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Maternal malnutrition programs pancreatic islet mitochondrial dysfunction in the adult offspring☆,☆☆

Nicolas Theys, Marie-Thérèse Ahn, Thomas Bouckenooghe, Brigitte Reusens, Claude Remacle*

Laboratory of Cell Biology, Institute of Life Sciences, Université catholique de Louvain, Louvain-la-Neuve, Belgium

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Abstract

Accumulating evidence has shown that maternal malnutrition increases the risk of metabolic disease in the progeny. We previously reported that prenatal exposure to a low-protein diet (LP) leads to mitochondrial dysfunction in pancreatic islets from adult rodent offspring that could relate physiological and cellular alterations due to early diet. We aim to determine whether mitochondrial dysfunction could be a common consequence of prenatal nutritional unbalances. Pregnant Wistar rats received either a global food restriction (GFR), consisting in the reduction by 50% of the normal daily food intake, or a high-fat diet (HF) throughout gestation. GFR or HF diet during pregnancy leads to a lack of increase in insulin release and ATP content in response to glucose stimulation in islets from 3-month-old male and female offspring. These similar consequences originated from impairment in either glucose sensing or glucose metabolism, depending on the type of early malnutrition and on the sex of the progeny. Indeed, the glucose transport across β-cell membrane seemed compromised in female HF offspring, since GLUT-2 gene was markedly underexpressed. Additionally, for each progeny, consequences downstream the entry of glucose were also apparent. Expression of genes involved in glycolysis, TCA cycle and oxidative phosphorylations was altered in GFR and HF rats in a sex- and diet-dependent manner. Moreover, prenatal malnutrition affected the regulators of mitochondrial biogenesis, namely, PPAR coactivator 1 alpha (PGC-1α), since its expression was higher in islets from GFR rats. In conclusion, programming of mitochondrial dysfunction is a consequence of maternal malnutrition, which may predispose to glucose intolerance in the adult offspring.

Keywords: Programming; Nutrition; Insulin secretion; Pancreatic islet

1. Introduction

Intra-uterine nutritional disturbances can lead to a cluster of metabolic abnormalities in the adult offspring. This concept is known as fetal programming [1]. Several epidemiological and experimental evidences have shown a convincing relationship between poor intra-uterine nutrition and the subsequent development of metabolic disorders like insulin resistance, obesity and cardiovascular disease in adulthood [2–8]. Nowadays, the mechanistic basis of this concept remains unclear.

Since a few years, attention has focused on the involvement of mitochondria as putative targets linking physiological and cellular consequences of biochemical disorders during early life [9–12]. Experimental studies have proposed that an adaptation of mitochondrial function may confer an advantage for the development of the fetus in an imbalanced nutritional intra-uterine milieu, but after birth, owing to the inconsistency of postnatal experience, the adaptation may become detrimental, rendering the individual more prone to developing metabolic diseases including type 2 diabetes [9–14].

Recently, we demonstrated that mitochondrial function was altered in pancreatic islets from adult offspring of dams fed a low-protein diet (LP) during gestation [15]. A lower insulin release and a blunted ATP content in response to glucose stimulation suggested mitochondrial dysfunction in pancreatic islets of LP offspring. These physiological consequences were associated with alterations in the expression of genes involved in mitochondrial energetic metabolism such as tricarboxylic acid cycle (TCA) and electron transport chain (ETC). Indeed, malate dehydrogenase (MD) and the subunit 6 of ATP synthase, which is encoded by the mitochondrial DNA (mtDNA), were found significantly down-regulated in islets from progeny of LP dams lowering their capacity for ATP biosynthesis.

Recent evaluations of the fetal origin of obesity, insulin resistance and vascular dysfunction revealed differences between sexes on the

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☆☆ NT, BR and CR have conceived and designed the experiments, analyzed the data and wrote the manuscript. NT, MTA and TB performed experiments and were involved in discussion of results.

* Corresponding author. Place Croix du Sud, 5 box 2, B-1348 Louvain-la-Neuve, Belgium. Tel.: +3210473522; fax: +3210473515.

E-mail address: claude.remacle@uclouvain.be (C. Remacle).

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issue [8,16–19], which could explain the sex-dependent time-course development of metabolic disorders. In early protein-restricted adult offspring, mitochondrial function was also altered in both sexes but to a greater extent in males [15], since the level of reactive oxygen species (ROS), which are produced mainly by the mitochondria, was specifically increased in male LP rats, the sex that develops earlier glucose intolerance [6,7].

Can different disturbances of the metabolic intrauterine milieu program mitochondrial dysfunction in pancreatic islets of the adult offspring or is this restricted to specific nutritional aberration such as protein restriction? We have thus used two contrasting models of maternal malnutrition in the rat. The first one was a global food restriction (GFR) and the second one, a high-fat diet (HF), given to pregnant rat from the first day of gestation until delivery. The GFR consisted in the reduction by 50% of the daily food intake of the pregnant rat throughout gestation. This undernutrition during fetal life is known to affect the development of the endocrine pancreas [20] and predisposes adult offspring to glucose intolerance [21]. The HF diet consisted in an increase in maternal dietary intake of animal fat during pregnancy. HF diet during early life induced metabolic abnormalities in adult offspring with shared characteristics of the metabolic syndrome in human [12,22–24]. Thus, this work aimed to determine whether mitochondrial dysfunction could be the common consequence of *in utero* imbalance and may predispose to glucose intolerance in adult offspring of malnourished dams. Mitochondrial function of pancreatic islets was assessed in 3-month-old offspring of both sexes, before the appearance of glucose intolerance, by measurement of ROS and ATP content in correlation with *in vitro* and *in vivo* analysis of islet function. These parameters were associated with molecular analysis of the expression of genes encoded by nuclear or mitochondrial DNA and involved in mitochondrial function. Moreover, mtDNA content was assessed as well as the expression of regulatory factors of mitochondrial biogenesis.

2. Methods and materials

2.1. Animals

Adult virgin 3-month-old female 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 access to their respective diets and to water. Three groups of pregnant rats were followed. The control group (C group) in which rats were fed *ad libitum* a control diet containing 20% protein; the global food restriction group (GFR group), where 11 g/day (147.3 kJ/day) of the 20% protein diet was given to the animals, corresponding to 50% of the daily food intake of control pregnant rats; females of a third group, which were fed *ad libitum* a diet rich in animal fat (high fat, HF group). The composition of the control diet has been described previously [25]; HF was composed of 10% simple sugars (w/w), 29% polysaccharide, 23% fat (17% animal lard), 23% protein and energy of 18.83 kJ/g. After parturition, all litters were sexed and standardized to eight pups (four males and four females when possible) and fed with the control diet. Food intake and body weight (BW) of dams were assessed every 3 days during gestation/lactation and also of offspring after weaning. Animals were housed individually until sacrifice by decapitation at 3 months of age. All procedures were carried out in accordance with the "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.

2.2. Islets collection

After obstruction of the junction of the common bile duct with the 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 Hank's Balanced Salt Solution (HBSS, 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).

2.3. Liver collection

Livers were removed rapidly after decapitation, weighted, washed in HBSS, frozen and stored at −80°C until utilized.

2.4. Oral glucose tolerance test

Insulinemic and glycemic responses to an oral glucose tolerance test (OGTT) were determined in 3-month-old male and female offspring after an overnight fast according to Merezak et al. [26]. After collection of the first blood sample, 0.7 ml/100 g BW of a 50% glucose solution (w/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 area under the curve (AUC) of glucose and insulin.

2.5. 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 $HClO_4$ (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 (Mercodia, Uppsala, Sweden). Triglycerides and total cholesterol concentrations were determined by using the TRF400CH and CTF400CH kits, respectively, following the manufacturer's instruction (Chema Diagnostica, Jesi, Italy).

2.6. Pancreas processing for immunohistochemistry and pancreatic insulin content

Pancreas was removed, weighed and dissected. The splenic part 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 [0.15 M HCl in 75% (v/v) ethanol in water] to extract insulin. Pancreatic insulin content (PIC) was determined using the High Range Rat Insulin ELISA (Mercodia).

2.7. Immunohistochemistry and morphometry measurements

Tissue sections were rehydrated and incubated for 1 h with a blocking buffer [0.1% (v/v) Tween 20–3% (v/v) BSA in Tris-buffered saline] before an overnight incubation at 4°C with mouse anti-insulin antibody (1/6000; Sigma, St. Louis, MO, USA). After washing, samples were incubated with biotin-conjugated anti-mouse secondary antibody (1/2000; Chemicon, Temecula, CA, USA). The complex was revealed using peroxidase-conjugated streptavidin (1/1000; Amersham Pharmacia Biotech Europe, Saclay, France). Peroxidase was detected using diaminobenzidine (Sigma-Aldrich, Bornem, Belgium). The insulin-positive area was morphometrically measured on six sections per animal using the Zeiss KS 400 3.0 software (Carl Zeiss GmbH, Jena, Germany). The β-cell mass was obtained by multiplying the volume density of β cells in pancreas by the weight of the pancreas.

2.8. ATP Measurements

Total cellular ATP content was determined in islets after 3 days of culture by chemiluminescence using a reagent based on luciferase reaction as described previously [15,27]. Briefly, islets were washed and incubated in Krebs-Ringer buffer (KRB; 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $NaHCO_3$) without glucose at 37°C in 5% CO_2/O_2 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 of incubation, islets were transferred in cold PBS 0.1 M and lysis buffer for ATP extraction. Samples were immediately placed on ice. Islets were sonicated during 30 s in a water bath to ensure complete lysis of cells. Islet lysate ATP content was measured using an ATP luminescent assay kit (Promega, Madison, WI, USA) using 50 μl in duplicate. ATP was calculated per microgram of protein in the islet homogenate.

2.9. Insulin secretion study

These experiments were performed using a KRB supplemented with 5 g/L bovine serum albumin (Fraction V, Calbiochem-Behring, San Diego, CA, USA) and glucose. This solution was gassed for 10 min with 95% O_2/5% CO_2 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 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 (30 s, 40 W) in 0.5-ml acid–ethanol [0.15 M 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 of the experiment to the amount of released insulin.

2.10. Fluorescence measurement of ROS

ROS content in islets was measured using chloro-methyl dichlorofluorescein 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 or 16.7 mmol/L glucose. Islets were then loaded with 10 μM CM-H_2DCFDA for 3 h at 37°C. At the end of the experiment, islets were rinsed and disrupted using PBS–1% Triton (Sigma). 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 microgram of islet protein.

2.11. 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 SYBR Green master mix according to the supplier protocols (Westburg, Leusden, The Netherlands). Primer sequences (Sigma) are presented in Table 1. The level of mRNA expression was calculated using the threshold cycle (C_t) value, the number of PCR cycles at which the fluorescence signal during the PCR reaches a fixed threshold. For each sample, the C_t value both for the gene of interest and for the internal standard housekeeping gene *Gapdh* was determined to calculate $\Delta C_{t,\text{sample}}$ ($C_{t,\text{ target gene}} - C_{t,\text{ housekeeping gene}}$). The expression level was reported to a calibrator consisting of cDNA from control rats. Subsequently, $\Delta\Delta C_t$ ($\Delta C_{t,\text{sample}} - \Delta C_{t,\text{calibrator}}$) was determined, and the relative expression levels were calculated from $2^{-\Delta\Delta C}{}_t$ according to the manufacturer's instructions (Applied Biosystems, Lennik, Belgium). RNA expression levels are thus indicated as arbitrary units±S.E.M.

2.12. Mitochondrial DNA analysis

We used quantitative real-time PCR to determine the amount of nuclear DNA (nDNA) relative to mtDNA. Total DNA was extracted from islets using the 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 1.

2.13. Statistical analysis

Results were reported as means±S.E.M. Statistical analyses were performed using *t* test. Two-way ANOVA followed by Bonferroni post-test was used to discriminate diet and glucose concentration effects (GraphPad Software, Inc., San Diego, CA, USA). A *P* value of less than .05 was considered as statistically significant.

3. Results

3.1. Dams: body weight and food intake during gestation and lactation

Before gestation, rats of the three experimental groups had a similar BW (C 285±6 g, GFR 290±7 g, HF 269±11 g, n=7–8). During pregnancy, maternal BW increased similarly in C and HF dams until Day 17. However, just before delivery, HF dams featured a significant lower BW than C dams (P<.01). GFR pregnant rats did not gain weight until 17 days of pregnancy and therefore presented a lower BW than C (P<.01) at this stage, which was recovered at Day 21, before delivery. No difference in litter size was observed between groups (on average, C 11.9±0.7, GFR 11.3±1.1, HF 10.3±1 pups per litter). After 1 week of lactation, BW was similar between the three groups and increased similarly until weaning (C 317±7 g, GFR 311±9 g, HF 301±11 g). Calorific intake during gestation was similar in C and HF dams (C 285.5±10 *vs.* HF 319±27 kJ/day), whereas it was experimentally significantly reduced for GFR rats (GFR 147.3 kJ/day). During lactation, daily food intake did not differ between C, GFR and HF groups (C 598.7±25, GFR 665.7±54, HF 552.6±25 kJ/day).

3.2. Offspring: body weight and food intake

At birth, both female and male GFR offspring showed growth retardation (females: C 6.6±0.06 *vs.* GFR 5.6±0.09 g, P<.001; males: C 6.95±0.08 *vs.* GFR 5.8±0.08 g, P<.001; n=27–41). The GFR rats caught up progressively with a normal BW and, at 3 months, no difference was observed anymore when compared with the C group. At birth and at weaning, HF neonates featured a BW similar to that of C animals. At 3 months, HF females were heavier than C (C 240.3±2.2 *vs.* HF 252±4.2 g, P<.05; n=21–22), whereas HF and C males presented a similar BW (C 391.4±7 *vs.* HF 363.6±15.3 g, n=12-15). No difference was observed in the food intake for both female and male GFR and HF offspring compared to C, from weaning to 3 months (data not shown).

3.3. Plasma analysis at 3 months

As shown in Table 2A, the maternal diet during early life had no effect on the fasting plasma glucose level of the adult male and female offspring. Fasting plasma insulin level was also similar in females of the three groups. Male GFR featured a normal plasma insulin level but it was significantly lower in HF males compared to controls. Maternal diet had no effect on triglyceride concentration of both sexes. No significant difference in cholesterol level was observed between C and

Table 1
Primer sequences for genes investigated, amplicon size and GenBank accession number

Gene	Primers		Amplicon size	Genbank
	Sequence (F)	Sequence (R)		
GAPDH	TGA CTC TAC CCA CGG CAA GTT	CTT CCC ATT CTC AGC CTT GAC T	52	NM_017008
PGC-1α	GTT CCC GAT CAC CAT ATT CCA	TGT GCG GTG TCT GTA GTG GC	104	NM_031347
NRF-1	CTC ACC CTC CAA ACC CAA CC	CCA GCG CAG ACT CCA GGT C	110	XM_231566
Tfam	GGC GAA GTG ATC TCA TCC G	TTC TTG TGC CCA ATC CCA A	101	NM_031326
SIRT-1	TGT TCG AGG ATC GGT GCC	CAG AGC ATC ACA CGC AAG CT	103	XM_001080493
COX1	ATC TAT AGG AGC AGT ATT CGC C	TGG GCT TTT GCT CAT GTG TC	96	U03388
ND4L	TCC CAA TTA CCA TTC TAG TTT T	AGG TTT TGT ACG TAG TCT GTT CCG T	103	EU104720
ATP6	TCG CTT CGC TTT GTA CTC TCT T	GGT CAG CAG TCT CTG TGC ATT T	122	BC059121
UCP2	CTC CCA ATG TTG CCC GAA A	GAG GTC GTC TGT CAT GAG GTT G	144	AB005143
PPARγ	CTT CAA ACT CCC TCA TGG CCA	GTT CTT CGG AAA AAA CCC TTG C	104	NM_013124
Glut 2	TGC TTC CAG TAC ATT GCG GA	AGG ACC ACC CCA GCA AAA A	65	NM_012879
Glucokinase	CCC TCC AAG AGA CCA CCC A	CCA GGT AGA GAG GCT GGC ATT	53	NM_012565
Citrate synthase	ACC CAG CCA ATA CAA ACT GCC T	TGA GAT TTT GCT CGG TTG TGG	106	NM_130755
Malate dehydrogenase	AGC CTT GGA GAA GTA CGC CAA	TCC AAT CGA GTC AGG CAA CTG	135	NM_033235.1

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; 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 oxidoreductase subunit 4L; ATP6, ATP synthase subunit 6; UCP2, uncoupling protein 2; PPARγ, peroxisome proliferator-activated receptor gamma.

Table 2
Plasma analytes measured in fasted female and male offspring at 3 months of age

	Females			Males		
	C (n=5-7)	GFR (n=4-7)	HF (n=6-8)	C (n=5-7)	GFR (n=6)	HF (n=6)
A						
Glucose (mmol/L)	5.01±0.15	4.80±0.19	5.14±0.18	5.06±0.14	5.19±0.15	5.26±0.18
Insulin (pmol/L)	103.6±17	94.4±13.7	104.1±21	153.3±14.8	138.7±7.8	85.9±4.9**
Triglycerides (mmol/L)	0.40±0.03	0.44±0.02	0.43±0.04	0.42±0.02	0.52±0.03	0.44±0.03 (n=3)
Cholesterol (mmol/L)	0.96±0.08	1.27±0.09*	0.97±0.08	0.73±0.07	1.04±0.09	0.83±0.13 (n=3)
B						
β-Cell mass (mg)	32.73±4.46	18.89±2.41*	52.7±6.1*	47.97±7.7	24.95±2.76*	45.02±3.84
PIC (pmol/mg)	17.68±0.8	20.26±2.2	18.86±2.26	16.68±1.4	23±2.17†	18.56±2.03

Values are expressed as means±S.E.M.
* P<.05.
** P<.01 *vs.* C.
† P=.06 *vs.* C.

HF animals, whereas cholesterol level was higher in GFR females and tended to be increased in GFR males (P=.05) compared to their respective controls.

3.4. Morphometrical analysis and pancreatic insulin content at 3 months

The pancreas of the 3-month-old offspring 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 PIC measurement. In GFR adult progeny, the β-cell mass (volume density of β cells*weight of pancreas) was strongly reduced in both sexes (Table 2B). β-Cell mass did not differ between HF and C males, whereas it was significantly higher for HF females compared to C (Table 2B). High-fat diet during gestation did not influence the PIC of the adult progeny. GFR and C females showed a similar PIC, whereas it tended to be higher in GFR males compared to C (P=.06).

3.5. Oral glucose tolerance test at 3 months

Plasma insulin and glucose concentrations measured at different times after an oral glucose challenge are shown in Fig. 1. Both sexes of HF and GFR progeny showed a normal plasma glucose concentration during the test and the AUC was similar to controls (data not shown). No difference was observed for the evolution of the plasma insulin level during the glucose challenge (two-way ANOVA, no diet effect), and the AUC was similar between the groups (data not shown).

3.6. In vitro insulin secretion

Insulin secretion was measured in islets after 3 days of culture (Fig. 2). At 3.3 mmol/L glucose, islets from female and male GFR offspring released significantly more insulin than their respective controls. In response to 16.7 mmol/L glucose, insulin release was significantly

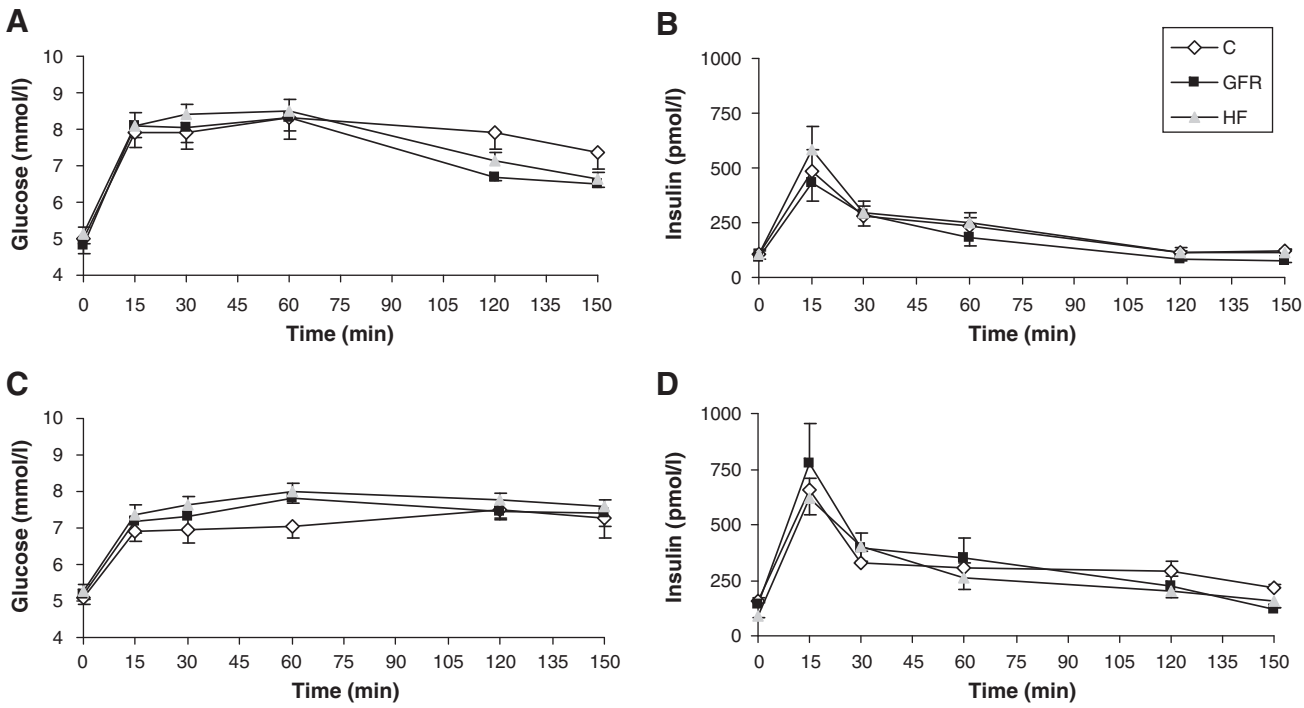

Fig. 1. Plasma glucose (A, C) and insulin (B, D) response after an OGTT performed in 3-month-old female (A, B) and male (C, D) rats (control, open diamond; GFR, black square; HF, grey triangle); n=6 per group.

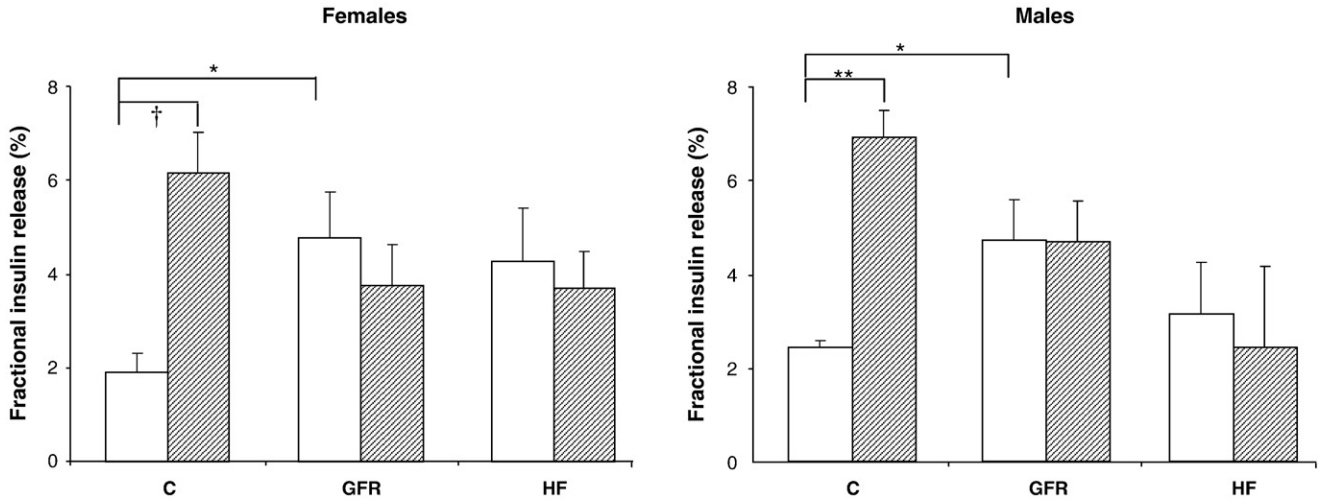

Fig. 2. *In vitro* insulin secretion by islets from female and male offspring of control, GFR and HF dams in response to 3.3 mmol/L (white bars) and 16.7 mmol/L (hatched bars) glucose. Data are means±S.E.M., expressed as fractional insulin release; n=4–7. *P<.05; **P<.01 LP *vs.* C; †P=.05; $$P$<.05 LP 16.7 *vs.* C 16.7.

increased in C male and female islets. This response to glucose was impaired in islets from HF and GFR male and female animals.

3.7. ATP Content

Under glucose stimulation (16.7 mmol/L), ATP content in the C islets of both sexes was significantly increased (Fig. 3). In contrast, GFR and HF male and female animals did not increase their islet ATP content in response to glucose, which was significantly lower than C (two-way ANOVA, glucose concentration effect). At low glucose concentration, ATP content was reduced in HF islets compared to C. This was true for female as well as for male progeny (two-way ANOVA, diet effect).

3.8. ROS Content

In female, no diet effect on ROS production was observed when islets were incubated in low glucose concentration, but a diet effect was detected by two-way ANOVA in high glucose concentration (Fig. 4). Indeed, ROS production at 16.7 mmol/L glucose was lower in both GFR and HF female islets compared to C. In male, in addition to a diet effect at 16.7 mmol/L glucose observed only for HF animals, the early malnutrition led to a higher ROS production at 5.5 mmol/L glucose in both GFR and HF islets.

3.9. Mitochondrial DNA content and expression of genes involved in mitochondrial biogenesis and function

In islets, mtDNA content was significantly increased in both female and male GFR animals compared to controls. In HF female, it tended to be higher (P=.07) than in C islet but was similar in HF male (Fig. 5A). We evaluated the expression of PPAR coactivator 1 alpha (PGC-1α), nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (Tfam) because these factors provide a molecular basis for the connection between environmental stimuli and mitochondrial biogenesis [28,29]. The diet during gestation affected differently the gene

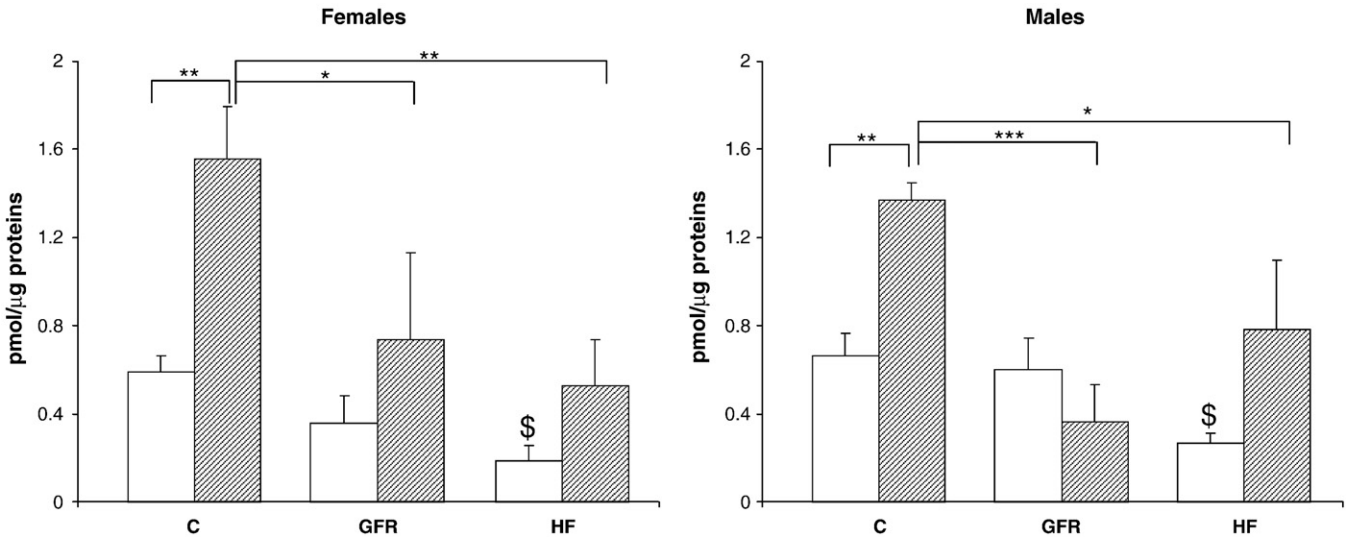

Fig. 3. *In vitro* ATP content in islets from female and male offspring of control, GFR and HF dams in response to 3.3 mmol/L (white bars) and 16.7 mmol/L (hatched bars) glucose. Data are means±S.E.M., expressed as picomoles of ATP per micrograms of proteins, n=4–7; *P<.05, **P<.01, ***P<.001. $$P$<.05 *vs.* C 3.3 mmol/L glucose.

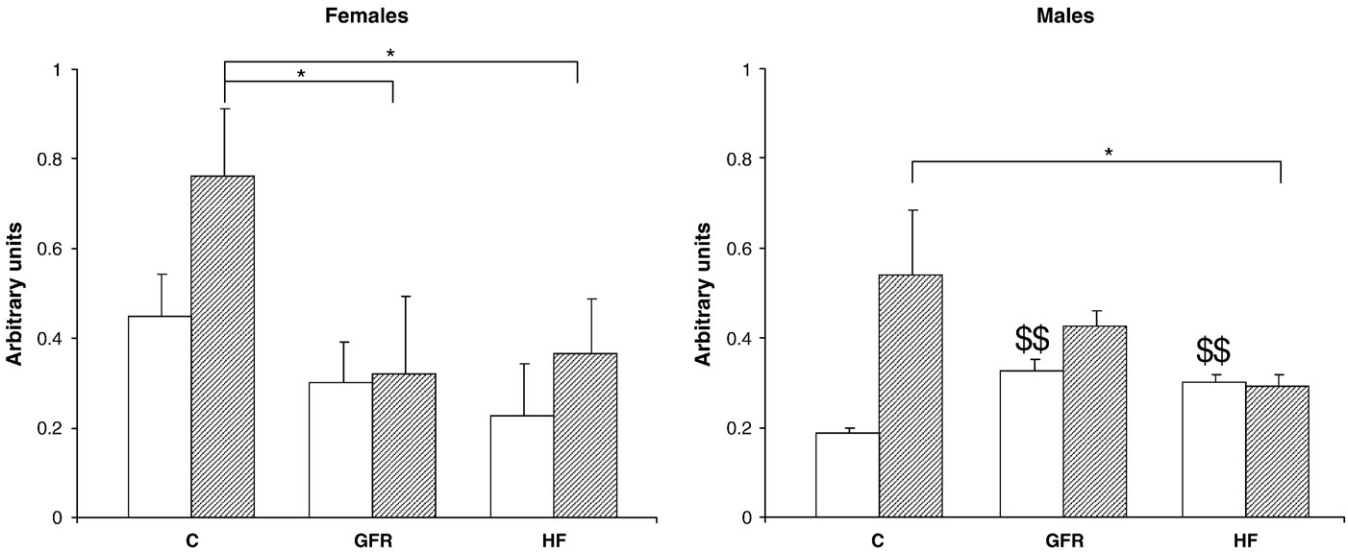

Fig. 4. ROS accumulation in the presence of 5.5 mmol/L (white bars) or 16.7 mmol/L (hatched bars) glucose in cultured islets of female and male offspring from C, GFR and OB dams. Data are means±S.E.M., expressed as fluorescence normalized to micrograms of protein; n=5–6. *P<.05, ***P<.001 LP *vs.* respective C. $^{\$\$}P$<.01 *vs.* C 3.3 mmol/L glucose.

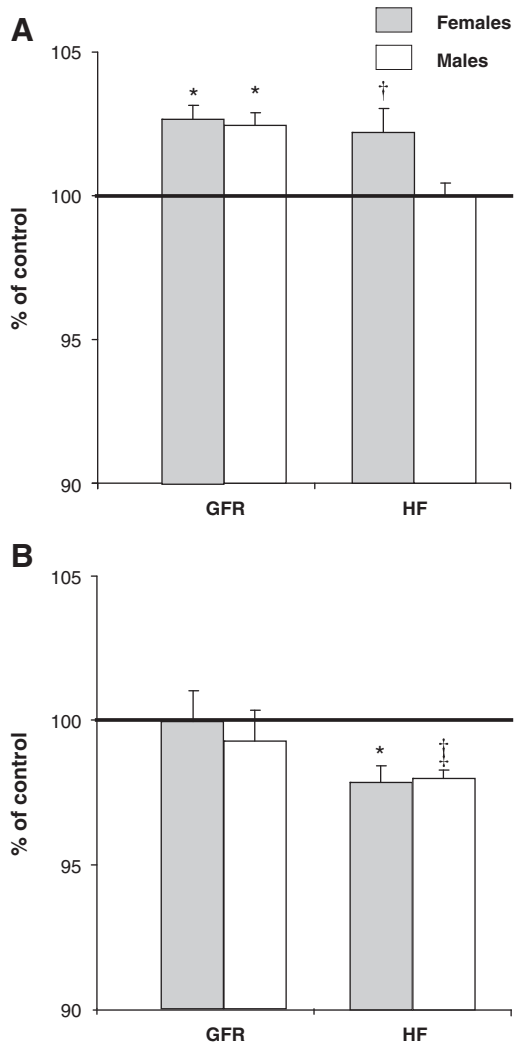

Fig. 5. Female and male mitochondrial DNA/nuclear DNA ratios in islets (A) and liver (B) from offspring of GFR and HF dams (males, white bars; females, grey bars). Data are expressed as percentage of C, and values are means±S.E.M.; n=5–7 (islets), n=4–6 (liver). *P<.05, $^{\dagger}P$=.07, $^{\ddagger}P$=.06 *vs.* C.

expression in organs of the adult progeny. In islets from GFR animals, PGC-1α mRNA was significantly up-regulated in males as well as in females, whereas expression of Tfam was significantly down-regulated (Table 3A). These two genes were, however, normally expressed in HF islets from both sexes. NRF-1 level was significantly higher in HF female than in C. Mitochondrial genome encodes for essential proteins which are all components of the ETC [30]. We have chosen to analyze the expression of three of them, namely, ND4L, COX-1 and ATP6, known to be affected in other models of fetal programming such as uterine artery ligation [9] and protein restricted diet [15]. ND4L is a subunit of Complex 1, whereas COX-1 is a subunit of Complex 4 of the ETC. ATP6 is a subunit of the mitochondrial pump ATP synthase [30]. Transcript level of mtDNA-encoded genes ND4L and COX-1 was unchanged in both GFR and HF male and female animals (Table 3A). However, the level of ATP6 expression was affected by the maternal malnutrition. Indeed, it dramatically declined in male and female GFR offspring as well as in HF females,

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 GFR and HF offspring

	Females		Males	
	GFR	HF	GFR	HF
A				
PGC-1α	1.43±0.12*	0.77±0.20	2.01±0.27**	1.28±0.18
NRF-1	0.78±0.22	1.74±0.24*	0.80±0.05	1.32±0.24
Tfam	0.72±0.06*	1.17±0.26	0.66±0.07**	1.14±0.09
SIRT-1	1.32±0.34	1.62±0.40	0.64±0.05	1.13±0.17
ND4L	1.38±0.12	0.81±0.11	0.78±0.12	0.98±0.08
COX1	0.81±0.06	0.90±0.11	0.97±0.08	0.89±0.07
ATP6	0.56±0.07**	0.45±0.05***	0.64±0.04**	1.75±0.15**
B				
UCP-2	1.11±0.08	0.62±0.05***	0.78±0.06*	0.61±0.12*
PPARγ	1.92±0.40 (P=.07)	1.74±0.33 (P=.09)	3.09±0.46**	4.30±1.20*
GLUT-2	1.21±0.10	0.17±0.08***	1.48±0.12*	0.96±0.20
Glucokinase	0.79±0.06	0.35±0.07**	0.38±0.04*	2.12±0.40
Citrate synthase	0.76±0.05	0.58±0.11*	1.65±0.18*	1.31±0.23
Malate dehydrogenase	0.52±0.04**	0.46±0.05***	1.45±0.07**	1.15±0.16

Numbers below 1 are down-regulated and numbers above 1 are up-regulated in GFR or HF islets *vs.* C; n=6 per group. *p <.05, **p <.01, ***p <.001 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 GFR and HF offspring

	Females		Males	
	GFR	HF	GFR	HF
A				
Tfam	0.75±0.09	0.44±0.01***	0.73±0.11	0.57±0.05
ND4L	1.01±0.23	0.66±0.02***	2.11±0.38**	2.16±0.26**
COX1	0.92±0.22	0.97±0.05	1.63±0.15**	1.59±0.14*
ATP6	0.80±0.19	0.49±0.01***	1.33±0.08	1.23±0.09
B				
PPARγ	0.65±0.06 (P=.06)	0.66±0.26	0.82±0.33	0.69±0.32
Citrate synthase	0.69±0.11*	0.55±0.05**	0.51±0.05**	0.52±0.13**
Malate dehydrogenase	0.88±0.10	0.35±0.15**	0.62±0.04**	0.66±0.05**

Numbers below 1 are down-regulated and numbers above 1 are up-regulated in GFR or HF islets *vs.* C; $n=5$ (males GFR) or 6 per group. *p <.05, **p <.01, ***p <.001 vs C.

whereas it was significantly increased in HF male compared to their respective controls.

In the liver, mtDNA content was significantly reduced in HF females compared to C (Fig. 5B). This reduction was associated with a lower Tfam gene expression (Table 4A). In HF male, liver mtDNA content tended to be lower but no change in Tfam mRNA level was observed (Fig. 5B). Expression of mtDNA-encoded genes was affected in HF liver but in an opposite way in males and females. Indeed, ND4L and ATP6 were underexpressed in female HF, whereas in male, ND4L and COX-1 were up-regulated (Table 4A). In GFR progeny, a mtDNA content similar to C was detected in both sexes (Fig. 5B). The Tfam transcript level was also similar in GFR and C liver in both sexes. In male GFR liver, the level of ND4L and COX-1 mRNA expression was higher compared to C, whereas no difference was observed for female GFR progeny. No difference in ATP6 expression was found in GFR for male and female progeny.

3.10. Expression of genes involved in metabolism

In general, female islets were more affected by the maternal HF diet, whereas male islets were more altered by the maternal GFR diet. The mRNA level for the glucose transporter GLUT-2 was reduced by more than 80% in HF female but was similar to C in HF male islets (Table 3B). In GFR offspring, islets from males showed a significantly higher expression of GLUT-2. The analysis of glucokinase, citrate synthase (CS) and MD expression should provide information concerning the rate-limiting steps of glycolysis and TCA and, therefore, on glucose energy metabolism. Glucokinase expression was significantly reduced in HF females and in GFR males compared to their respective C. Expression level of genes involved in the TCA cycle was not affected in HF males but was modified in HF females for which a reduction in CS and MD transcripts was found. In GFR animals, CS and MD were up-regulated in males, whereas MD expression was significantly lower in female islets than in C. UCP-2 expression was evaluated for its function in the decreased metabolic efficiency of mitochondrial ATP synthesis [31] and PPARγ for its involvement in pathway leading to altered mitochondrial function in β cell [32]. Islet UCP-2 mRNA was significantly reduced in HF females and GFR males. PPARγ expression was significantly up-regulated in both GFR and HF males and tended to be higher in GFR and HF females.

In the liver, CS and MD expression were significantly lower in both HF female and male rats (Table 4B). A reduction was also found for GFR offspring except for the MD expression in liver from GFR female. No significant difference in PPARγ expression was detected in the liver between the three groups, either for male or female.

4. Discussion

It is only recently that attention was paid to the involvement of mitochondria as putative targets for the fetal programming of adult diseases because these organelles may relate the physiological and the cellular consequences of environmental disturbances during early life [33,34]. This was demonstrated episodically in kidney, liver, muscle, aorta and even in pancreatic islets, as a consequence of different experimental conditions during gestation [9–14]. Recently, we investigated more extensively islets from adult offspring of dams fed a low-protein diet throughout gestation, where β-cell secretion and mitochondrial function appeared altered [27]. Here, we found that, like protein deficiency during pregnancy, prenatal exposure to a global food restriction (GFR) or to a high-fat diet (HF) had important *in vitro* long-term consequences, which could be linked to mitochondrial dysfunction. Both types of maternal malnutrition led indeed to a lower response of insulin secretion and ATP content to glucose stimulation in islets from male and female adult offspring. For easy reading, our main results are summarized in Table 5, together with those we have obtained previously in case of maternal LP diet [15].

Abnormal islet function was observed in offspring of dams fed an imbalanced diet. Contrary to control islets, which increased their insulin release in response to glucose simulation, islets from both GFR and HF progeny did not enhance their insulin secretion. This could result from dysfunctions in energy metabolism, located for a large part in the mitochondria [35,36]. The mitochondria are intimately involved in the glucose sensing by β cells and defects in their function are associated with impaired glucose-stimulated insulin secretion (GSIS) [36]. Insulin release requires an increase in cytosolic ATP production to allow the closure of ATP-sensitive K^+ channels, the depolarization of the plasma membrane and, subsequently, the elevation of cytosolic Ca^{2+} concentration, which will trigger the

Table 5
Summary of the consequences of malnutrition during pregnancy for the 3-month-old offspring

	FEMALES			MALES		
	LP	GFR	HF	LP	GFR	HF
Oral glucose tolerance test						
Insulin response	↓	=	=	↓	=	=
Glucose response	=	=	=	=	=	=
β-Cell mass	↓	↓	↑	↓	↓	=
In vitro insulin secretion						
3.3 mM Glucose	↑	↑	=	=	↑	=
16.7 mM Glucose	=	=	=	=	=	=
Response (δ 5.5 *vs.* 16.7 mM)	↓	↓	↓	↓	↓	↓
ROS content						
5.5 mM Glucose	=	=	=	↑	↑	↑
16.7 mM Glucose	=	↓	↓	↑	=	↓
ATP content						
3.3 mM Glucose	=	=	↓	=	=	↓
16.7 mM Glucose	↓	↓	↓	↓	↓	↓
Mitochondrial biogenesis						
Mt DNA content	=	↑	↑	=	↑	=
PGC-1α mRNA	=	↑	=	=	↑	=
NRF-1 mRNA	↓	=	↑	=	=	=
Tfam mRNA	↓	↓	=	=	↓	=
Glucose metabolism						
GLUT-2 mRNA	↑	=	↓	=	↑	=
Glucokinase mRNA	=	=	↓	=	↓	=
Citrate synthase mRNA	↓	=	↓	↓	↑	=
Malate dehydrogenase mRNA	↓	↓	↓	↓	↑	=
UCP-2 mRNA	=	=	↓		↓	=
Mt DNA encoded genes						
ND4L mRNA	=	=	=	↑	=	=
COX-1 mRNA	=	=	=	↑	=	=
ATP6 mRNA	↓	↓	↓	↓	↓	↑

Results concerning the protein restriction (LP) were published previously [15].
↑, increased; ↓, decreased; =, similar compared with C.

exocytosis mediating the insulin release [37–39]. Whereas control islets exposed to 16.7 mmol/L glucose increased their ATP content, our results showed that it was completely blunted after glucose challenge in both GFR and HF progeny. The correlation between inadequate adaptation of insulin release and ATP production after glucose challenge was observed previously in adult offspring of dams fed a LP diet [15] or as a consequence of uteroplacental insufficiency during late gestation [9].

Defect in glucose-evoked insulin release and ATP content could first be due to impairment in glucose sensing that requires, as an initial event, an adequate expression of GLUT-2 [40,41]. Glucose is transported by this high-capacity transporter into the β cell where it can enter the glycolysis [42]. Female offspring of dams fed a HF diet presented a marked reduction in GLUT-2 expression, which may participate in the loss of ability of GSIS and ATP production, as described for islets from weanlings of dams fed a HF diet during gestation [43]. However, male HF progeny and both GFR offspring did not feature down-regulation of this glucose transporter. Thus, in these animals, impairment in ATP biosynthesis appeared to take its origin downstream of the glucose transport across the β-cell membrane.

For this reason, we have investigated the gene expression of key enzymes that regulate ATP production through the three consecutive biochemical pathways: glycolysis, TCA cycle and oxidative phosphorylation. The last two take place within the mitochondria and generate about 98% of β-cell ATP production [44]. The phosphorylation of glucose to glucose-6-phosphate by glucokinase (GK) determines the rate of glycolysis and, consequently, the rate of available pyruvate that can enter the mitochondrial metabolism [42,45]. In male GFR rats, glycolysis could be affected since the GK gene was found to be strongly underexpressed. A mutation in the GK gene leads to impaired insulin secretion [46] and GK is the rate-limiting step of glucose utilization in the β cell [45,46]. The expression of CS and MD, two enzymes involved in regulatory steps of the TCA cycle, was up-regulated in GFR males, suggesting that the mitochondria tried to compensate for the reduced glycolytic rate with an optimal use of pyruvate through the TCA cycle. An increased CS and MD expression was previously observed in fetuses (21.5 days) of dams fed a low-protein diet [27]. However, in these fetuses, the increased expression of such genes was associated with higher than normal ATP production in basal glucose condition. In male GFR rats, the increased CS and MD expression was not accompanied by elevated ATP content, suggesting that, in addition to defective glycolysis, other alterations may concern the final step of ATP production constituted by the mitochondrial ETC (see below). Contrary to males, female offspring of dams exposed to GFR during gestation presented an unaffected CS expression, whereas MD level was found to be lower. In HF females, a lower expression of GK, MD and CS probably contributes to the reduced ATP content already observed in basal glucose condition. Previous observations of female offspring of dams fed a low-protein diet showed already an association between a lower expression of MD and a blunted ATP production [15] (Table 5). In male HF rats, although the same alterations in ATP content than in other offspring of malnourished dams were found, we did not observe any change in the transcript level of genes involved in the glycolysis and the TCA cycle.

Glucose oxidation through glycolysis and TCA cycle generates NADH and $FADH_2$, which provides electrons to the mitochondrial ETC. The ETC generates by itself approximatively 90% of ATP production [44]. Briefly, consequently to the oxidation of NADH and $FADH_2$, protons are leaked along the ETC but reenter the mitochondrial matrix *via* ATP synthase to generate by ATP. The ATP synthase is composed of several subunits and the mtDNA encodes for the subunit 6 of this complex (ATP6) [30]. This subunit was down-regulated in GFR rats as well as in HF females and may certainly participate in the impairment of ATP production in these animals. The expression of other mtDNA-encoded genes, ND4L and COX-1, was unchanged in the GFR and HF progeny of both sexes. It should be noted that each model of fetal programming investigated until now featured an altered expression of ATP6 in pancreatic islets [9,12,15]. In the case of *in utero* placental insufficiency at the end of gestation, the disrupted ATPase activity was attributed to accumulation of mtDNA mutations caused by oxidative stress and randomly distributed along the ATP6 gene from growth-restricted offspring [9]. The analysis of mtDNA mutations could be particularly interesting for male HF rats because a higher ROS production and an overexpression of ATP6 were found, although, like all of the other progenies, they showed marked reduction in ATP production.

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 [28]. We evaluated the expression of PGC-1α, NRF-1 and Tfam because these factors provide a molecular basis for the connection between environmental stimuli and mitochondrial biogenesis [28,29]. PGC-1α can affect mtDNA levels by modulating the transcription of Tfam gene, through physical interaction with NRF-1 [28,30]. Although maternal malnutrition showed a modification in the expression of these genes, this could not be correlated with the small increase in mtDNA content observed in the islet of both GFR offspring and HF females. Moreover, this increase appeared too weak to foresee physiological implication. We may thus suggest that mitochondrial biogenesis might not be targeted at this stage. Fetuses presenting growth retardation due to uteroplacental insufficiency had blunted mitochondrial function associated with higher mtDNA content [9]. In such progeny, the mtDNA content decreased after birth to become lower than in control animals at 7 weeks. It is conceivable that in case of *in utero* undernutrition, without the strong reduction of oxygen supply caused by uterine artery ligation [9], the possible process of reduction in mtDNA may be delayed, so that the decline might appear later, the age of 3 months then being too early to detect such an effect.

In addition to intervening in the mitochondrial biogenesis, some transcriptional regulators such as PGC-1α may have additional biological function. Indeed, an elevated PGC-1α level was described as deleterious in β cell and was associated with defective insulin secretion [47,48]. Interestingly, islets overexpressing PGC-1α presented a basal insulin secretion higher than normal, but both the early and the late phase of insulin release were negatively affected following 16.7 mM glucose stimulation [47]. These findings are in agreement with our observations regarding the *in vitro* insulin release in GFR rats. Furthermore, higher PGC-1α level was found in several animal models of type 2 diabetes [47,48], but to a much higher level than in GFR rats. This could influence later development of metabolic disorders in GFR offspring [21].

Independently of the type of maternal malnutrition, we observed that the expression of PPARγ was increased in pancreatic islets from GFR and HF male progeny. PPARγ is a transcription factor involved in numerous cellular processes going from the control of cell cycle to the regulation of metabolic pathways. PPARγ also appears essential for glucose homeostasis. Indeed, PPARγ ligands reduced insulin levels because the insulin gene is a target of PPARγ [49]. Improvement of mitochondrial biogenesis was also associated with enhanced PPARγ function in adipose tissue [50]. We may postulate that PPARγ might play the role of a compensatory pathway in islets from adult offspring of malnourished dams. Such overexpression was also observed in the LP model of fetal programming [15].

The lower expression of UCP-2 observed only in HF and GFR male progeny may participate in the higher ROS production and in the impairment of β-cell function. Although the biological function of UCP-2 in β cell is subject to debate [31,51–53], accumulating evidence suggests that UCP-2 is not a physiologically relevant "uncoupling protein" but may participate in the control of mitochondria-derived ROS [52,54]. The absence of UCP-2 caused oxidative stress in pancreatic islets and decreased GSIS [52], whereas *in vitro*

overexpression of UCP-2 in clonal β cell showed protection against overt oxidative damage [55]. The ROS concentration influences islet secretory function through a mechanism that remains unexplained [56,57]. Thus, reduction of islet ROS content in HF and GFR females could potentially contribute to the absence of insulin response to glucose challenge. At the opposite, the higher ROS accumulation, under basal glucose concentration, in islets from GFR males could participate in their higher β-cell insulin secretion. Indeed, a strong correlation between ROS and insulin release was recently proposed using GK rats where lower ROS accumulation in diabetic rats was associated with an abolished GSIS and, consequently, with diabetes development [58]. However, this concept failed to explain our observations at least for HF males. It appears important to determine to what extent maternal diet may change islet antioxidant defenses in HF and GFR animals. As proposed previously [58], modification with age of antioxidant potential could be involved in β-cell secretory dysfunction and early malnutrition can change antioxidant capacity in an age-dependent process [59].

If the programming of mitochondrial dysfunction in islets appears as a common feature of maternal malnutrition, the majority of the alterations depend on the type of prenatal malnutrition and on the sex of the progeny (Table 5). In males, the restriction of nutrients seemed to provoke larger disturbances since β-cell mass as well as the expression of genes coding for proteins involved in energy metabolism and TCA was found altered to a greater extent in LP and GFR rats than in HF animals. Conversely, a maternal diet enriched with animal fat was more pernicious for females because HF females presented much more damages than LP and GFR females. The effect of steroid hormones on mitochondrial biogenesis and function in pancreatic islets is not known. However, in other cell types that have high demands of energy through mitochondrial metabolism, there is increasing evidence that mtDNA is one of the major targets for the direct action of steroid hormones, estrogens in particular [60]. Thus, we may postulate that steroid hormones play a role in the later issues at adulthood of the fetal programming, by modulating molecular targets in a sex-dependent manner.

In conclusion, the present study showed that inadequate diet during gestation led to similar functional consequences on islets *in vitro* which appear through an absence of increased ATP production and insulin release in response to glucose stimulation. Together, these alterations suggest an absence of islet sensitivity to glucose, which may originate from the glucose sensing or in the different biochemical pathways of glucose metabolism, depending on the type of prenatal malnutrition and sex. Indeed, although similar final alterations were found in both GFR and HF rats, sex- and diet-specific consequences are apparent and the molecular pathways leading to physiological impact differed in the two genders. These findings, in addition to previous experiments [9,12,14,15], clearly indicated that mitochondrial abnormality is a common consequence of fetal programming, which may predispose to the development of metabolic disorders at adulthood, such as glucose intolerance later in life.

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