Maternal low protein diet alters pancreatic islet mitochondrial function in a sex-specific manner in the adult rat

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ABSTRACT

Mitochondrial dysfunction may be a long-term consequence to poor nutritional environment during early life. Our aim was to investigate whether maternal low protein diet may program mitochondrial dysfunction in islets of adult progeny before glucose intolerance ensues. To address this, pregnant Wistar rats were fed isocaloric diets containing either 20% protein (C) or 8% protein (LP) throughout gestation. From birth, offspring received the control diet. The mitochondrial function was analyzed in islets of 3 month-old offspring. Related to their basal insulin release, cultured islets from both male and female LP offspring presented a lower response to glucose challenge and a blunted ATP production compared to C offspring. The expression of malate deshydrogenase as well as the subunit 6 of the ATP synthase encoded by mitochondrial genome (mtDNA) was lower in these islets, reducing the capacity of ATP production through the Krebs cycle and oxidative phosphorylation. However, mtDNA content was unchanged in LP islets compared to C. Several consequences of protein restriction during fetal life were more marked in male offspring. Only LP males showed an increased ROS production associated to a higher expression of mitochondrial subunits of the electron transport chain ND4L, an overexpression of PPARγ and UCP-2 and a strongly reduced β-cell mass. In conclusion, mitochondrial function is clearly altered in islets from LP adult offspring in a sex-specific manner. That may provide a cellular explanation for the earlier development of glucose intolerance in male than in female offspring of dams fed a LP diet.

KEYWORDS

Developmental programming, malnutrition, metabolic syndrome, insulin secretion
INTRODUCTION

Epidemiological and experimental evidence shows convincing relationship between poor intra-uterine nutritional environment and the subsequent development of metabolic disorders like insulin resistance, obesity and cardiovascular disease in adulthood (11; 12). The original concept of fetal programming of Type 2 diabetes mellitus (T2DM) was proposed by Barker et al. (12) who found in a cohort of adult men who had been smaller at birth, that they were six times more prone to develop T2DM compared with individuals heavier at birth. These first epidemiological clues of programming have been largely replicated in many studies throughout the world (17; 27; 61). Nowadays, this concept is widely accepted but the mechanistic basis is unclear.

Since few years, attention focused on the involvement of mitochondria as putative targets linking physiological and molecular consequences of environmental disorders during early life. Some experimental studies revealed that a key adaptation enabling the fetus to survive in an imbalanced intra-uterine milieu might be a programming of the mitochondrial function (36; 37; 39; 53; 55; 59). Uterine artery ligation in rat showed that such uteroplacental insufficiency, where both the supply of oxygen and of crucial nutrients were restricted, provoked growth retardation and altered mitochondrial function in liver and muscle of the male offspring (39; 53). In this offspring, mitochondrial dysfunction also occurred in endocrine pancreas, leading to an increased production of reactive oxygen species (ROS), a reduced ATP production, and a decline in expression of mitochondrial DNA encoded-genes involved in electron transport chain (55). These studies propose a molecular mechanism underlying the link between mitochondrial dysfunction secondary to an environmental insult and the development of T2DM in the adult.

Although animal models of maternal malnutrition also feature mitochondrial abnormalities (36; 59), the concept of a “nutritional mitochondrial programming” is not as well established,
particularly for the endocrine pancreas. Since many years, our studies focused on the
development of pancreatic islets from progeny of dams fed an isocaloric low protein diet (LP)
containing 8% protein (as opposite to 20% in the controls) during early life. Maternal LP diet
reduced the \( \beta \)-cell mass by decreasing proliferation, increasing the apoptotic rate (40) and by
modifying the amino-acid profile, with a marked reduction of taurine in plasma of dams and
pups (46). Suzuki et al. (58) reported that taurine could critically affect the function of
mitochondria. Indeed, taurine is a constituent of mitochondrial tRNAs and the loss of these
tRNAs would explain the molecular pathogenesis of some mitochondrial diseases (24; 58). In a
proteome analysis of fetal LP islets, we found that the level of proteins involved in the
mitochondrial energy transfer, glucose metabolism, RNA and DNA metabolism was changed
(57). Moreover, we have recently shown that intrauterine LP diet changed the gene expression
pattern in fetal islets among which 10% were genes coding for mitochondrial proteins (47). Later
in life, the net result of the early protein deficiency was a progressive loss of pancreatic islets
function and development of insulin resistance in the adult with sex-specific time-course (8; 13;
41).

It is increasingly obvious that the programming is sex specific. Recent clinical and
experimental evaluations of the fetal origins of obesity (20; 33), insulin resistance (33) and
vascular dysfunction showed a role of sex in the issue (5; 50; 52). Until now, the sex-specific
consequence was rarely taken into account in the evaluation of the long-term cellular and
molecular effects of early poor protein restriction.

The present study was designed to determine if maternal protein restriction may change the
mitochondrial function of pancreatic islets in both male and female adult progeny. We have
investigated the 3 month-old offspring in which a low protein diet was given throughout
gestation. After delivery, each progeny was fed with the control diet containing 20% protein. Mitochondrial function was assessed by measurement of ROS and ATP production, mitochondrial DNA content, expression of genes involved in mitochondrial biogenesis and function, in correlation with *in vitro* and *in vivo* analysis of islet function.
MATERIALS AND METHODS

Animals- Adult virgin females Wistar rats (Janvier, Le Geneste St. Isle, France), were caged overnight with males (four females to one male), and copulation was verified the next morning by detection of spermatozoa in the vaginal smear. Midnight was considered as the time of mating. Pregnant females were then housed singly under controlled conditions (25°C; 14:10-h light-dark cycle) and had free access to their respective diets and to water. Rats were fed either a control diet containing 20% protein (C group) or an isocaloric low protein diet containing 8% protein during gestation (LP group). The composition of these diets has been described previously (56). After parturition, all litters were sexed and standardized to 8 pups (4 males and 4 females when possible) and fed with the control diet. Food intake and body weight of dams were assessed every three days during gestation/lactation and after weaning, on offspring, where animals were housed individually until sacrifice by decapitation at 3 months of age. All procedures were carried out in accordance with “Principles of laboratory animal care” (NIH publication no.85-23, revised 1985) and with the approval of the animal ethics committee of the Université catholique de Louvain, Belgium.

Islets collection- After obstruction of the junction of the common bile duct with duodenum, a catheter was introduced into the bile duct. Collagenase P (Roche, Mannheim, Germany) was injected into the duct to distend the pancreas. The pancreas was laid down into a tube and placed in 37°C water bath to allow digestion of the exocrine tissue. After washing in cold HBSS (Hank’s Balanced Salt Solution, pH 7.4), islets were isolated by hand picking and were cultured for 3 days in RPMI 1640 culture medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% PenStrepFungizone (300 U/ml penicillin, 300 µg/ml Streptomycin, 0.750 µg/ml fungizone – CambrexBioScience, Walkerville, MO, USA).
Liver collection- Livers were removed rapidly after decapitation, weighted, washed in HBSS, frozen and stored at -80°C until utilized.

Oral Glucose Tolerance Test (OGTT)- Insulinemic and glycemic responses to an OGTT were determined in 3 month-old male and female offspring after an overnight fast according to Merezak et al. (32). After collection of a basal blood sample, 0.7 ml/100g body weight of a 50% glucose solution (wt/v) was administered by gavage. Tail venous blood samples were collected in ice-cold heparinized tubes at 15, 30, 60, 120 and 150 min to measure glucose and insulin. Glucose homeostasis was assessed by calculating the AUC of glucose and insulin.

Assays- Blood samples were collected from 3 month-old rats in tubes containing heparin and used for preparation of plasma. For glucose concentration analysis, proteins were precipitated by addition of 150 µl HClO₄ (0.33N) to 15 µl blood and concentration was determined by the glucose oxidase colorimetric method (Stanbio, Boerne, TX, USA). Insulin concentration was measured by ELISA test using the Mercodia Ultrasensitive Rat Insulin ELISA Kit (Uppsala, Sweden). Triglycerides and total cholesterol concentrations were determined by using respectively TRF400CH and CTF400CH kits following the manufacturer’s instruction (Chema Diagnostica, Jesi, Italy).

Pancreas processing for immunohistochemistry and pancreatic insulin content- Pancreas was removed, weighted and dissected. The splenic part of pancreases was fixed in 0.2% glutaraldehyde – 2% paraformaldehyde in phosphate buffer solution, dehydrated and embedded in paraffin. Tissue sections (7 µm) were collected in poly-L-lysine coated glass slides. The duodenal part was placed in 5 ml acid-ethanol [0.15M HCl in 75% (v/v) ethanol in water] to extract insulin. Pancreatic insulin content was determined using the high range rat insulin ELISA (Mercodia, Uppsala, Sweden).
**Immunohistochemistry and morphometry measurements** - Tissue sections were rehydrated and incubated 1 h with a blocking buffer [0.1% (v/v) Tween20 – 3% (v/v) BSA in Tris-buffered saline] before an overnight incubation at 4°C with mouse anti-insulin antibodies (1/6,000; Sigma, St Louis, MO, USA). After washing, samples were then incubated with biotin-conjugated anti-mouse secondary antibodies (1/2,000; Chemicon, Temecula, CA, USA). The complex was revealed using peroxidase-conjugated streptavidin (1/1,000; Amersham Pharmacia Biotech Europe, Saclay, France). Peroxidase was detected using diaminobenzidine (Sigma-Aldrich, Bornem, Belgium). The insulin-positive area was morphologically measured on 6 sections per animal using the Zeiss KS 400 3.0 software (Carl Zeiss GmbH, Jena, Germany). The beta cell mass was obtained multiplying the volume density of beta cells in pancreas by the weight of the pancreas.

**ATP measurements** - Total cellular ATP was determined in islets after 3 days of culture by chemiluminescence using a reagent based on luciferase reaction as described previously (47). Briefly, islets were washed and incubated in Krebs-Ringer buffer (KRB) without glucose at 37°C in 5% CO₂/O₂ air for 60 min. Islets were then divided into batches of 50 islets and transferred into dishes containing KRB with 3.3 or 16.7 mmol/l glucose. After 2 h incubation, islets were transferred in cold PBS 0.1M and lysis buffer for ATP extraction. Samples were immediately placed on ice. Islets were sonicated during 30 sec to ensure complete lysis of cells. Islet lysate ATP content was measured using ATP luminescent assay kit (Promega, Madison, WI, USA) using 50 µl in duplicate. ATP was calculated per µg of protein in the islet homogenate.

**Insulin secretion study** - These experiments were performed using a KRB containing: 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM NaHCO₃ supplemented with 5 g/l bovine serum albumin (Fraction V, Calbiochem-Behring, San Diego, CA, USA) and glucose.
This solution was gassed 10 min with 95% O₂/5%CO₂ to maintain a pH of 7.4. After 3 days of culture, batches of 10 islets were incubated for 60 min at 37°C in KRB containing 2.5 mmol/l glucose. Islets were then incubated at 37°C in a shaking water bath in 1 ml KRB containing glucose at 3.3 mmol/l or 16.7 mmol/l. After 120 min, the incubation medium was removed and placed in a watch glass to verify that no islet had been taken; then the medium was frozen at -20°C until insulin release measurement. To determine insulin content, islets were collected under microscopic observation and homogenized by sonification (30s, 40W) in a 0.5 ml acid-ethanol (0.15M HCl in 75% (v/v) ethanol in water) to extract insulin. Insulin secretion during incubation was expressed as a percentage of insulin content at the start of the incubation. The latter was obtained by adding the content measured at the end to the amount of released insulin.

**Fluorescence measurement of ROS-** ROS production in islets was measured using chloro-methyl dichlorofluorescin diacetate (Molecular Probes, Eugene, OR, USA). After 3 days of culture, batches of 50 islets were incubated for an additional 24 h in either 5.5 mmol/l or 16.7 mmol/l glucose. Islets were then loaded with 10 µM CM-H₂DCFDA for 3h at 37°C. At the end of the experiment, islets were rinsed and disrupted using PBS-1% Triton (Sigma, St. Louis, MO, USA). After centrifugation, islet supernatants were collected. Fluorescence was monitored using a Fluoroskan Ascent FL (Labsystems, Ramsey, MN, USA) and corrected by subtracting parallel blanks in which islet cells were not loaded with probes. Data were expressed per µg of islet protein.

**Real-Time RT-PCR-** The level of mRNA expression was measured by real-time reverse transcription (RT)-PCR. Total RNA was extracted from islets/liver with the NucleoSpin® RNAII/L (Macherey-Nagel, Hoerdt, France); RNA was extracted as described by the manufacturer and stored at -80°C before use. All RNA used for quantitative real-time gene
analysis met the minimum of at least 1.8 ratio of 18S:28S rRNA. First-strand cDNA was generated from 1 µg of DNA-free total RNA by RT using random hexamers and SuperScript III (Invitrogen, Merelbeek, Belgium). Final reaction mixture was divided into aliquots and stored at -20°C until used. Quantitative real-time PCR was performed using SybrGreen master mix according to the supplier protocols (Westburg, Leusden, The Netherlands) and design primers (Sigma, St. Louis, Mo, USA). Primers sequences are presented in Table 1. The level of mRNA expression was calculated using the threshold cycle (Ct) value, the number of PCR cycles at which the fluorescence signal during the PCR reaches a fixed threshold. For each sample, the Ct both for the gene of interest and for the internal standard housekeeping gene Gapdh were determined to calculate \( \triangle C_{t, \text{sample}} \) (\( C_{t, \text{target gene}} - C_{t, \text{housekeeping gene}} \)). The expression level was reported to a calibrator consisting of cDNA from control rats. Subsequently, \( \triangle \triangle C_{t} \) (\( \triangle C_{t, \text{sample}} - \triangle C_{t, \text{calibrator}} \)) was determined, and the relative expression levels were calculated from \( 2^{-\triangle \triangle C_{t}} \) according to the manufacturer’s instructions (Applied Biosystems, Lennik, Belgium). RNA expression levels are thus indicated as arbitrary units ± SEM.

**mtDNA analysis**- We used quantitative real-time PCR to determine the amount of nuclear DNA (nDNA) relative to mitochondrial DNA (mtDNA). Total DNA was extracted from islets using DNAeasy® Blood & Tissue Kit (Qiagen, Venlo, Netherlands). The mtDNA content was obtained by dividing the mtDNA signal for ATPase6 by the nDNA signal for glyceraldehyde-3-phosphate dehydrogenase. The ratio was expressed as a percentage of controls set at 100%.

**Statistical analysis**- Results were reported as means ± SEM. Statistical analyses were performed using t-test. Two-way ANOVA followed by Bonferroni post-test were assessed to discriminate diet and glucose concentration effects or sex and diet interaction (GraphPad
software INC, San Diego, CA, USA). A $p$ value of less than 0.05 was considered as statistically significant.
RESULTS

Dams: body weight gain and food intake during gestation and lactation- At day 1 of gestation, dams of the two experimental groups had a similar body weight (C 285 ± 6 vs LP 288 ± 12 g, n=7). During gestation, LP dams featured a similar weight gain (C 410 ± 12 vs LP 370 ± 18 g, p=0.08) despite an unchanged calorific intake (C 68.2 ± 2.6 vs LP 70.6 ± 4.6 kcal/day) and no difference in litter size (on average C 11.9 ± 0.7 vs LP 11.7 ± 0.5 pups per litter). After one week of lactation, the body weight was similar in LP and C animals and it increased similarly until weaning (C 317 ± 7 vs LP 281 ± 25 g). Calorific intake during lactation did not differ in LP and C groups (C 143 ± 6 vs LP 139 ± 19 kcal/day).

Offspring: body weight and food intake- At birth, both female and male LP offspring showed early growth retardation (females: C 6.6 ± 0.06 vs LP 5.72 ± 0.10 g, p<0.001; males: C 6.95 ± 0.08 vs LP 6.19 ± 0.09 g, p<0.001) and remained smaller at weaning. Rats recuperated progressively a normal body weight at 3 months (females: C 240 ± 2 vs LP 239 ± 7 g; males: C 391 ± 7 vs LP 385 ± 11 g). No difference was observed in the daily food intake for both female and male LP offspring compared to C, from weaning to 3 months.

Plasma analysis at 3 months- As shown in Table 2, fasting plasma insulin level tended to be reduced in females (p=0.06) and males (p=0.08) of dams fed a LP diet during gestation, but the diet had no effect on the fasting plasma glucose level. Triglycerides concentration was significantly increased in male LP animals compared to C whereas cholesterol level was unchanged. In females, no significant difference was observed between controls and LP animals for both triglycerides and cholesterol levels.

Morphometrical analysis and pancreatic insulin content- The pancreas was dissected and weighed. The splenic part was used for immunohistochemistry and morphometrical analysis
whereas the duodenal part was placed in acid-ethanol to extract insulin for pancreatic insulin content measurement. In LP adult progeny, the β-cell mass (Volume density of β-cells*Weight of pancreas) was strongly reduced in males whereas the reduction was not significant in females (Table 2). When insulin content was reported to the pancreatic weight, it was significantly increased in both LP sexes (Table 2).

Oral glucose tolerance test- Plasma insulin and glucose concentrations measured at different times after an oral glucose challenge are shown in Fig. 1. Both LP sexes showed a normal plasma glucose concentration during the test, and the area under the curve (AUC) was similar to controls. No statistically significant difference was observed for the evolution of the plasma insulin level during the glucose challenge (Two-way ANOVA). However, the AUC tended to be reduced for the male and female LP rats, because the early response was lower.

In vitro insulin secretion- Insulin secretion by islets was measured after 3 days of culture (Fig. 2). In response to 16.7 mmol/l glucose, insulin release was significantly increased in both C (p<0.01) and LP groups (p<0.05, females; p=0.05, males). Islets from LP females released more insulin in response to high glucose concentration than islets from controls. However, related to their basal insulin secretion, islets from LP females increased only 2.4 times their insulin released whereas control islets released 3.2 folds more insulin. In islets from LP males, insulin secretion increased 2.2 times between 3.3 mmol/l and 16.7 mmol/l glucose conditions compared to 2.8 times for control islets under the same challenge.

ATP production- At low glucose concentration, no difference in ATP production was observed between LP and C islets (Fig. 3). This was true for male as well as female progeny. When stimulated by 16.7 mmol/l glucose, ATP production by control islets of both sexes was
increased. In contrast, LP male and female islets did not increase their ATP production in response to glucose.

*ROS production*- In female islets, ROS production was increased by glucose stimulation (p<0.05), but no diet effect was detected by two-way ANOVA (Fig. 4). In contrast, in addition to the glucose effect (p<0.05), a diet effect was shown in male islets (p<0.01) in which ROS production was higher in LP than control group, in both low and high glucose concentrations.

*Expression of genes involved in mitochondrial biogenesis and function; mtDNA content*- We evaluated the expression of genes encoded by the nuclear and the mitochondrial genomes in islets (Table 3A) and liver (Table 4A). The expression of PGC-1α, NRF-1 and Tfam was assessed because these factors provide a molecular basis for the connection between environmental stimuli and mitochondrial biogenesis and respiration (15; 43). LP diet affected differently males and females, as confirmed by two-way ANOVA showing the interaction between sex of the offspring and the consequence of prenatal diet. In islets from LP females, both nDNA encoded genes NRF-1 and Tfam were significantly downregulated whereas they were similarly expressed in males (Sex/diet interaction, p=0.07, p<0.05, respectively). The expression of PGC-1α was unchanged. Mitochondrial genome encodes for essential proteins which are all components of the electron transport chain (ETC) (64). We have chosen to analyze the expression of three of them, known as affected in another model of fetal programming (55). ND4L is a subunit of the complex I whereas COX-1 is a subunit of the complex III of the ETC. ATP6 is a subunit of the mitochondrial pump ATP synthase (64). Although unchanged in LP females, the transcript level of mtDNA-encoded genes ND4L and COX-1 were higher in LP males (Sex/diet interaction, p=0.001, p<0.01 respectively). The level of ATP6 expression declined in male and female LP offspring. Furthermore, no difference in islet mtDNA content
was observed between C and LP rats (Fig. 5). In the liver, similar expression was detected between the groups in the expression of mtDNA-encoded genes (Table 4A). However, lower mtDNA/nDNA ratio was obtained in male LP animals compared to controls (Fig. 5), although the reduction in Tfam expression was not significant (p=0.10; Table 4A).

Expression of genes involved in metabolism- The mRNA level for the glucose transporter GLUT-2 was higher in LP female but similar to controls in LP male islets (Table 3B). The analysis of glucokinase, citrate synthase and malate deshydrogenase expression should give information concerning rate-limiting steps of glycolysis and tricarboxylic acid cycle (TCA) and then, on glucose energy metabolism. Expression level of genes involved in the TCA cycle was affected in LP animals with a reduction in malate deshydrogenase transcript in islets of males and females (Table 3B). Citrate synthase mRNA was significantly lower in LP males whereas the decrease did not reach statistical difference in LP females (p=0.09). Glucokinase expression was similar between groups. UCP-2 expression was evaluated for its function in the decreased metabolic efficiency of mitochondrial ATP synthesis (4) and PPARγ for its involvement in pathway leading to altered mitochondrial function in β-cell (38). In islets, both UCP-2 and PPARγ were upregulated in LP males whereas they were similar in LP and C females (Sex/diet interaction, p=0.001, p<0.001 respectively). In liver, PPARγ expression was enhanced in LP females, whereas citrate synthase and malate deshydrogenase expression was significantly downregulated (Table 4B). In male, no significant difference in gene expression was detected in liver (Sex/diet interaction, p<0.05).
DISCUSSION

In our previous research, we demonstrated that the metabolism of progeny of dams fed a low protein diet during gestation was altered (6; 10; 31; 32; 47; 57). Although the concept of fetal programming is widely accepted (7; 12; 45; 67), the mechanism is unknown, but the influence of early protein restriction on the pancreatic islet mitochondrial function of offspring may be critical (47; 57). In this study, we have examined some aspects of mitochondrial function and biogenesis in islets from 3 month-old offspring of dam fed a LP diet during gestation (LP progeny), both in males as well as in females.

Abnormal mitochondrial function can lead to glucose intolerance most often by affecting insulin secretion (62; 65). In vitro, male and female islets from control as well as LP offspring increased their insulin secretion in response to glucose but the increase was less marked in LP offspring. This smaller response could be associated with mitochondrial dysfunction since our results showed clearly that ATP production was blunted after glucose challenge in islets of both male and female LP progeny. Insulin release requires an increase in cytosolic ATP production which promotes the closure of ATP-sensitive K⁺ channels and the depolarization of the plasma membrane (14). As a consequence, cytosolic Ca²⁺ rises which will trigger the exocytosis of insulin (1; 23; 51). However, insulin secretion at 16.7 mmol/L glucose, despite a blunted ATP production lets think to alternative pathways in male and female LP islets. The classical ATP elevation-induced insulin closure of K⁺ channels may not be the only player for triggering insulin secretion (14; 29). Evidence that ROS might contribute to glucose-stimulated insulin secretion (GSIS) is emerging (25; 42). Leloup et al. (25) strongly suggested that ROS are stimulators of insulin secretion independently of other products linked to glucose metabolism such as ATP. Therefore, the considerable production of ROS in islets from male LP offspring could participate
to the insulin release observed at 16.7 mmol/l glucose in absence of higher ATP production. Moreover, it is conceivable that modification of redox state regulates insulin secretion at the exocytosis level (16). Beside the role of ATP or ROS for islet insulin secretion, other amplifying signals are proposed (18; 28; 66). The putative involvement of regulatory messengers must be clarified, such as cAMP, that we previously highlighted for its involvement in the low insulin release in islets from LP fetuses (6). In vivo, the plasma insulin level was lower in LP offspring in response to an oral glucose challenge, but in presence of a normal glucose concentration. This may be explained by the better sensitivity of the target tissues to insulin (34; 35; 54).

The higher ROS production in male islets from LP offspring is congruent with observations demonstrating the influence of early protein restriction on the adult antioxidant potential. Indeed, islets from male LP rats showed a harsh unbalance between superoxide dismutase and hydrogen peroxide-inactivating systems, which may favor hydrogen peroxide production (60).

The reduction of ATP production in LP islets can be explained by modifications in metabolic pathways. In β-cell, glucose is phosphorylated by glucokinase, which determines the rate of glycolysis and the generation of pyruvate (30). In mitochondria, pyruvate enters the TCA cycle which produces reducing equivalents that activate ATP formation (64). Glucokinase expression was similar in LP and C rats, for both sexes. However, genes coding for enzymes involved in rate-limiting steps of the mitochondrial TCA cycle, citrate synthase and malate deshydrogenase, were underexpressed in male LP animals whereas only malate deshydrogenase expression was lower in LP females. Moreover, the subunit 6 of the mitochondrial ATP synthase (ATP6), encoded by mtDNA, was downregulated in both adult LP offspring. Although differences in gene expression do not obligatorily reflect modifications in enzyme activity, these anomalies may account, at least in part, for the impairment of ATP production caused by early protein
malnutrition. Using microarray analysis, we previously described that the LP diet altered the energy metabolism in neoformed islets of LP fetuses and that maternal taurine supplementation restored all the altered gene expression (47). Contrary to what we observed in the present study, citrate synthase and malate deshydrogenase were found upregulated and ATP production was significantly increased in these LP fetal islets compared to controls. It is not the first time that perturbations during fetal life programs alterations in organ that emerge in one direction at an early age and in the opposite way later in life. The passage from a more anaerobic life such as in utero to an aerobic metabolism after birth, in which ATP synthesis through TCA in mitochondria becomes predominant, should be taken into account in this dissimilarity between fetus and adult. Those differences may also testify more plasticity in the effects of the LP diet compared to uteroplacental insufficiency where most of the consequences on mitochondrial metabolism were already acquired in the fetus (55).

The mtDNA content in pancreatic islets was similar in C and LP islets from both sexes. Our results are thus not in agreement with those reported by Park et al. (36). However, their data associating a significant reduction of mtDNA content in pancreas from LP male progeny to a lower insulin release should be reappraised. Indeed, these findings were collected using the totality of the pancreas whereas in our study, the analysis was restricted to the endocrine fraction representing only 1-2 percent of the pancreatic tissue. Lower mtDNA has been reported in adult offspring in a model of fetal growth retardation induced by uteroplacental insufficiency in rat (55). In this model, although normal mtDNA content was reported 1 week after birth, higher mtDNA content was observed in fetal stage but continuously decreased thereafter to become significantly reduced already at 7 weeks. It is conceivable that in case of maternal LP diet, without modification of oxygen supply, the process may be delayed, so that the age of 3 months
was too early to detect any effect on the mtDNA content. The control of mitochondrial biogenesis is a complex biological process that requires the interaction of multiple factors to orchestrate the programs of mitochondrial and nuclear gene expressions (43). Mitochondrial biogenesis is modulated by PPARγ coactivator-1 alpha (PGC-1α), a transcriptional coactivator of nuclear receptors and other transcription factors, including nuclear respiratory factor NRF-1. PGC-1α also affects mtDNA levels by modulating transcription of the mtDNA transcription factor A gene (Tfam) (43; 64). The expression of PGC-1α, NRF-1 and Tfam was preserved in pancreatic islets of LP male offspring whereas NRF-1 and Tfam were less expressed in LP females without consequence on the mtDNA content. Difference of desacetylase SIRT-1 expression would have explained modification in mtDNA content in LP progeny because it can enhance the role of PGC-1α in the mitochondrial biogenesis (9; 15; 48). However, SIRT-1 was similarly expressed in male and female islets of LP and C animals.

Several genes showed a sex-dependant opposite regulation. The effect of testosterone and estrogen on mitochondrial biogenesis and function in islets are not known. In brown fat cells, these hormones play a role in the control of mitochondrial biogenesis by modifying the mRNA expression of several transcription factors and other upstream stimulators of specific signaling pathways (49). A striking sex-specific difference was the mRNA level for ND4L, subunit of the complex I of the mitochondrial electron transport chain respectively, which was modified in male but unchanged in female LP offspring compared to their respective controls. These data can be linked to the ROS production that was also only significantly increased in male LP progeny. Therefore, the defective mitochondrial gene expression resulted in overproduction of ROS which in turn initiates many oxidative reactions that lead to oxidative damage (64) and may contribute to the 50% reduction of β-cell mass observed only in male LP rats. Indeed, defective
mitochondrial gene expression results in ROS production and apoptosis in mammalian cells (19) and a higher apoptotic rate was described previously in 3 month-old LP offspring (10; 32).

Another sex-specific observation was the eight times higher expression of PPARγ in male LP islets only. PPARγ is a nuclear transcription factor involved in many biochemical processes like cell cycle control, or regulation of enzymes implicated in metabolism. This strong PPARγ expression might increase ROS production through enhanced lipid uptake in cells not metabolically adjusted to handle this challenge (44). PPARγ has been reported to induce expression of UCP-2 in β-cells (38) and we observed this induction in LP male islets. In β-cells, UCP-2 transcription is modulated by various environmental factors like glucose, fatty acids (38) and hydrogen peroxide (26). UCP-2 participates in cellular defense against oxidative stress in β-cells by uncoupling substrate oxidation, and as such, it may be beneficial for male LP islets. However, by decreasing ATP biosynthesis, UCP-2 impairs GSIS and as such is detrimental to β-cell function (2; 3). Thus, modification of PPARγ and UCP-2 levels in islets from LP males may sustain the physiological consequences observed such as the increased ROS production, the blunted ATP production and weaker insulin release after glucose challenge.

Nutrient restriction in early life affected mitochondria, more in male than in female islets. This observation was already made in skeletal muscle where mitochondrial metabolic characteristics and biogenesis were altered to a greater extent in male compared to female rats following in utero placental insufficiency (21; 22; 63). Moreover, long-term consequences of maternal LP diet on glucose tolerance develop earlier in male than in female offspring (8; 13).

In conclusion, the present study underlies mitochondrial dysfunction in pancreatic islet at adult age as a consequence of a protein restriction during prenatal life. Moreover, we found that mitochondrial function is altered in LP offspring in a sex-specific manner. Although islets from
female and male LP rats presented mitochondrial dysfunction, the consequences of protein restriction during early life were more evident in males. The early protein restriction alters mitochondrial function in the absence of overt change in glucose tolerance but may provide a cellular explanation for the development of Type 2 diabetes later in life.

PERSPECTIVES AND SIGNIFICANCE

Our study shows that molecular mechanism which can explain the fetal programming due to maternal protein restriction is located, at least in part, in mitochondria. Since normal mitochondrial function is strictly required for the β-cell to secrete insulin in response to glucose challenge, alteration in mitochondrial function could trigger glucose intolerance in adult offspring of dams fed a LP diet during gestation. Furthermore, the programming appeared sex-specific since early protein deprivation affected male offspring to a greater extent than females. The involvement of mitochondrial dysfunction in LP animals when glucose intolerance is appearing requires further investigation.
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40. **Petrik J, Reusens B, Arany E, Remacle C, Coelho C, Hoet JJ and Hill DJ.** A low protein diet alters the balance of islet cell replication and apoptosis in the fetal and
neonatal rat and is associated with a reduced pancreatic expression of insulin-like growth factor-II. *Endocrinology* 140: 4861-4873, 1999.


FIGURE LEGENDS

**Fig. 1.** Plasma glucose (A, C) and insulin (B, D) response after an oral glucose tolerance test performed in 3 month-old female (A, B) and male (C, D) rats, [C, open diamond; LP, black square]; n=6 per group. Incremental areas under curves (iAUC) are shown for each graph.

**Fig. 2.** *In vitro* insulin secretion by islets from control and LP animals in response to 3.3 mmol/l (white bars) and 16.7 mmol/l (hatched bars) glucose. Data are means ± SEM, expressed as fractional insulin release; n=4-5, $ p<0.05 $ LP 16.7 vs C 16.7. Folds of increase insulin secretion between 3.3 mmol/l and 16.7 mmol/l are indicated for each experimental group.

**Fig. 3.** *In vitro* ATP concentration in control and LP islets after 3.3 mmol/l (white bars) and 16.7 mmol/l (hatched bars) glucose challenge. Data are means ± SEM, expressed as pmol ATP per µg of proteins, n=4-5; **p<0.01.

**Fig. 4.** ROS concentration in the presence of 5.5 mmol/l (white bars) or 16.7 mmol/l (hatched bars) glucose in cultured islets of C and LP rats. Data are means ± SEM, expressed as fluorescence normalized to µg of protein; n=5-6. *p<0.05, ***p<0.001 LP vs respective C.

**Fig. 5.** Mitochondrial DNA/nuclear DNA ratio in islets and liver from LP rats, males (white), females (grey). Data are expressed as percentage of control and values are means ± SEM; n=5-7 (islets), n=4-6 (liver). *p<0.05.
<table>
<thead>
<tr>
<th>Gene</th>
<th>5'-Primer (F)</th>
<th>3'-Primer (R)</th>
<th>Amplicon size</th>
<th>Accession number</th>
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<tr>
<td>GAPDH</td>
<td>TGA CTC TAC CCA CGG CAA GTT</td>
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<td>UCP2</td>
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<td>GAG GTC GTC TGT CAT GAG GTT G</td>
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<tr>
<td>Citrate synthase</td>
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<td>Malate deshydrogenase</td>
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<td>TCC AAT CGA GTG AGG CAA CTG</td>
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<td>NM_033235.1</td>
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</table>

GAPDH, Glyceraldehyde-3-phosphate deshydrogenase; PGC-1α, PPAR coactivator 1 alpha; NRF-1, nuclear respiratory factor 1; Tfam, mitochondrial transcription factor A; SIRT1, mammalian silencing information regulator 2α; COX-1, cytochrome c oxidase subunit 1; ND4L, NADH-ubiquinone oxireductase subunit 4L; ATP6, ATP synthase subunit 6; UCP2, uncoupling protein 2; PPARγ, peroxisome proliferator-activated receptor gamma;
Table 2. Plasma analytes, beta-cell mass and PIC measured in fasted female and male offspring at 3 months of age

<table>
<thead>
<tr>
<th></th>
<th>Females</th>
<th></th>
<th>Males</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>C</td>
<td>LP</td>
<td>P value</td>
<td>Diet effect</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.01 ± 0.15</td>
<td>4.80 ± 0.21</td>
<td>ns</td>
<td>5.06 ± 0.14</td>
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<tr>
<td>Insulin (pmol/l)</td>
<td>103.6 ± 17.2</td>
<td>63.75 ± 3.5</td>
<td>ns</td>
<td>153.3 ± 14.8</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.40 ± 0.03</td>
<td>0.42 ± 0.01</td>
<td>ns</td>
<td>0.42 ± 0.02</td>
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<tr>
<td>Cholesterol (mmol/l)</td>
<td>0.96 ± 0.08</td>
<td>1.00 ± 0.05</td>
<td>ns</td>
<td>0.73 ± 0.07</td>
</tr>
<tr>
<td>Beta-cell mass (mg)</td>
<td>32.73 ± 4.46</td>
<td>21.82 ± 3.7</td>
<td>ns</td>
<td>47.97 ± 7.7</td>
</tr>
<tr>
<td>PIC (pmol/mg)</td>
<td>17.68 ± 0.8</td>
<td>23.28 ± 2.8</td>
<td>p&lt;0.001</td>
<td>16.68 ± 1.4</td>
</tr>
</tbody>
</table>

PIC, pancreatic insulin content. Values are expressed as means ± SEM; ns, not significant.
Table 3. Expression of genes involved in mitochondrial biogenesis and function (A) and metabolism (B), in pancreatic islets from 3 month-old male and female LP offspring

<table>
<thead>
<tr>
<th></th>
<th>Females</th>
<th></th>
<th></th>
<th>Males</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LP (n=6)</td>
<td>P value</td>
<td>LP (n=5)</td>
<td>P value</td>
<td>Diet effect</td>
</tr>
<tr>
<td><strong>A</strong></td>
<td></td>
<td>Diet effect</td>
<td></td>
<td></td>
<td>Diet effect</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>0.79 ± 0.14</td>
<td>ns</td>
<td>1.07 ± 0.20</td>
<td>ns</td>
<td></td>
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<tr>
<td>NRF-1</td>
<td>0.57 ± 0.06</td>
<td>p&lt;0.01</td>
<td>1.03 ± 0.10</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Tfam</td>
<td>0.65 ± 0.07</td>
<td>p&lt;0.05</td>
<td>1.78 ± 0.47</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>SIRT-1</td>
<td>0.93 ± 0.10</td>
<td>ns</td>
<td>0.67 ± 0.09</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>ND4L</td>
<td>0.79 ± 0.16</td>
<td>ns</td>
<td>2.19 ± 0.27</td>
<td>p&lt;0.01</td>
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<tr>
<td>COX1</td>
<td>0.68 ± 0.06</td>
<td>ns</td>
<td>1.42 ± 0.18</td>
<td>ns (p=0.05)</td>
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<tr>
<td>ATP6</td>
<td>0.49 ± 0.05</td>
<td>p&lt;0.01</td>
<td>0.60 ± 0.10</td>
<td>p&lt;0.05</td>
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<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>UCP-2</td>
<td>0.79 ± 0.08</td>
<td>ns</td>
<td>1.42 ± 0.17</td>
<td>p&lt;0.05</td>
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<tr>
<td>PPARγ</td>
<td>1.49 ± 0.28</td>
<td>ns</td>
<td>8.45 ± 2.94</td>
<td>p&lt;0.01</td>
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<td>GLUT-2</td>
<td>1.30 ± 0.11</td>
<td>p&lt;0.05</td>
<td>0.82 ± 0.08</td>
<td>ns</td>
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</tr>
<tr>
<td>Glucokinase</td>
<td>0.99 ± 0.16</td>
<td>ns</td>
<td>0.69 ± 0.07</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>0.72 ± 0.08</td>
<td>ns</td>
<td>0.51 ± 0.03</td>
<td>p&lt;0.05</td>
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</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>0.67 ± 0.08</td>
<td>p&lt;0.05</td>
<td>0.67 ± 0.07</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Numbers below 1 are downregulated and numbers above 1 are upregulated in LP islets vs C.
Table 4. Expression of genes involved in mitochondrial biogenesis and function (A) and metabolism (B), in liver from 3 month-old male and female LP offspring

<table>
<thead>
<tr>
<th></th>
<th>Females</th>
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<tbody>
<tr>
<td></td>
<td>LP (n=6)</td>
<td>P value Diet effect</td>
</tr>
<tr>
<td>Tfarm</td>
<td>0.85 ± 0.07</td>
<td>ns</td>
</tr>
<tr>
<td>ND4L</td>
<td>0.89 ± 0.07</td>
<td>ns</td>
</tr>
<tr>
<td>COX1</td>
<td>1.02 ± 0.09</td>
<td>ns</td>
</tr>
<tr>
<td>ATP6</td>
<td>1.25 ± 0.17</td>
<td>ns</td>
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<table>
<thead>
<tr>
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<tr>
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<td>p&lt;0.05</td>
</tr>
<tr>
<td>Citrate synthase</td>
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<td>p&lt;0.05</td>
</tr>
<tr>
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<td>0.78 ± 0.03</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

Numbers below 1 are downregulated and numbers above 1 are upregulated in LP islets vs C.