (11).

Two key mechanisms important in maintaining glucose homeostasis have been identified previously *in vivo* which may help explain such gender differences. Firstly, differences in pancreatic insulin secretion could exist between males versus females (48). Secondly sex-specific differences also exist in the activation of the insulin-dependent pathways responsible for glucose disposal in liver (20) and muscle (21). These pathways involve activation of protein kinase B (PKB/Akt), one of the key kinases in the insulin signaling pathway that contributes to nutrient metabolism, cell growth and inhibition of apoptosis. PKB/Akt activation downstream of phosphoinositide-3 kinase (PI3K) triggers a cascade of proteins that induces glucose transporters to translocate to the membrane allowing glucose transfer into the cell (17, 19). It has been previously demonstrated that early nutritional imbalance can change the phosphorylation of PKB/Akt signaling and consequently alter normal glucose homeostasis permanently, leading to metabolic disease (20–22). However, to date there is no information that specifically identifies the earliest time point when these alterations in PKB/Akt signaling occur or how they might differ with gender.

The pathogenic mechanisms leading to type 2 diabetes are still controversial, and it is not certain whether the primary events are related to insulin resistance, insulin deficiency, or both. The present study introduces the concept that substantial sexual dimorphism exists and suggests that it may be involved in the primary mechanisms that lead to glucose intolerance as a result of a dietary insult in early life.

Methods

Animals. All procedures were performed with the approval of the Animal Care Committee of the University of Western Ontario in accordance with the guidelines given by the Canadian Council of Animal Care. Female and male Wistar rats were purchased at breeding age (250 g) from Charles River (La Salle, St-Constant, Quebec) and left to acclimatize in our animal care facility at the Lawson Health Research Institute. For 3 weeks the female reproductive

cycle was followed to identify the day of fertility. Animals were mated at the onset of pro-estrus. Impregnation was confirmed by the presence of sperm in the vaginal smear the next morning.

Rats were housed in individual cages and maintained at 22°C on a 12:12-h light–dark cycle. Starting on day 1 of gestation, the rats were fed with one of two different diets varying in protein content, but containing equivalent calories. The control diet (C) contained 20% protein, and the low protein diet (LP) contained 8% protein. In the LP diet, the balance of calories was supplied by an increase of carbohydrates with normal fat content (Bio-Serv, Frenchtown, NJ, USA). The detailed composition of this diet has been published previously (23). At birth, litter size was restricted to 8 animals (4 males and 4 females) with weights closest to the litter mean. Gender was determined by anatomical differences. In the neonatal rat the male has a greater anogenital distance and larger genital papillae. Pups were weaned at day 21 of lactation and were fed with the (C) diet. Food and water was provided *ad libitum*.

At least three to four independent litters were considered per dietary treatment. Food intake, pregnancy and post-natal weight were recorded during the course of the experiment.

Intraperitoneal Glucose Tolerance Test (IGTT). Intraperitoneal glucose tolerance tests were performed weekly after the onset of puberty up to 130 days of age after 15 hours fasting in order to determine changes in insulin sensitivity (24). Approximately 8–10 rats from independent litters were tested. To establish basal values of glucose and insulin, blood samples were taken by lancing the tail vein before glucose challenge (time 0). They then received a single bolus of 2 g/kg glucose *i.p.* Blood samples were taken at 0, 15, 30, 60, 90 and 120 minutes from the tail vein. Glucose from whole blood (2 μ l sample) was measured with a handheld glucose monitor (Dex-II, Bayer). Serum was separated (100 μ l) and kept at –20°C for measurement of insulin levels by radioimmunoassay. The incremental area under the glucose tolerance curve (iAUC) was calculated as the integrated area under the curve above the basal value (time 0) over the 120-minute sampling period using Prism 4 for Windows (25). HOMA IR (fasting insulin (μ U/ml) $\times$ fasting glucose (mmol/l) / 22.5) and HOMA β -cell (20 $\times$ fasting insulin (μ U/ml) / fasting glucose (mmol/l) – 3.5) were also calculated (26).

Radioimmunoassays. The serum collected before and during the glucose tolerance test was assayed for insulin levels using a sensitive rat insulin RIA kit (Linco Research, St. Louis, MO, USA). The sensitivity of the assay was 0.1 ng/ml. The intra-assay coefficient of variation was 7.5% and within assay 5%. Serum testosterone and estradiol levels were assayed by ELISA. Testosterone Bioassay ELISA Kit T2949 and Estradiol Bioassay ELISA Kit E3550–02 (US Biological, Swampscott, MA) were used following the instructions provided by the manufacturer after ethyl ether

extraction of the samples. The intra- and inter-assay coefficients of variation were < 10%.

Intraportal Insulin Challenge. Three to four animals from different litters fed with C or LP diet were challenged with an acute intra-portal insulin bolus at 130 days of age to evaluate the insulin sensitivity in liver, adipose tissue and muscle by analyzing phosphorylation of Akt (18, 27). Rats were anaesthetized with Ketamine (100 mg/kg) and Xylazine (5–10 mg/kg) i.p. The abdominal wall was dissected and the portal vein exposed and ligated distally. Insulin 2 U/kg (Humulin R, Lilly) was then infused over 10 seconds and animals sacrificed after 1 minute. Control animals were infused with saline and were used to analyze the basal levels of Akt phosphorylation. A portion of the medial lobe of the liver, soleus muscle and visceral fat were immediately harvested, snap frozen in liquid nitrogen and kept at -80°C for further protein analysis. Body weight, total visceral fat and liver weight were also recorded.

Western Blots. Proteins were extracted from tissues (liver, adipose and muscle) of rats subjected to intraportal insulin challenge as described previously (28). Briefly, 1 ml of lysis buffer (20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 2 mM EDTA, 25 mM β -glycerophosphate, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 20 μM leupeptin, 1 mM benzamidine, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 μM microcystin) was added to approximately 50 mg of frozen tissue. Livers were disrupted by sonication (using a Branson 450 Sonifier). Tissues were rocked for 40 minutes at 4°C . Detergent-insoluble material was precipitated by centrifugation at 12,000 g for 10 minutes at 4°C and lipid layers were removed. Whole protein concentrations from the supernatant were determined by the Micro BCA protein assay (Pierce, IL, USA) using bovine serum albumin as standard protein, as per manufacturer directions. Protein samples were diluted in the SDS sample buffer, and the mixture was boiled for 5 minutes. Proteins (50 μg) were separated on an 8% SDS-PAGE and transferred to nitrocellulose membranes using Tris buffered saline containing Glycine and 20% Methanol as transfer buffer for 60 minutes at 70 volts. After this, membranes were blocked for 60 minutes at room temperature in Tris buffered saline containing 0.05% Tween 20 (TTBS) and 5% non-fat dry milk or 5% BSA, and they were probed overnight at 4°C with 1:1000 anti-phospho Akt (Ser473) (pAkt S-473) mouse monoclonal antibody or 1:1000 anti Akt (pan-E11E7) rabbit monoclonal antibody (Cell Signalling Technology, New England Biolabs, Beverly, MA), according to the recommendations of the manufacturer. The dilution buffer for the antibodies had 5% non-fat dry milk in TTBS. After incubation with the primary antibodies, membranes were washed in TTBS 3 to 5 times for 5 minutes at room temperature on a rocking platform. The proteins were detected by enhanced chemiluminescence (Pierce, IL, USA) with horseradish peroxidase-labelled secondary antibodies

(Sigma, St. Louis, MO). The optical density of the bands was quantified with a Bio Imaging Gel System (Chemii Genius II, Syngene) with Gene Tools software. Each membrane was first probed with antibody against pAkt S-473, then incubated in stripping buffer (Pierce, IL, USA) and re-probed with an antibody against Akt to normalize the results, and results were expressed as ratio to optical density values of the corresponding Akt.

Immunohistochemistry. Pancreata from 130 day-old animals fixed in 10% formalin were embedded in paraffin. Sections of 5 μm were cut and mounted on Superfrost-plus slides (Fisher Scientific, Toronto, ON, Canada). Immunohistochemistry was performed using a modified avidin-biotin peroxidase method (29) as we have described previously (30). Antibody against insulin (polyclonal human anti-insulin antibody 1:200 dilution; Santa Cruz, CA, USA) was used.

Morphometric Analysis. Images of pancreatic sections and islets immunostained for insulin were taken using a Carl Zeiss transmitted light microscope at a magnification of $\times 25$ and $\times 400$. Morphometric analysis of pancreatic sections was performed by Northern Eclipse, version 6.0 software (Empix Imaging, Mississauga, ON, Canada) where tissue and islet areas were measured. The number of small (< 5000 μm^2), medium (5000–10,000 μm^2) and large (> 10,000 μm^2) islets, and the percent of area immuno positive for insulin within the pancreas was calculated for each group at 130 days of age ($n = 3-4$).

Statistical Analysis. Data are presented as mean $\pm$ SEM from at least 3–4 independent litters. Differences between mean values for variables within individual experiments were compared statistically by two-way analysis of variance to analyze gender and diet followed by multiple comparisons by Holm-Sidak method. To analyze differences between diets in glucose and insulin measurements after glucose challenge, a two-way repeated measures ANOVA was performed followed by multiple comparisons using the Holm-Sidak method upon significant interaction. P values of 0.05 and lower were considered significant.

Results

Body and Organ Weight from Gestation to Adulthood: Relationship to Food Intake. Pregnant female rats fed with LP or C diet had comparable food intake (Fig. 1A). Interestingly, a significant decrease in body weight gain was observed during the last week of pregnancy in the rats fed a LP diet (Fig. 1B) although the litter sizes were not significantly different (data not shown). Mothers fed a LP diet during gestation and lactation produced offspring with a lower birth weight and a lower weight gain later in life as seen previously (31) regardless of gender. At birth, the body weights of both LP female and male offspring were significantly reduced ($P < 0.01$) compared with their control-fed counterparts (Male LP 5.00 ± 0.11 , n

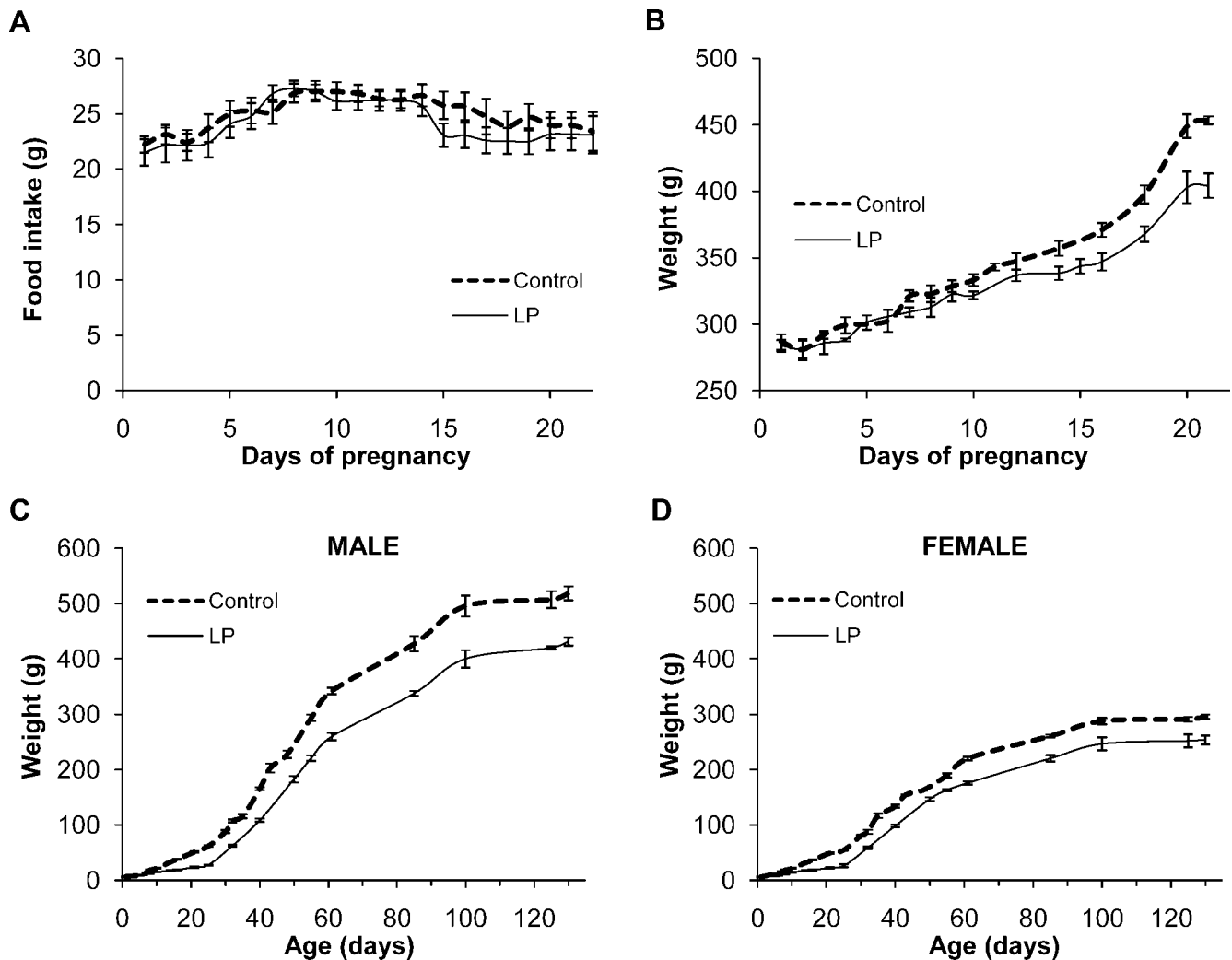


Figure 1. Total food intake during pregnancy (A) and weight gain from conception to birth dates of rats fed with LP or C diets (B). Body weight from birth to 130 days of age of male (C) and female (D) offspring treated with LP or C diet before weaning. Animals fed an LP diet during pregnancy and lactation had a significantly reduced body weight ($P < 0.05$). Values are means $\pm$ SEM. $n = 3$ –4 rats per litter. Three to four independent litters were analyzed per dietary treatment.

= 18 animals; control 6.40 ± 0.13 , $n = 32$; Female LP 4.99 ± 0.10 , $n = 20$; control 5.99 ± 0.11 , $n = 30$ (mean $\pm$ SEM). These offspring were switched to a C diet at weaning, and although they showed a similar daily food intake for both males and females (data not shown), they exhibited a lower weight gain and body weight ($P < 0.05$) at 130 days of age

compared with animals receiving C diet in early life (Fig. 1C and 1D). Body weight, visceral fat and liver weights at 130 days of age are shown in Table 1. Although liver weight was significantly reduced in LP males ($P < 0.001$), there was no change when it was calculated relative to body weight. The ratio of visceral fat weight relative to body

Table 1. Summary of Body, Liver and Visceral Fat Weight in Male and Female C vs. LP Rats Taken at 130 Days of Age^a

Weight (g)	Male		Female	
	C	LP	C	LP
Body	544 $\pm$ 16	460 $\pm$ 13*	300 $\pm$ 8	256 $\pm$ 6*
Liver	20 $\pm$ 1	16 $\pm$ 1*	10 $\pm$ 1	9 $\pm$ 1
Visceral fat	16 $\pm$ 1	24 $\pm$ 4*	8 $\pm$ 2	10 $\pm$ 2
% visceral fat/body weight	3.35 $\pm$ 0.26	5.23 $\pm$ 0.71*	2.82 $\pm$ 0.59	3.62 $\pm$ 0.50

^a Percentage of visceral fat to body weight was compared between the groups.

*, significant between treatments.

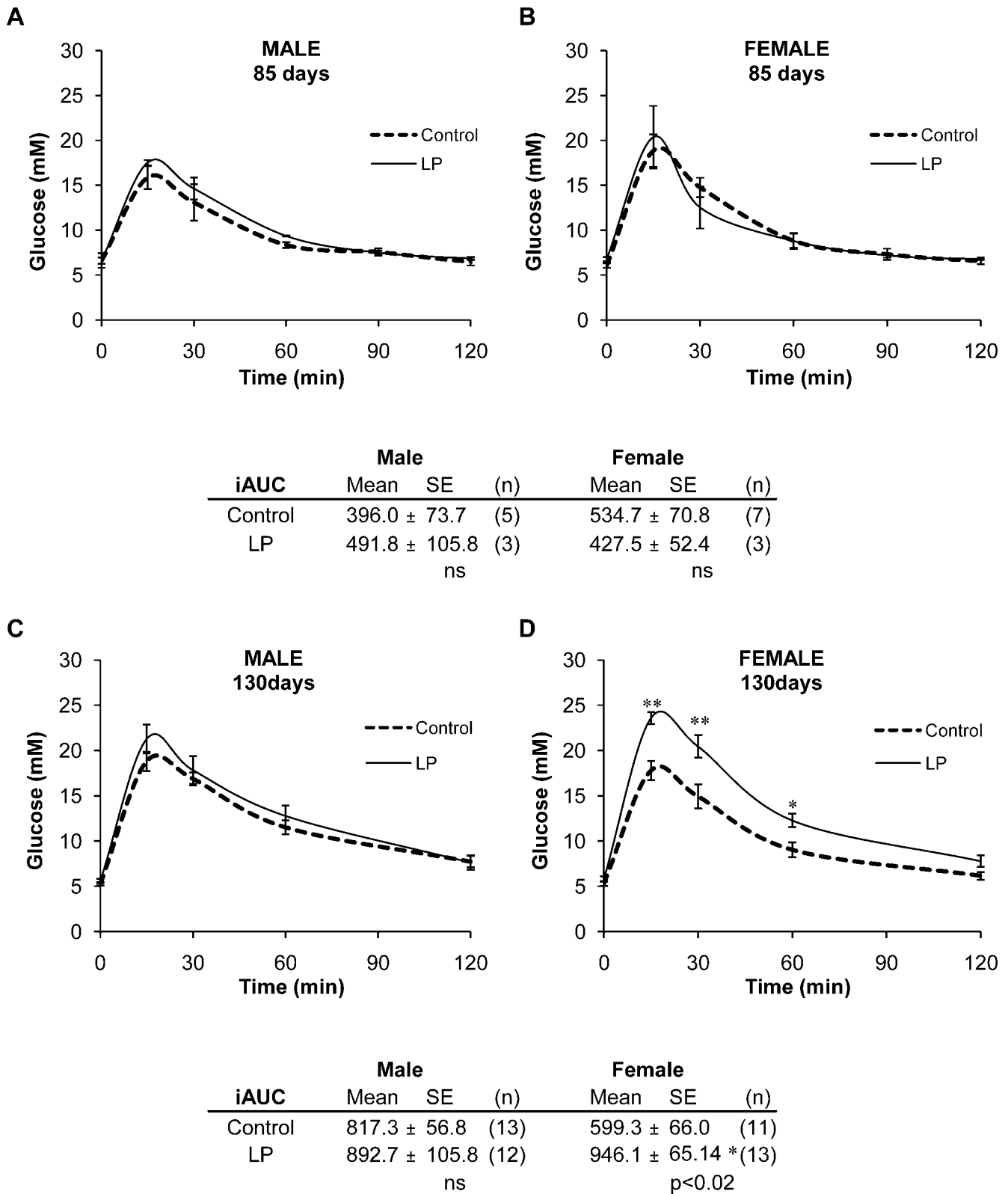


Figure 2. Intraperitoneal glucose tolerance test (IGTT) after overnight fasting at 85 (A, B) and at 130 days of age (C, D) for male and female rats fed C and LP diets. $n = 8-10$ litters per gender and treatment. Values are means $\pm$ SEM. Mean incremental area under the curve (iAUC) for each corresponding treatment shown in the table below the graphs.

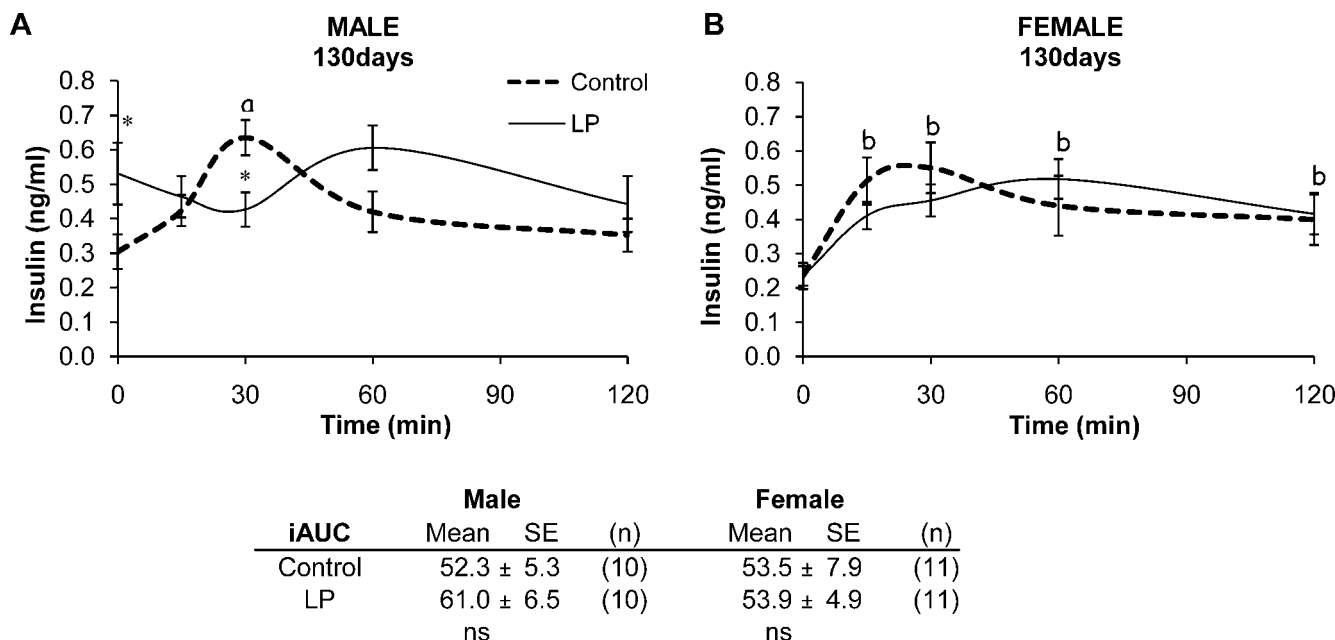


Figure 3. Insulin levels at different time points during IGTT of male and female C- and LP-fed rats at 130 days of age. 0 min time point = before glucose load. $n=8-10$ rats per gender and treatment. Values are means $\pm$ SEM. * $P < 0.05$ for LP vs. control at same time point. A, $P < 0.05$ for 30 minutes vs. all other time points. B, $P < 0.05$ values at any time point are higher than the mean values at time 0 for each group.

weight was significantly increased in males. No such changes were seen in LP females.

Intraperitoneal Glucose Tolerance Test (IGTT). Weekly IGTTs performed after sexual maturation showed a normal and comparable response curve between the two dietary groups. No gender differences were observed. Data observed at 85 days is shown (Fig. 2A and 2B). By 130 days of age, the AUC of the IGTT was similar in males fed with C or LP diets (Fig. 2C). Fasting insulin levels were significantly increased in male rats fed LP diet ($P < 0.05$), when compared with those on C diet (Fig. 3A). After glucose challenge, the C males showed an increase in insulin at the 30 minutes time point ($P < 0.05$) not observed in the LP males and recovered to normal levels by 60 minutes. No significant changes over time were observed in the LP males. Both HOMA IR and HOMA β -cell parameters were significantly increased ($P < 0.01$ and $P < 0.05$, respectively) in LP males at 130 days (Table 2),

Table 2. Parameters Used to Determine the Diabetic State in C vs. LP Male and Female Rats at 130 Days^a

		HOMA IR	HOMA β -cell
Male	C	2.53 $\pm$ 0.73	100.1 $\pm$ 15.5
	LP	7.47 $\pm$ 2.55 ^{*,&}	172.0 $\pm$ 43.3 ^{*,&}
Female	C	2.15 $\pm$ 0.76	94.6 $\pm$ 18.8
	LP	1.84 $\pm$ 0.32	70.4 $\pm$ 13.3

^a Homa IR = (Fasting insulin (mU/ml) $\times$ Fasting glucose (mmol/L)) / 22.5, Homa β -cell = 20 $\times$ (Fasting insulin (mU/ml) / (Fasting glucose (mmol/L) - 3.5) in C and LP treated male and female rats. ^{*} $P < 0.05$ and [&] $P < 0.01$ vs. Female, ^{*} $P < 0.05$ and ^{**} $P < 0.01$ vs. Control. $n=8-10$ in each group.

suggesting that LP males at this age show signs of insulin resistance.

In 130 day-old LP female rats, the iAUC after IGTT was increased ($P < 0.002$) compared to female controls (Fig. 2D). Neither fasting insulin levels nor levels during the IGTT showed significant changes between the two treatments (Fig. 3B). Both HOMA IR and HOMA β -cell parameters did not show significant differences between female groups (Table 2).

Glucose Tolerance and Gender Dimorphism. We measured serum testosterone and estradiol levels by radioimmunoassay at 85 days of age when no changes in glucose tolerance were seen in either sex treated with LP diet, and at 130 days when clear differences were first observed. At 85 days no changes in sex steroid levels were observed when compared to the control groups, although at 130 days of age LP males had significantly less

Table 3. Blood Levels of Testosterone and Estradiol (ng/ml) in Males and Females at 130 Days and Testosterone at 85 Days of Age in Males. Control vs. LP^a

		ng/ml	Male	Female
85 days	Testosterone	C	3.36 $\pm$ 0.43	
		LP	2.94 $\pm$ 0.64	
130 days	Testosterone	C	4.77 $\pm$ 0.34	1.38 $\pm$ 0.10
		LP	3.70 $\pm$ 0.21 [*]	1.40 $\pm$ 0.08
	Estradiol	C	0.86 $\pm$ 0.08	0.83 $\pm$ 0.10
		LP	0.71 $\pm$ 0.02	0.79 $\pm$ 0.04

^a Mean $\pm$ SEM in ng/ml.

^{*} $P < 0.05$ vs. Control, t test. $n=6-7$ animals per group.

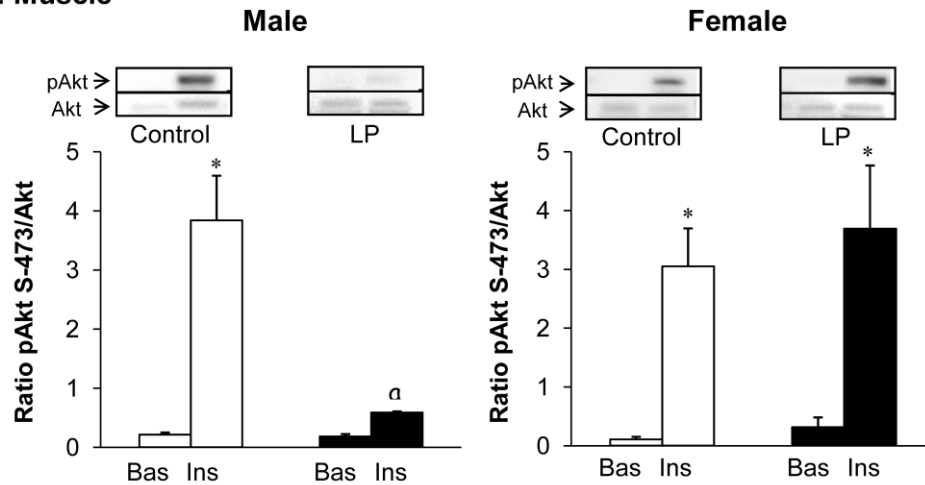
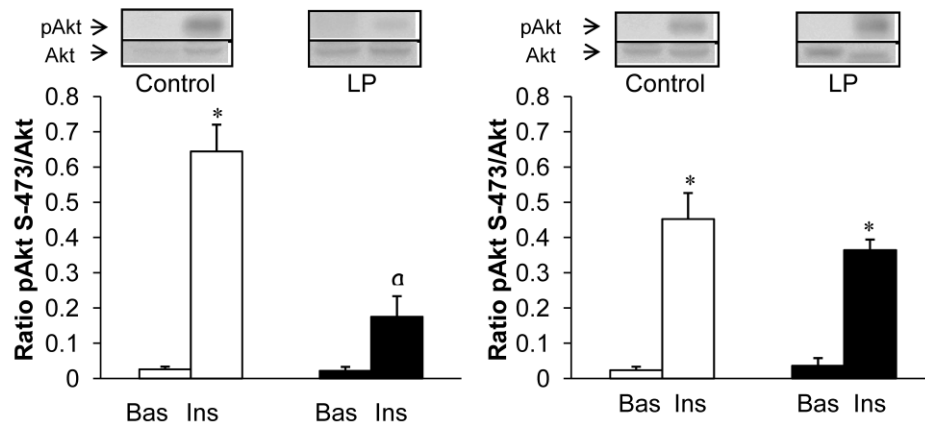
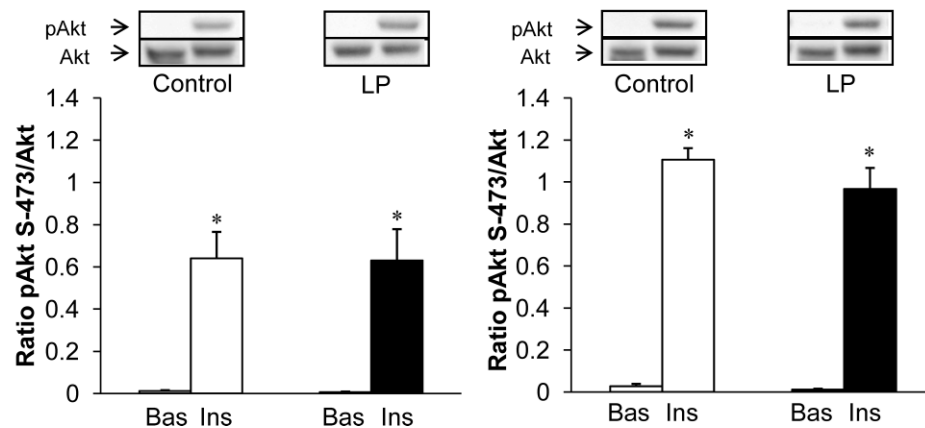
A. Muscle**B. Adipose tissue****C. Liver**

Figure 4. Western Blot Analysis of pAKB/AKT/S-473 ratio in basal conditions (Bas) and after Insulin challenge (Ins) in both male and female rats fed with C and LP diets. (A) Muscle. (B) Visceral adipose tissue. (C) Liver. Tissues of three to four animals were analyzed from 3 independent litters per dietary treatment. The molecular weights of Akt and pAkt are 60 kD and bands were quantified by densitometry. * $P < 0.001$ for Ins vs. Bas levels. ^a $P < 0.05$ for LP vs. Control. Data represents 3 separate immunoblots.

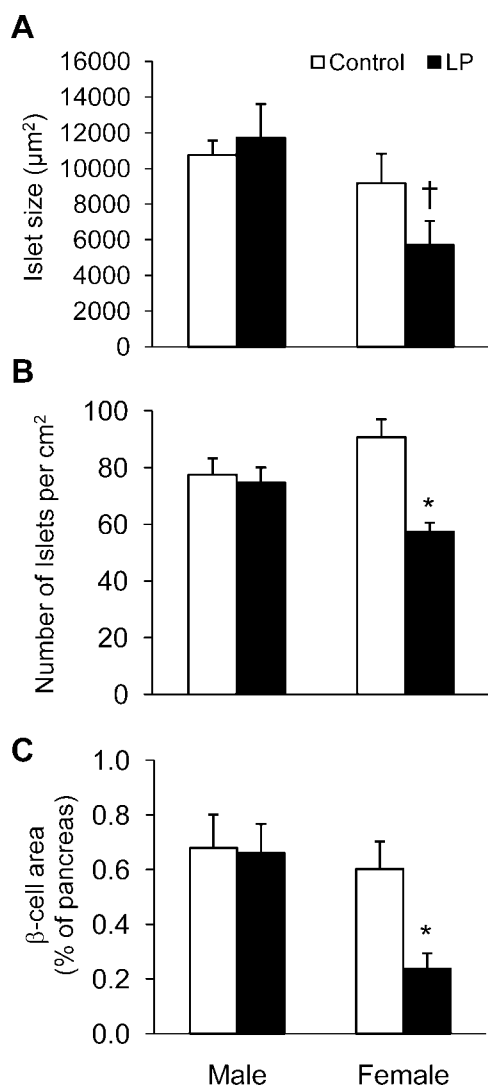


Figure 5. Pancreas Histomorphometry in male and female rats fed with C and LP diets at 130 days of age. (A) Islet size. (B) Number of islets per cm². (C) β-cell area (% of pancreas). *n* = 3–4 independent litters were studied per dietary treatment. Values are means ± SEM. * *P* < 0.05 for LP vs. Control. † *P* < 0.05 for female vs. male LP.

testosterone (Table 3). These results are comparable to recent human studies demonstrating that reduced levels of testosterone in older men are linked with insulin resistance and hyperglycemia (32–34).

Intraportal Insulin Challenge to Assess Insulin Signaling Pathway. Infusion of insulin via the portal vein was assessed by Western immunoblotting. In the C males Akt phosphorylation was significantly increased (*P* < 0.001) by 4–6 fold in muscle and visceral adipose tissue after 1 minute. In contrast, LP males did not respond to the insulin-infusion when compared to control (Fig. 4A and 4B). These results further support the likelihood that LP-treated males are insulin resistant at 130 days of age, since not only do they have high fasting basal insulin, but their response to external insulin is also reduced in both adipose and muscle tissues.

In females, AKT phosphorylation in response to the insulin challenge did not differ with dietary treatments (Fig. 4A and 4B), suggesting that the LP females maintained their response to insulin in peripheral insulin-dependent tissues (muscle, adipose tissue) and the Akt phosphorylation was unaltered (Fig. 4A–C). Akt phosphorylation in the liver at 130 days of age in response to insulin challenge showed a similar response in males and females previously exposed to either diet, suggesting that this insulin signaling mechanism was not affected by diet (Fig. 4C).

Pancreatic Morphometry. Image analysis revealed that both the number of islets per cm² of pancreas and the percentage of pancreas area accounted for by β-cells area were reduced significantly in LP females at 130 days of age (*P* < 0.05) (Fig. 5A). Moreover, the islet distribution by size in the LP females showed a significant reduction in the number of large islets (> 10,000 μm²) per pancreatic area and a reduction in the β-cell area of small (< 5000 μm²) and large islets (Fig. 6A). These morphological changes in islet area and β-cell area support the functional results of insulin deficiency shown in Figure 3. In contrast, males did not show any differences in islet number or size between either diet (Figs. 5 and 6).

Discussion

Animal studies have shown that maternal malnutrition during fetal and neonatal life can impede normal cell division, organ growth and differentiation in the fetus and neonate, resulting in permanent changes in tissue composition and cell size during adulthood (14). Such maternal programming of the structure and physiology of fetal organs also applies to the endocrine pancreas and insulin-sensitive target tissues such as adipose, muscle and liver (2, 35, 37).

The incorporation of fat-rich diets after weaning to promote normal growth following a dietary deficiency in early life has been previously demonstrated to predispose the male offspring to develop obesity, and metabolic disorders in later life (38, 39). Conversely, if the restricted diet continues into adulthood, no metabolic changes are observed and pathologies associated with chronic diseases are prevented (40). Similarly in humans, fetal growth restriction has been shown to impact insulin action in adulthood (41). Data obtained from epidemiological studies have shown that subjects who had low birth weight or were thin at birth have a higher prevalence of developing insulin resistance syndrome or syndrome X in adulthood, accompanied by the co-existence of glucose intolerance, hypertension and hypertriglyceridemia (35, 36, 38, 42, 43). Depending on the length and timing of the dietary insult during pregnancy, outcomes varied with gender and were linked to decreased glucose tolerance and obesity in adults (44).

In this study, we have shown that rats weaned onto a C diet after having received a LP diet during gestation and lactation had lower body weight after weaning and up to

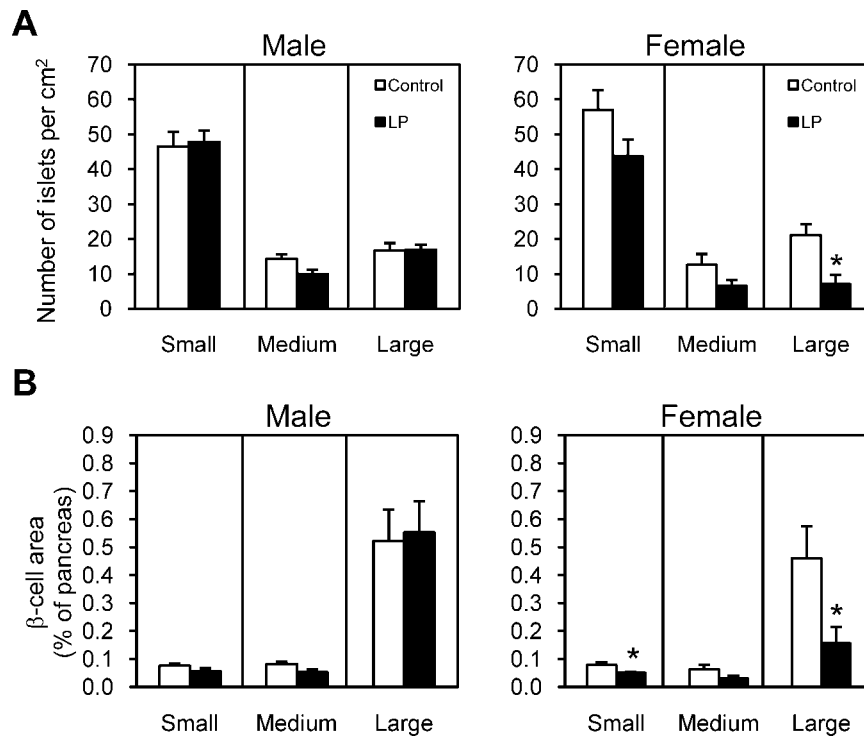


Figure 6. Distribution of islets by size in male and female fed with C and LP diets at 130 days of age. (A) Number of islets per cm². (B) β-cell area (% of pancreas). Values are means $\pm$ SEM. $n = 3-4$ independent litters were studied per dietary treatment. t test, * $P < 0.05$ vs. control.

130 days of age compared to continuous C-fed controls despite similar food intake for both females and males. However, by 130 days the LP male group had higher relative visceral adiposity, low testosterone levels and higher basal insulin levels. Previously, we showed a widespread upregulation of genes involved in carbohydrate, lipid and protein metabolism within visceral adipose tissue obtained from 130 day-old adult male rats previously fed LP diet (45). Insulin is a strong inducer of lipogenesis through activation of lipogenic and glycolytic enzymes. Higher basal insulin levels in male rats fed LP diet increased accumulation of visceral fat and this may correlate with the etiology of insulin resistance. Several human studies have reported that greater fat deposition around the waist is associated with insulin resistance (6, 7) and is considered a predictor of metabolic syndrome (46). It has also been suggested that men with low testosterone are at a greater risk of developing type 2 diabetes (32, 33, 47).

Animal studies showed that male offspring fed a LP diet prior to birth, but weaned onto a C diet, were more glucose tolerant at 3 months of age (14) than C-fed animals, but became glucose intolerant by 15 months of age. A similar study (48) showed insulin hypersensitivity in females at 110 days of life compared with controls. Depending on the length and timing of the dietary insult during pregnancy, outcomes varied with gender and were linked to decreased glucose tolerance and obesity in adults (44). In the present study we performed longitudinal analyses and found that glucose intolerance was not

detectable before 130 days of age in LP treated animals and that for males there was evidence of hyperinsulinemia associated with insulin resistance. Although glucose tolerance was maintained in the LP male at 130 days of age, they had an increased fasting circulating insulin level paralleled by a reduction in insulin sensitivity in muscle and adipose tissue, with relative visceral obesity and low testosterone levels. Collectively, all of these factors may contribute to the onset of glucose intolerance in later life. In LP-fed males, the two major classic peripheral insulin-responsive tissues, muscle and adipose, displayed a diminished response to insulin resulting in decreased disposal of excess circulating glucose and fatty acids. Increased fat mass, visceral fat mass and an adverse lipid profile are all observed in the human metabolic syndrome which is characterized by decreased insulin sensitivity whereby the degree of insulin resistance is related to the percentage fat mass (49).

In contrast, at 130 days of age LP-fed female offspring had a significantly altered glucose tolerance, but maintained insulin sensitivity in muscle and adipose tissue. Female LP-fed offspring showed no visceral obesity or impairment of insulin signaling in adipose or muscle, no change in HOMA, but had impaired glucose tolerance compared to C-fed animals. Moreover, LP-fed females possessed fewer β-cells and had fewer small and large pancreatic islets, and accordingly higher hyperglycemia and less serum insulin concentration than their male counterparts. Therefore the primary reason for the impaired glucose tolerance in the females is likely due to a relative deficiency of pancreatic β-

Table 4. Summarized Results of LP Males and Females Compared to Control Animals at 130 Days of Age^a

	Male LP	Female LP
Body weight	↓	↓
Liver weight	↓	—
Adipose tissue weight	↑	—
% fat/body weight	↑	—
Estradiol	—	—
Testosterone	↓	—
β-cell		
HOMA β-cell	↑	—
Number of islets	—	↓
% β-cell in pancreas	—	↓
Number of large islets	—	↓
Insulin sensitivity		
HOMA IR	↑	—
IGTT (AUC)	—	↑
Insulin IGTT (time 0)	↑	—
Phosphorylation of Akt		
Muscle	↓	—
Adipose	↓	—
Liver	—	—

^a Parameters analyzed were decreased (↓), increased (↑) or unchanged (—) when compared to Control animals at the same age.

cells. Dahri *et al.* previously showed that LP-fed female rats had larger pancreatic islets at 84 days of age than C-fed rats, with lower insulin content but normal fasting glucose levels (8, 9). We did not have comparable data on pancreatic morphology at that age, but taking this data into account would suggest that for females, the relative failure to maintain β-cell mass must occur between 84 and 130 days of age. These gender-specific findings are consistent with observations in humans that showed that men are more insulin resistant than women (5, 7).

In the LP diet model the diet was made isocaloric with the control diet through the addition of carbohydrate, and it should be considered whether the changes in pancreas morphology or insulin sensitivity observed in the offspring were primarily due to the effects of reduced protein or increased carbohydrate. Protein content was reduced from 20% to 8% corresponding to a reduction of 40%. The increase in carbohydrate content represented only a 20% change suggesting that the changes in glucose homeostasis were more likely to result from the reduction in protein.

Others had observed significant sex differences for the associations between endogenous testosterone and risk of type 2 diabetes. High testosterone levels were associated with higher risk of type 2 diabetes among women, while simultaneously associated with decreased risk of type 2 diabetes among men (8). We also found this association in this animal model. Other rodent models of diabetes also show differences in insulin sensitivity and secretion with gender. The spontaneous incidence of type 1 diabetes in the

non-obese diabetic mice is 85% in females and 25% in males (50). Development of diabetes in response to STZ treatment in animals receiving nicotinamide is more severe in female rats than in males, as females develop the disease more rapidly (51).

In the present study, we show that the insulin signaling pathway in the liver of the LP rat was maintained and no gender differences were observed at 130 days of age regardless of diet. Previous studies have shown that at 3 months of age there was a decrease in hepatocyte proliferation and liver growth with altered morphology in the offspring of LP-restricted rats (52). Our data show that LP-fed males had a decreased liver weight but that this was appropriate for body weight. Others have shown that there was a decrease in IGF-I production in hepatocytes derived from fetuses exposed to a LP diet through gestation (53). However, the livers had larger lobules and an increased expression of the insulin receptor at 3 months of age and it was proposed that glucokinase expression might be altered (54). It would appear that in liver of rats previously fed LP diet there may be a compensatory maintenance of insulin sensitivity despite alterations in liver size and morphometry.

In summary, this rat model suggests that LP diet during intrauterine development and lactation affects metabolic function, contributing to later glucose intolerance, but this does not become apparent until maturity at 130 days of age. It also suggests that there is a gender difference in the mechanism by which these alterations occur. Females become relatively more insulin deficient while males are more insulin resistant. Our results suggest that treatment strategies for type 2 diabetes in humans could also take into consideration gender differences along with age.

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