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ORIGINAL PAPER

Effect of maternal protein restriction on liver metabolism in rat offspring

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Abstract Consequences of gestational protein restriction (GPR) on liver metabolism in rat offspring were investigated. Pregnant dams were divided into groups: normal (NP, 17 % casein) or low-protein diet (LP, 6 % casein). Livers were collected from 30-day-old offspring (d30) for analysis or isolation of mitochondria. At d30, hepatic and muscle glycogen was increased in LP group. Mitochondrial swelling and oxygen uptake (recorded with a Clark-type electrode) were significantly reduced in NP female and LP pups. Thiobarbituric acid reactive substances production was lower in females (NP or LP), suggesting significant inhibition of lipid peroxidation. Measurement of mitochondrial respiration (states 3 and 4 stimulated by succinate) showed a higher ADP/O ratio in LP pups, particularly females, suggesting higher phosphorylation efficiency. In the 1st month of life, under our experimental conditions, GPR protects liver mitochondria against oxidative stress and females seem to be more resistant or more suitable for survival.

Keywords Metabolism · Development · Gestational protein restriction · Rat liver mitochondria · Oxidative stress

Introduction

Maternal nutrient restriction during intrauterine development has been recognized as a cause of mortality at birth [1] and is associated with postnatal kidney dysfunction [2] and an increased risk of developing hypertension and cardiovascular disease in adulthood [3–5]. An estimated 53 % of perinatal mortality is attributed to low birth weight [6]. Thus, there is now widespread acceptance that the period of pregnancy and the first years of life play a crucial role in the development of metabolic and cardiovascular diseases in adulthood [7].

The concept of fetal programming has been used to explain the occurrence of events during the prenatal phase that lead to changes in fetal development and that can cause physiopathological alterations in adulthood [8] although the mechanisms are still not completely understood. In animal models of programming, sex differences are also apparent, with males most often affected more severely. In the rat, moderate maternal dietary protein restriction during pregnancy results in hypertension in adult male offspring, but not in females [9]. The severe protein restriction (6 % protein diet compared to 20 % in controls) causes hypertension in both males and female offspring but the effect is more pronounced in the males [10] and suggests that sex hormones may play an important role in modulating cardiovascular responses to an adverse fetal environment. A recent report showed that prenatal exposure to testosterone was able to alter important aspects of sexual and social behavior in adult male rats [11]. Many published data report the effect of gestational protein restriction (GPR) on adults but not on rats in the first month of life.

Some studies suggest that mitochondria are targets for programming in response to a number of insults, and that mitochondrial oxidative stress is exacerbated in complicated pregnancies, such as cases of preeclampsia or diabetes, in which production of reactive oxygen species (ROS) is increased and antioxidant enzymes are decreased

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[12–14]. Mitochondrial dysfunction has been linked to many of the diseases associated with early-life programming, including atherosclerosis, hypertension, renal dysfunction, and diabetes [15]. The basis underlying these effects of maternal diet is unclear and, while programming is widely believed to be driven by epigenetic mechanisms [16], there is no specific evidence for the differential regulation of genes that determine the balance between antioxidant systems and ROS production. One hypothesis is that early-life programming is achieved through altered regulation of coupling efficiency [17]. Mitochondria are intracellular organelles that produce ATP by oxidative phosphorylation. Parameters for evaluating mitochondrial integrity include: (a) respiration and phosphorylation [18]; (b) the ability of mitochondria to capture and retain calcium [19, 20], and (c) protons ejected from the matrix during respiration generating an electrical membrane potential [21].

In order to assess whether mitochondrial uncoupling could justify the harmful effects of GPR for offspring such as low birth weight and hypertension, the purpose of this study was to determine the effects of GPR on liver mitochondrial bioenergetics. To avoid interference of sex steroid hormones and to clarify the gender difference during development, the effect of maternal protein restriction on the mitochondrial oxidative stress in male and female offspring was examined separately in the first month of life. Since the liver is the organ with the largest number of functions, the aim of the present study was to evaluate the effects of GPR on liver structure and function of male and female rat offspring by measuring mitochondrial energy parameters in isolated liver mitochondria on day 30 after birth (d30). We specifically investigated oxygen uptake, mitochondrial swelling, thiobarbituric acid reactive substance (TBARS) production, and mitochondrial phosphorylation efficiency. Serum glucose, insulin, total protein and lipid levels, muscle and liver glycogen, and changes in hepatic morphology and weight were analyzed. In order to evaluate the effects of GPR in adult life, the oxygen consumption measurements were performed on mitochondria isolated from livers of adult male rats at 45, 60, 120, 180, 240 and 300 days old.

Materials and methods

Animal care

All experiments were conducted in strict agreement with the National Institute of Health Guidelines for Humane Treatment of Animals. Virgin female Wistar rats weighing 180–225 g were mated with stud males. After confirmation of pregnancy by the presence of sperm in a vaginal smear, female rats were randomly allocated to be fed one of two isocaloric and normal-sodium semi-synthetic diets (17 % casein, NP; 6 % casein, LP) as described previously [22, 23]. The animals were maintained at a controlled temperature (21 ± 1 °C) on a 12 h light/dark cycle, with free access to water. Pregnant rats ($n = 36$) were fed the semi-synthetic diets (NP, $n = 18$; LP, $n = 18$) until they delivered pups at 22 days of gestation. After delivery, the mothers and pups received a standard laboratory diet (Nuvilab CR-1, Nuvital, Colombro, PR, Brazil) containing 21.6 % protein and 4.0 % lipid. The anogenital distance was measured in all pups [24] and litters were culled to a maximum of 8 pups (4 males and 4 females) to minimize variation in nutrition during the suckling period. At d30, for mitochondrial isolation, the rat offspring (NP, $n = 6$ dams; LP, $n = 6$ dams) were sacrificed after a 12-h fast by cervical dislocation by a technician experienced in the procedure. For biochemical analyses, offspring blood samples (NP, $n = 6$ dams; LP, $n = 6$ dams) were obtained after anesthesia by heart puncture and sera were stored at −20 °C. Liver tissues were collected and weighed and fragments were processed for biochemical and histological analysis. To study the mitochondrial oxidative stress in adult rats, the experiments were performed on isolated liver mitochondria of male rats, aged between 45 and 300 days after birth (NP, $n = 6$ dams; LP, $n = 6$ dams). Adult female rats were not employed in these experiments to avoid interference of sex steroid hormones.

Biochemical analysis

Serum glucose, total protein, cholesterol and triacylglycerol levels were measured using a kit according to manufacturer's instructions (Laborlab, São Paulo, Brazil). Hepatic and muscle glycogen was determined as described elsewhere [25]. Total liver lipids were extracted according to the method of Folch et al. [26].

Histology and morphometric analysis

The livers were removed, fixed in 10 % paraformaldehyde in Millonig's buffer, pH 7.4, for 24 h at room temperature and embedded in Paraplast (Merck). Sections (6 μm thick) were stained with periodic acid Schiff (PAS) for glycogen morphometry, and hematoxylin & eosin for morphological analysis. For glycogen quantification, the periodate of the PAS method oxidizes the hydroxyl moieties of glucose residues to aldehydes, which in turn react with the Schiff reagent, generating a purple-magenta color. Five representative fields from at least three different liver fragments per rat were analyzed by light microscopy (Leica DM 2000 Photomicroscope) and captured with a digital video camera. Each digital image was photographed with the 40×

objective and formatted at fixed pixel density (8 × 10 inches at 150 dpi) using Sigma Scan Pro (v.6.0). Each digital image was then analyzed for histomorphometric analysis. The glycogen signal (standard color) was expressed as a percentage of total tissue area analyzed (10^4 μm^2). The area of glycogen signal was calculated in relation to total tissue area [27].

Isolation of rat liver mitochondria

Rat liver mitochondria (RLM) were isolated from Wistar rat pups fasted overnight by conventional differential centrifugation at 4 °C according to Schneider and Hogeboom [28]. For this purpose, the livers were quickly weighed after sacrifice, chopped and minced, and then homogenized in ice-cold isotonic isolation medium (250 mM sucrose, 1.0 mM EGTA, and 10 mM HEPES buffer, pH 7.2). The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was diluted in 250 mM sucrose and 10 mM HEPES buffer, pH 7.2. RLM samples were homogenized for the determination of protein content. All experiments using isolated mitochondria were conducted within 1 h of isolation.

Standard incubation procedure

The RLM experiments were carried out at 28 °C in a reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 1 mM inorganic phosphate, and 10 μM $CaCl_2$. Sodium succinate (2 mM) was used as respiratory substrate. Rotenone (5.0 μM) was added to prevent respiration by endogenous site-I substrates. Rotenone, sodium succinate, and HEPES were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Determination of mitochondrial swelling

Mitochondrial swelling was estimated based on the decrease in absorbance at 540 nm measured with a Genesys 10UV spectrophotometer (Thermo Electron Corporation, Madison, Wisconsin, USA) according to Nicholls and Åkerman [29]. RLM (0.5 mg/ml) isolated from d30 male and female offspring were added to a temperature-controlled (28 °C) chamber under gentle stirring and incubated in a standard reaction medium. Inner mitochondrial membrane permeabilization induced by Ca^{2+} can be assessed by the classical mitochondrial swelling technique to monitor the net influx of osmotic support associated with a non-specific increase in membrane permeability [30]. In the presence of low concentrations of calcium (10 μM $CaCl_2$), the mitochondrial permeability transition can be stimulated with inorganic phosphate [31] and this effect is inhibited in the presence of the Ca^{2+} chelator EGTA. To evaluate a possible role of GPR in liver oxidative stress, RLM were incubated in a standard reaction medium and the variation in absorbance at 540 nm and oxygen uptake were monitored for 10 min. Inorganic phosphate (2 mM) was added after 1 min of mitochondrial preincubation and absorbance was recorded over a period of 10 min. The results are reported as the mean ± standard deviation (SD) of the variation in absorbance obtained for at least six different animals. The mitochondrial swelling and oxygen uptake experiments were performed simultaneously using the same preparation of isolated RLM under the same experimental conditions.

Measurement of oxygen uptake

RLM (0.5 mg/ml) isolated from d30 male and female offspring were added to a temperature-controlled (28 °C) chamber equipped with a Clark-type electrode (Oxytherm System, Hansatech Instruments, Norfolk, UK) under gentle stirring and incubated in a reaction medium according to Robinson and Cooper [32]. The experiments were carried out at 28 °C in a reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 1 mM inorganic phosphate, and 0.5 mM EGTA. Sodium succinate (5.0 mM) was used as respiratory substrate. Rotenone (4.0 μM) was added to prevent respiration by endogenous site-I substrates. Oxygen tension was monitored for 1–2 min (state 2 respiration) prior to the addition of 200 nmol ADP to initiate state 3 respiration. Mitochondrial respiration (oxygen uptake) was recorded over a period of 10 min, assuming a solubility of 210 μmol/ml at 28 °C. Respiration rates, expressed as natom O/min/mg protein, were measured during state 3 and then state 4 (after all ADP is consumed). We also evaluated the integrity of the inner mitochondrial membrane by calculation of the ADP/O ratio (stoichiometry between the number of molecules of ADP phosphorylated per atom of oxygen consumed). The efficiency of oxidative phosphorylation can be measured in isolated mitochondria by the respiration rate in states 3 and 4 after the addition of 200 nmol ADP using succinate as respiratory substrate. The ADP/O ratio was calculated from the amount of ADP added and oxygen consumed during state 3. The results are reported as the mean ± SD ($n = 6$).

Measurement of thiobarbituric reactive substances

TBARS production by mitochondria was quantified [33] in a reaction medium containing 250 mM sucrose, 10 mM HEPES buffer (pH 7.2), 4.0 μM rotenone, 0.5 μM antimycin A, and 2.0 mM citrate. After 1 min of mitochondrial preincubation, 50 μM Fe^{2+} was added. The optical density

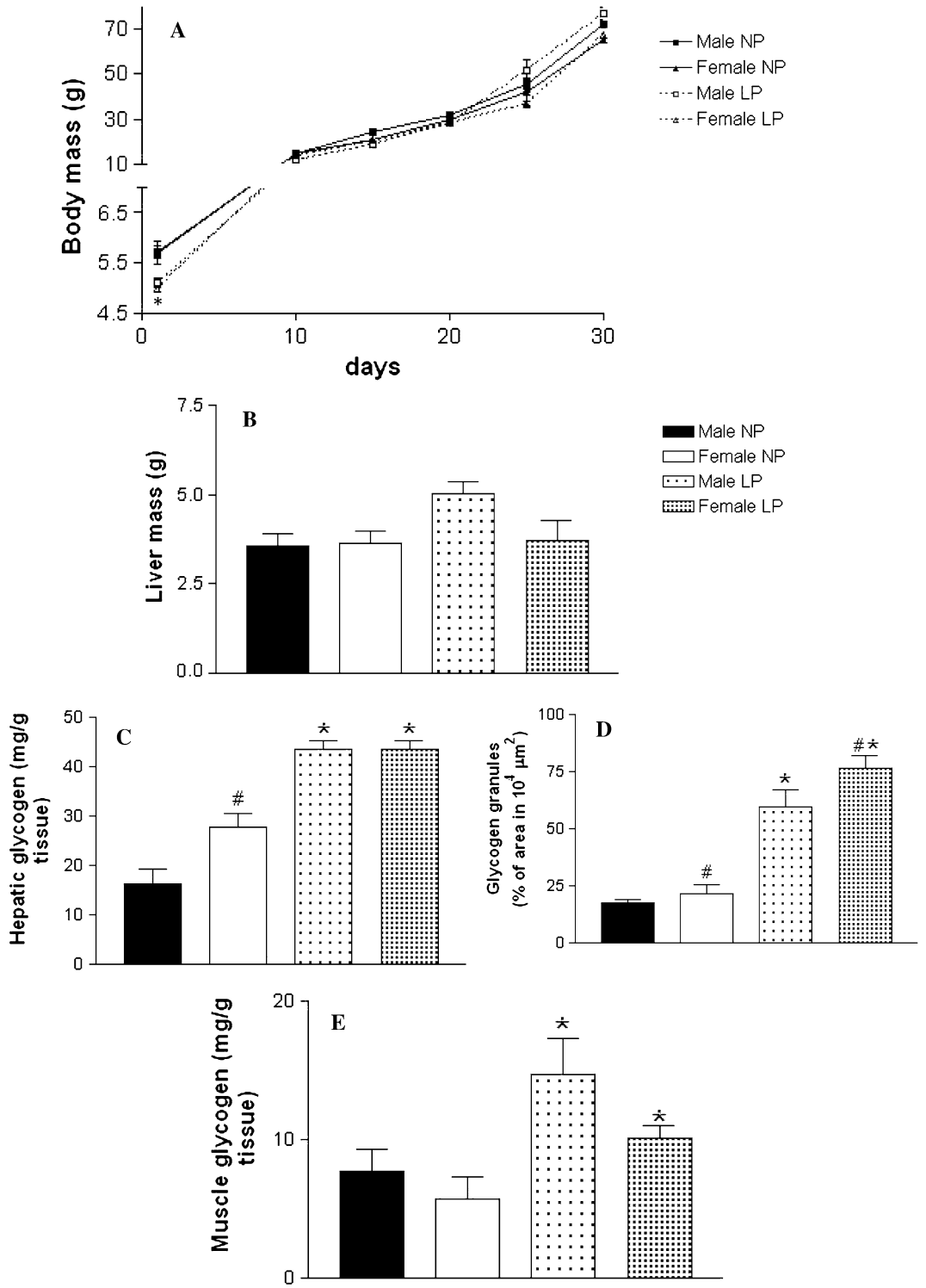

Fig. 1 **a** Growth curve of offspring: the 1st to the 30th day after birth. The body weight gain (g) of the offspring of mothers fed the normal (NP) or low-protein diet (LP) animals were determined for male NP pups (*full square symbols*, $n = 10$), female NP pups (*full triangle symbols*, $n = 14$), male LP pups (*empty square symbols*, $n = 6$) and female LP pups (*empty triangle symbols*, $n = 10$). **b** Liver weight of pups on day 30 after birth. The values (g) were determined in the male NP pups (*full bars*, $n = 18$); in the female NP pups (*empty bars*, $n = 23$); in the male LP pups (*dotted bars*, $n = 18$) and in the female LP pups (*grid bars*, $n = 16$). **c** Hepatic glycogen of pups on d30 (mg/g tissue). **d** Glycogen granules of pups on d30 (% of area in 10^4 µm^2). **e** Muscle glycogen of pups on d30 (mg/g tissue). Mean ± SD ($n \geq 6$) $^*p < 0.05$ LP vs NP (ANOVA); $^{\#}p < 0.05$ male vs female (Student's *t*-test)

of the supernatant was determined at 535 nm. Under these conditions, the molar extinction coefficient used to calculate TBARS concentration was 1.56×10^5 M^{-1} cm^{-1}.

Data analysis

Figures 1 and 3 report the mean ± SD of measurements from 6 to 9 different animals. Data were compared by one-way ANOVA followed by Tukey's post hoc test performed by GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA) adopting a level of significance of 5 % ($p < 0.05$). When one parameter was compared between two groups, male vs female, Student's *t*-test was used.

Results

Animal characteristics

The body weight gain of the animals was analyzed over a period of 30 days after birth (Fig. 1a). Recently we have found that GPR alters the placental structure on the 19th gestational day [34]. Analysis of the weight of pups on day 1 after birth (previously published in [34, Table 2] to validate the GPR model) showed that male (5.114 ± 1.4 g, $n = 6$, empty square symbols) and female (5.001 ± 2.2 g, $n = 10$, empty triangle symbols) offspring of mothers fed a low protein diet (LP) were significantly lighter than offspring of mothers fed the normal protein diet (NP) (male 5.726 ± 1.5 g, $n = 10$, full square symbols; female 5.701 ± 2.5, $n = 14$, full triangle symbols). With respect to body weight gain, the body weight of male and female offspring of NP or LP dams was similar on day 25 after birth. On d30, there was no significant difference between offspring of NP or LP dams (males LP, 76.66 ± 13.77 g, $n = 18$; NP, male NP 71.41 ± 6.64 g, $n = 18$; female LP 67.46 ± 10.24 g, $n = 16$; female NP 64.99 ± 5.20 g, $n = 23$). In the same group, NP or LP, there was no significant difference between body mass of male or female pups.

Biochemical and histological parameters

The liver weights of rats on d30 are shown in Fig. 1b. The values (g) found were 3.401 ± 0.82, $n = 18$, in the male NP pups (full bars); 3.393 ± 0.87, $n = 23$, in the female NP pups (empty bars); 4.235 ± 1.49, $n = 18$, in the male LP pups (point bars) and 3.642 ± 1.10, $n = 16$, in the female LP pups (grid bars). No significant differences were observed between groups.

Figure 1c shows a significant increase of hepatic glycogen observed on d30 in LP pups, which was more pronounced in male animals ($^*p < 0.05$ vs NP); female NP pups also showed significant increase compared to male NP pups ($^{\#}p < 0.05$). These data were demonstrated by histological analysis (Fig. 2) and the number of glycogen granules was counted. In fact, these were confirmed by the results of the area of glycogen signal (% of area in 10^4 μm^2; Fig. 1d), which was 17.5 ± 3.2 in the male NP pups (full bars); 21.6 ± 9.4 in the female NP pups (empty bars, $^{\#}p < 0.05$ vs male NP); 59.3 ± 18.8 in the male LP pups (point bars, $^*p < 0.05$ vs male NP); 76.5 ± 12.9 in the female LP pups (grid bars, $^*p < 0.05$ vs female NP). Muscle glycogen also was increased on d30 in LP animals (Fig. 1e). The values (mg/g tissue) found were 7.68 ± 4.79 in the male NP pups; 5.66 ± 4.92 in the female NP pups; 14.68 ± 7.98 in the male LP pups ($^*p < 0.05$ vs male NP); 10.09 ± 2.73 in the female LP pups ($^*p < 0.05$ vs female NP). There were no significant differences on d30 between male and female NP or LP pups in muscle glycogen.

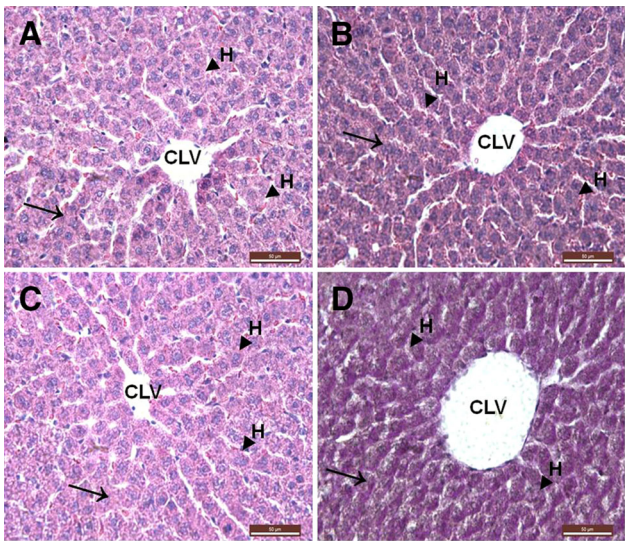

Fig. 2 Photomicrographs of rat liver specimens obtained on day 30 after birth. The sections were stained by the periodic acid Schiff method. *Top panels* male offspring born to dams receiving a normal (**a**) or low-protein diet (**b**). *Bottom panels* female offspring born to dams receiving a normal (**c**) or low-protein diet (**d**). Centrolobular vein (CLV), hepatocytes (H) and glycogen granules (*arrows*) *Bar* 50 μm

The results of biochemical analysis on d30 (shown in Table 1) of serum glucose, cholesterol, triacylglycerol, total protein, liver triacylglycerol, or cholesterol showed no significant differences between all groups.

Effect of GPR on liver mitochondrial metabolism on day 30 after birth

To evaluate a possible role of GPR in liver oxidative stress, the RLM swelling and oxygen uptake were monitored simultaneously. The results show that Ca^{2+} induced mitochondrial swelling was significantly inhibited in

Table 1 Biochemical parameters on day 30 after birth of offspring born to dams fed a normal (NP, 17 % casein) or low-protein (LP, 6 % casein) diet during pregnancy

Parameter	NP males	NP females	LP males	LP females
Glucose (mg/dl)	101.8 ± 9.032	104.3 ± 5.377	96.00 ± 12.52	102.3 ± 2.986
Cholesterol (mg/dl)	90.87 ± 13.83	107.8 ± 17.93	107.0 ± 13.39	95.61 ± 12.95
Triacylglycerol (mg/dl)	58.34 ± 13.14	72.21 ± 15.48	83.41 ± 23.4	94.33 ± 56.99
Total protein (g/dl)	3.893 ± 0.823	3.516 ± 0.707	4.095 ± 0.9	4.337 ± 0.902
Liver cholesterol (mg/g tissue)	0.163 ± 0.052	0.144 ± 0.019	0.165 ± 0.026	0.173 ± 0.045
Mitochondrial swelling	0.2743 ± 0.06	0.2217 ± 0.045$^{\#}$	0.1853 ± 0.005*	0.0703 ± 0.0245$^{\#}$*
Oxygen uptake (nmol/mg/min)	185.7 ± 15.57	124.7 ± 4.509$^{\#}$	150.3 ± 5.508*	101.3 ± 10.07$^{\#}$*
ADP/O	1.370 ± 0.061	1.494 ± 0.35	1.508 ± 0.094*	1.878 ± 0.474*
TBARS (nmol/mg protein/10 min)	3.973 ± 0.005	3.147 ± 0.002$^{\#}$	3.047 ± 0.078*	2.850 ± 0.123*

Rat liver mitochondria (0.5 mg/ml) isolated from d30 offspring were added to a standard reaction medium. Mitochondrial swelling and oxygen uptake were monitored for 10 min at 28 °C. Inorganic phosphate (2 mM) was added after 1 min of mitochondrial preincubation (*$p < 0.05$, NP vs LP). TBARS production and ADP/O were measured as described in 'Materials and methods'. The experiments were performed simultaneously on the same day using the same RLM preparation under the same experimental conditions. All experiments using isolated mitochondria were conducted within 1 h of isolation. Mean ± SD ($n \geq 6$) *$p < 0.05$ LP vs NP; $^{\#}p < 0.05$ female vs male (ANOVA)

isolated RLM from offspring of mothers fed a low protein diet (LP) (Table 1). Under the same experimental conditions, a significant reduction in oxygen uptake was observed in isolated RLM from female NP pups and all LP pups when compared to male NP pups, and this inhibition of respiration was proportional to the inhibition observed in the mitochondrial swelling experiments. The ADP/O ratio was increased in male and female LP pups. Analysis of TBARS production (nmol/mg protein/10 min) of isolated RLM showed that female NP pups and male and female LP pups were significantly lower than male LP pups.

Effect of GPR on liver mitochondrial metabolism in adult male rats

Oxygen consumption experiments performed in isolated RLM from adult male rats (on day 45–day 300 after birth) under the same experimental conditions (Fig. 3) showed significant increase in oxygen uptake on isolated RLM from offspring of mothers fed a low protein diet (LP) on day 45 (d45), on day 60 (d60) and on day 120 (d120) after birth. There was no significant difference in oxygen consumption on day 180 (d180), on day 240 (d240) or day 300 (d300) between groups NP or LP.

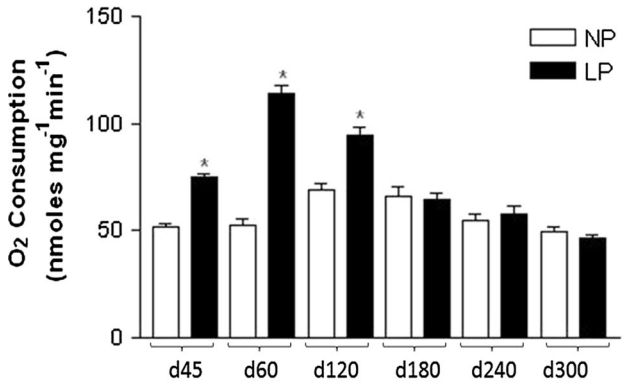

Fig. 3 Effect of gestational protein restriction (GPR) on the oxygen consumption of adult male rat liver mitochondria. RLM (0.5 mg/ml) isolated from offspring of mothers fed a normal (NP) or low-protein diet (LP). The 45-, 60-, 120-, 180-, 240- or 300-day-old male rat livers were added to a standard reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer, pH 7.2; 10 μM $CaCl_2$, 5.0 mM succinate and 4.0 μM rotenone, and the oxygen consumption followed for 10 min. Inorganic phosphate (2 mM) was added after 1 min of mitochondrial preincubation. All experiments were conducted within 1 h of isolation. Mean ± SD ($n \geq 6$) *$p < 0.05$ LP vs NP (Student's *t*-test)

Discussion

Studies have shown that change in dietary protein is deleterious to the formation and development of the fetus, causing malnutrition and low birth weight of offspring [34–36]. In the present study, differences were observed in animals submitted to GPR. As expected and previously published [34], offspring of mothers fed a low protein diet (LP) were significantly lighter than offspring of mothers fed the normal protein diet (NP). These results validate the GPR model showing that restricted animals presented lower intrauterine weight gain than controls not submitted to restriction [37].The observation of a lower birth weight in offspring of LP dams and higher weight on d30 when compared to NP pups is known as "catch-up growth". This phenomenon is characterized by a growth rate above the normal range for age after the transitional period of intrauterine growth inhibition and increases the risk of cardiovascular disease [38–40]. As evident from their body

weight at d30, restoration of protein in the maternal diet at birth resulted in complete catch-up growth in LP pups, with no changes in the liver to body weight ratio.

Biochemical analysis showed no differences in serum glucose, cholesterol, triacylglycerol, total protein, liver triacylglycerol, or cholesterol. The similar total protein levels of the groups might be attributed to the period of lactation of the pups during which the mothers again received a diet containing 21.6 % protein. Furthermore, the pups were fed a diet with 21.6 % protein after weaning. The present results agree with the literature, i.e., the lipid profile was characterized by similar levels of circulating cholesterol and triacylglycerol and hepatic cholesterol and triglycerides in all groups of animals [41]. A significant increase of hepatic and muscle glycogen was observed in LP pups. This increased glycogen storage is mainly due to an increase of insulin sensitivity despite reduced secretion of this hormone [42]. Another hypothesis is that increased glycogen observed in fetuses submitted to GPR is a consequence of energy conservation due to increased gluconeogenesis and glycolysis in response to a reduction of nutrient availability [43, 44].

The study of inner mitochondrial membrane permeability (MMP) induced by Ca^{2+} can be associated with a nonspecific increase in membrane permeability that stimulates respiratory rates and decreases the coupling between oxygen consumption and oxidative phosphorylation [31]. Surprisingly the results suggest that RLM on d30 from female NP and both male and female LP pups were less susceptible to the same MMP transition conditions than male NP pups. For the same stimulus of oxidative damage (10 μM $CaCl_2$ and 2 mM inorganic phosphate), RLM from male NP animals exhibit a higher respiration rate to compensate for the increased production of ROS or reduced production of antioxidant enzymes. In this respect, GPR seems to increase the protection of offspring liver from conditions of oxidative stress by increasing the production of antioxidant enzymes or reducing the production of ROS. The ADP/O ratio was increased in LP pups. This finding indicates that mitochondrial membrane integrity is maintained in female and male LP pups, respiration and phosphorylation are coupled, and the process of oxidative phosphorylation is more efficient.

Changes in the respiration rate indicate variations in mitochondrial ROS production [45]. To quantify the production of ROS in this experimental model, we evaluated inner MMP induced by lipid peroxidation and protein fragmentation based on TBARS production. Castilho et al. [46] demonstrated that mitochondrial membrane permeability is mediated by lipid peroxidation and protein fragmentation is due to the attack of ROS in the presence of calcium and the Fe^{2+} citrate free radical generating system. The results shown in Table 1 indicate that female NP and LP pups produce fewer reactive products with thiobarbituric acid, suggesting the inhibition of lipid peroxidation, and fewer free radicals and ROS than NP males or females. These data agree with those obtained in the mitochondrial swelling and respiration experiments.

As expected, the uncoupling effect on mitochondrial respiration can be observed by the results of liver oxygen uptake in adult male LP pups (Fig. 3). The results indicate that RLM respiration was faster in the LP group, on d45–d120, in order to compensate the increased ROS production. This increase can be estimated by the rate of mitochondrial oxygen uptake, which promotes uncoupling between mitochondrial respiration and oxidative phosphorylation. Qasem et al. [47] showed that maternal protein restriction during pregnancy and lactation reduces the lipid content selectively in the male offspring on d65–d150. Opposite data were published by Sohi et al. [48]. Could the lipid alteration change the lipid/protein content of the inner mitochondrial membrane, promoting the increase of ROS by the electron transport chain and change mitochondrial respiration rate? Further studies are needed to test this hypothesis. From d180, the liver of the animal has recovered from the harmful effects of GPR and liver tissue returns to its normal physiological conditions.

Zambrano et al. [49] suggest that a key principle of developmental programming is that the consequences of cellular and molecular changes that occur during development may lie dormant to emerge in adulthood. Thus changes occurring from fetal development to puberty can affect later life stages.

Taken together, the present results suggest that GPR induces greater protection of the liver from conditions of oxidative stress in d30 pups by reducing the production of ROS and increasing mitochondrial coupling between respiration and oxidative phosphorylation. This effect is more pronounced in female LP pups. Female NP pups are more resistant than male NP pups of the same age. GPR causes low birth weight in rat offspring but increases muscle and liver glycogen, and protects RLM from different conditions of oxidative stress, with female RLM producing less ROS. Conversely, GPR induces uncoupling between mitochondrial respiration and oxidative phosphorylation in adult male rat liver mitochondria. It can be suggested that GPR protects pups during the 1st month after birth, preparing them for adverse conditions of life. The livers of these offspring contain energy reserves and are protected from conditions of oxidative stress. Hepatic mitochondria produce lower amounts of ROS and the integrity of the inner mitochondrial membrane is maintained. Female NP pups and both sex LP pups seem to be more protected against the adverse effects of oxidative stress on first month of life.

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References

1. Barker DJ, Osmond C (1986) Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1:1077–1081
2. Vanpée M, Blennow M, Linné T, Herin P, Aperia A (1992) Renal function in very low birth weight infants: normal maturity reached during early childhood. J Pediatr 121:784–788
3. Williams S, St George IM, Silva PA (1992) Intrauterine growth retardation and blood pressure at age seven and eighteen. J Clin Epidemiol 45:1257–1263
4. Langley SC, Jackson AA (1994) Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. Clin Sci (Lond) 86:217–222
5. Barker DJ, Shiell AW, Barker ME, Law CM (2000) Growth in utero and blood pressure levels in the next generation. J Hypertens 18:843–846
6. de Onis SM, Blössner M, Borghi E, Frongillo EA, Morris R (2004) Estimates of global prevalence of childhood underweight in 1990 and 2015. JAMA 291:2600–2606
7. Wintour EM, Johnson K, Koukoulas I, Moritz K, Tersteeg M, Dodic M (2003) Programming the cardiovascular system, kidney and the brain—a review. Placenta 24:S65–S71
8. Seckl JR (1998) Physiologic programming of the fetus. Clin Perinatol 25:939–962
9. Woods LL, Ingelfinger JR, Rasch R (2005) Modest maternal protein restriction fails to program adult hypertension in female rats. Am J Physiol Regul Integr Comp Physiol 289:R1131–R1136
10. Sathishkumar K, Elkins R, Yallampalli U, Yallampalli C (2012) Protein restriction during pregnancy induces hypertension in adult female rat offspring—influence of estradiol. Br J Nutr 107(5):665–673. doi:10.1017/S0007114511003448
11. Dela Cruz C, Pereira OC (2012) Prenatal testosterone supplementation alters puberty onset, aggressive behavior, and partner preference in adult male rats. J Physiol Sci 62(2):123–131. doi:10.1007/s12576-011-0190-7
12. Wang Y, Walsh SW, Kay HH (1992) Placental lipid peroxides and thromboxane are increased and prostacyclin is decreased in women with preeclampsia. Am J Obstet Gynecol 167:946–949
13. Giugliano D, Ceriello A, Paolisso G (1996) Oxidative stress and diabetic vascular complications. Diabetes Care 19:257–267
14. Myatt L (2006) Placental adaptive responses and fetal programming. J Physiol 572:25–30
15. Leduc L, Levy E, Bouity-Voubou M, Delvin E (2010) Fetal programming of atherosclerosis: possible role of the mitochondria. Eur J Obstet Gynecol Reprod Biol 149:127–130
16. Langley-Evans SC, Sculley DV (2006) The association between birth weight and longevity in the rat is complex and modulated by maternal protein intake during fetal life. FEBS Lett 580:4150–4153
17. Engeham S, Mdaki K, Jewell K, Austin R, Lehner AN, Langley-Evans SC (2012) Mitochondrial respiration is decreased in rat kidney following fetal exposure to a maternal low-protein diet. J Nutr Metab 12(2012):989037. doi:10.1155/2012/989037
18. Kennedy EP, Lehninger AL (1949) Oxidation of fatty acids and tricarboxylic acid cycle intermediates by isolated rat liver mitochondria. J Biol Chem 179:957–972
19. Nicholls DG (1978) Calcium transport and proton electrochemical potential gradient in mitochondria from guinea-pig cerebral cortex and rat heart. Biochem J 170:511–522
20. Nicholls DG, Åkerman KE (1982) Mitochondrial calcium transport. Biochim Biophys Acta 683:57–88
21. Åkerman KE, Wikström MKF (1976) Safranine as a probe of the mitochondrial membrane potential. FEBS Lett 68:191–197
22. Woods LL, Weeks DA, Rasch R (2004) Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis. Kidney Int 65:1339–1348
23. Reeves PG, Nielsen FH, Fahey GC Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123:1939–1951
24. Zambrano E, Bautista CJ, Deás M, Martínez-Samayoa PM, González-Zamorano M, Ledesma H et al (2006) A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. J Physiol 571:221–230
25. Lo S, Russell JC, Taylor AW (1970) Determination of glycogen in small tissue samples. J Appl Physiol 28:234–236
26. Folch J, Lees M, Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509
27. Diniz MF, Dourado VA, Silva ME, Pedrosa ML, Bezerra FS and Lima WG (2013) Cigarette smoke causes changes in liver and spleen of mice newborn exposed during pregnancy. J Cytol Histol 4:1 http://dx.doi.org/10.4172/2157-7099.1000168
28. Schneider WC, Hogeboom GH (1950) Intracellular distribution of enzymes V. Further studies on the distribution of cytochrome c in rat liver homogenates. J Biol Chem 183:123–128
29. Nicholls DG, Åkerman KEO (1982) Mitochondrial calcium transport. Biochim Biophys Acta 683:57–88
30. Gunter TE, Pfeiffer DR (1990) Mechanisms by which mitochondria transport calcium. Am J Physiol 258:755–786
31. Kowaltowski AJ, Castilho RF, Vercesi AE (1996) Opening of the mitochondrial permeability transition pore by uncoupling or inorganic phosphate in the presence of Ca^{2+} is dependent on mitochondrial-generated reactive oxygen species. FEBS Lett 378:150–152
32. Robinson J, Cooper JM (1970) Method of determining oxygen concentrations in biological media, suitable for calibration of the electrode. Anal Biochem 33:390–399
33. Buege JA, Aust SD (1978) In biomembranes, part C, biological oxidations, microsomal, cytochrome P-450, and other hemoprotein systems. In: Fleicher S, Packeer L (eds) Methods in enzymology, vol 52. Academic Press, New York
34. Rebelato HJ, Esquisatto MA, Moraes C, Amaral ME, Catisti R (2013) Gestational protein restriction induces alterations in placental morphology and mitochondrial function in rats during late pregnancy. J Mol Histol 44:629–637. doi:10.1007/s10735-013-9522-7
35. Fernandez-Twinn DS, Ozanne SE, Ekizoglou S, Doherty C, James L, Gusterson B (2003) The maternal endocrine environment in the low-protein model of intra-uterine growth restriction. Br J Nut 90:815–822

36. Ballen MLO, Moretto VL, Santos MP, Gonçalves TSS, Kawashita NH, Stoppiglia LF et al (2009) Protein restriction in pregnancy: effects related to dam metabolism. Arq Bras Endocrinol Metab 53:87–94
37. Muaku SM, Beauloye V, Thissen JP, Underwood LE, Ketelslegers JM, Maiter D (1995) Effects of maternal protein malnutrition on fetal growth, plasma insulin-like growth factors, insulin-like growth factor binding proteins, and liver insulin-like growth factor gene expression in the rat. Pediatr Res 37:334–342
38. Muaku SM, Thissen JP, Gerard G, Ketelslegers JM, Maiter D (1997) Postnatal catch-up growth induced by growth hormone and insulin-like growth factor-I in rats with intrauterine growth retardation caused by maternal protein malnutrition. Pediatr Res 42:370–377
39. Woodall SM, Johnston BM, Breier BH, Gluckman PD (1996) Chronic maternal undernutrition in the rat leads to delayed postnatal growth and elevated blood pressure of offspring. Pediatr Res 40:438–443
40. Tarry-Adkins JL, Martin-Gronert MS, Fernandez-Twinn DS, Hargreaves I, Alfaradhi MZ, Land JM, Aiken CE et al (2013) Poor maternal nutrition followed by accelerated postnatal growth leads to alterations in DNA damage and repair, oxidative and nitrosative stress, and oxidative defense capacity in rat heart. FASEB J 27:379–390
41. Erhuma A, Salter AM, Sculley DV, Langley-Evans SC, Bennett AJ (2007) Prenatal exposure to a low-protein diet programs disordered regulation of lipid metabolism in the aging rat. Am J Physiol Endocrinol Metab 292:E1702–E1714
42. Ramadan WS, Alshiraihi I, Al-Karim S (2013) Effect of maternal low protein diet during pregnancy on the fetal liver of rats. Ann Anat 195:68–76
43. Li C, Schlabritz-Loutsevitch NE, Hubbard GB, Han V, Nygard K, Cox LA et al (2009) Effect of maternal global nutrient restriction on fetal baboon hepatic insulin-like growth factor system genes and gene products. Endocrinology 150:4634–4642
44. Vo TX, Revesz A, Sohi G, Ma N, Hardy DB (2013) Maternal protein restriction leads to enhanced hepatic gluconeogenic gene expression in adult male rat offspring due to impaired expression of the liver X receptor. J Endocrinol 218(1):85–97. doi:10.1530/JOE-13-0055
45. Kowaltowski AJ, Souza-Pinto NC, Castilho RF, Vercesi AE (2009) Mitochondria and reactive oxygen species. Free Radical Biol Med 47(4):333–343. doi:10.1016/j.freeradbiomed.2009.05.004
46. Castilho RF, Meinicke AR, Almeida AM, Hermes-Lima M, Vercesi AE (1994) Oxidative damage of mitochondria induced by Fe(II)citrate is potentiated by Ca^{2+} and includes lipid peroxidation and alterations in membrane proteins. Arch Biochem Biophys 308:158–163
47. Qasem RJ, Cherala G, D'Mello AP (2010) Maternal protein restriction during pregnancy and lactation in rats imprints long-term reduction in hepatic lipid content selectively in the male offspring. Nutr Res 30(6):410–417
48. Sohi G, Marchand K, Revesz A, Arany E, Hardy DB (2011) Maternal protein restriction elevates cholesterol in adult rat offspring due to repressive changes in histone modifications at the cholesterol 7alpha-hydroxylase promoter. Mol Endocrinol 25(5):785–798. doi:10.1210/me.2010-0395
49. Zambrano E, Guzmán C, Rodríguez-González GL, Durand-Carbajal M, Nathanielsz PW (2014) Fetal programming of sexual development and reproductive function. Mol Cell Endocrinol 382(1):538–549. doi:10.1016/j.mce.2013.09.008