Low-protein diet in adult male rats has long-term effects on metabolism

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Abstract

Nutritional insults during developmental plasticity have been linked with metabolic diseases such as diabetes in adulthood. We aimed to investigate whether a low-protein (LP) diet at the beginning of adulthood is able to program metabolic disruptions in rats. While control rats ate a normal-protein (23%; NP group) diet, treated rats were fed a LP (4%; LP group) diet from 60 to 90 days of age, after which an NP diet was supplied until they were 150 days old. Plasma levels of glucose and insulin, autonomous nervous system (ANS), and pancreatic islet function were then evaluated. Compared with the NP group, LP rats exhibited unchanged body weight and reduced food intake throughout the period of protein restriction; however, after the switch to the NP diet, hyperphagia of 10% ($P < 0.05$), and catch-up growth of 113% ($P < 0.0001$) were found. The LP rats showed hyperglycemia, insulin resistance, and higher fat accretion than the NP rats. While the sympathetic tonus from LP rats reduced by 28%, the vagus tonus increased by 21% ($P < 0.05$). Compared with the islets from NP rats, the glucose insulinotropic effect as well as cholinergic and adrenergic actions was unaltered in the islets from LP rats. Protein restriction at the beginning of adulthood induced unbalanced ANS activity and fat tissue accretion later in life, even without functional disturbances in the pancreatic islets.

Key Words

- ▶ adulthood
- ▶ protein restriction
- ▶ autonomous nervous system
- ▶ pancreatic islets

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Introduction

The developmental origins of health and disease (DOHaD) concept originates from the epidemiological, clinical, and experimental evidence that insults during early life can program metabolic dysfunctions later in life and cause great impacts on world health and links the action of poor nutrition at critical development stages with the onset of metabolic diseases later in life. Many metabolic diseases with high worldwide prevalence such as obesity, type 2 diabetes, and hypertension can have their etiology during fetal development (Barker 2004).

One very potent insult is malnourishment, including protein restriction, during pregnancy, which causes intrauterine growth restriction leading to newborns with low body weight (BW) and/or small size. These neonates are at high risk for developing metabolic diseases later in life (Greenwood & Bell 2003). In addition, protein restriction during gestation and lactation has been observed in both humans and experimental animals to cause obesity during adulthood (Barker & Bagby 2005, Cripps & Ozanne 2006).

During fetal development, nutrients, including amino acids, are crucial to brain maturation, which allows neurons to connect to each other and build the neuronal network, enabling the brain to control body functions such as metabolism. Hypothalamic immaturity caused by protein malnourishment during fetal development has been observed in both children and rodents, and these findings were associated with disruptions of metabolic homeostasis in adulthood, demonstrating the important role of the hypothalamus in the regulation of energy metabolism (Plagemann *et al.* 2000a, Bouret *et al.* 2008). Protein restriction during lactation induces metabolic reprogramming in adult rats (de Oliveira *et al.* 2012a). Indeed, it has been shown in rodents that the milk-suckling phase is pivotal for producing neuronal network connections (Lesage *et al.* 2006). Recently, our laboratory has shown that protein malnourishment during adolescence can also cause metabolic dysfunctions during adulthood (de Oliveira *et al.* 2012b), leading us to conclude that the peripubertal phase could be another window for metabolic programming beyond perinatal development. It is important to note that metabolic programming can also be setup even before conception. Indeed, adult male rats that were fed a high-fat diet were able to transmit their impaired glucose/insulin homeostasis phenotype to their female offspring (Ng *et al.* 2010). Disruption of glycemic homeostasis and pancreatic β -cell dysfunction, among other metabolic alterations, are often observed later in life as a consequence of metabolic programming (Ng *et al.* 2010).

It has been reported that the acute effects of food as well as protein deprivation in adulthood were able to induce changes in glucose–insulin homeostasis and energy metabolism due to imbalances in the autonomous nervous system (ANS; Leon-Quinto *et al.* 1998). In addition, hypothalamus malfunctions and behavioral abnormalities induced by protein restriction during adulthood have been observed (Alamy & Bengelloun 2012); however, these effects were observed immediately after the diet-restriction treatment and not after diet recovery. Beyond the hypothalamus, it is striking that pancreatic β -cell are one among several other targets of early injuries, which can manifest as dysfunction in adulthood (Barbosa *et al.* 2002, Reusens & Remacle 2006). Taken together, these studies indicate that metabolic programming is not merely an adaptation and can influence lifelong health. However, it has not been determined whether protein restriction during early adulthood can affect metabolism later in life. To address this question, our current study tests whether protein malnourishment during early adulthood can provoke

changes in metabolism, including pancreatic islet dysfunction, later in adulthood.

Materials and methods

Animals and protein malnourishment

Male 60-day-old Wistar rats from 12 different litters were randomly chosen for the study and distributed into two groups (6 litters/group). One group of rats was supplied with a normal-protein (NP; 23% w/w protein) diet and allowed to feed *ad libitum* (Nuvital, Curitiba, PR, Brazil) throughout the experimental period (the NP group), while the other group of rats (the low-protein (LP) group) received a LP (4% w/w protein) diet containing the same amount of calories as the normal diet from 60 to 90 days of age, as described previously (de Oliveira *et al.* 2011). After 90 days of age, the LP group received a NP diet for another 60 days to allow dietary recovery. Because sex differences between insulin levels and glucose tolerance have been observed in some early poor-protein feeding studies (Lopes Da Costa *et al.* 2004), only male rats were used in the experiments. Throughout the experimental period, the rats (five rats per cage) were kept under controlled conditions, including temperature ($22 \pm 2^\circ\text{C}$) and photoperiod (0700–1900 h), and allowed to eat and drink *ad libitum*.

The rats were supplied by the Central Animal Facility of the State University of Maringá. The Ethical Committee for Animal Experiments of the State University of Maringá, which adheres to Brazilian Federal Law, approved this protocol.

BW and food intake

BW and chow consumption were recorded every 2 days during the poor diet treatment from 60 to 90 days of age. Data collection was continued until the end of the experimental protocol when rats were 150 days old, at which point all animals were used for biochemistry and physiological analysis. Food intake (FI) values were calculated as the difference between the amount of food remaining (final diet (Df)) and the total food available (initial diet (Di)), divided by the number of days and the number of rats in the cages: $\text{FI (g)} = (\text{Df} - \text{Di}) / (2/5)$. The BW gain was also calculated by subtracting the daily BW value throughout the experimental period by the starting BW value at 60 days old (beginning of the diet treatment) of all the rats in both experimental groups. The area under the curve (AUC) of the entire observation period (60–150 days) for BW gain and food intake was also calculated.

Intravenous glucose tolerance test

When the rats were 150 days old, animals in both groups underwent a surgical procedure under ketamine and xylazine anesthesia (3 and 0.6 mg/100 g of BW respectively) to implant a silicone cannula into the right jugular vein that attached in the dorsal region of the neck. The cannula was treated with heparinized saline solution (50 IU heparin/ml; 0.9% w/v of saline solution) before implantation to avoid blood clots. After a 12 h fast (1900–0700 h) and without anesthesia, a glucose load (1 g/kg BW) was infused through the cannula. Blood samples were collected immediately before glucose infusion (0 min) and at 5, 15, 30, and 45 min after infusion.

The blood samples collected previously (0 min) to the intravenous glucose tolerance test (ivGTT) were used to assess the fasting levels of glucose and insulin. Plasma obtained from the other blood samples was stored at -20°C for subsequent determination of the glucose concentration by the glucose oxidase method (Trinder 1969), with a commercial kit (Gold Analisa, Belo Horizonte, MG, Brazil). Insulin level was determined by RIA (Scott *et al.* 1981), with a gamma counter (Wizard² Automatic Gamma Counter-2470, PerkinElmer, Shelton, CT, USA), using human insulin as the standard, an anti-rat insulin antibody (Sigma–Aldrich), and ^{125}I -labeled recombinant human insulin (PerkinElmer). The insulin intra- and interassay coefficients of variation were respectively 12.2 and 9.8, and the limit of detection was 0.006 ng/ml.

Insulin sensitivity index

Because the insulin sensitivity index (ISI) used by Matsuda & DeFronzo (1999) gives a reasonable approximation of the whole-body insulin sensitivity, we used this index, with small modifications, to measure the body insulin sensitivity in our experimental model. To calculate ISI, we used the following calculation: $\text{ISI} = 10^4 / \sqrt{((\text{fasting glycemia} \times \text{fasting insulinemia}) \times (\text{AUC}_{\Delta\text{glycemia}} \times \text{AUC}_{\Delta\text{insulinemia}}))}$. The glycemia (Δ_{glycemia}) and insulinemia increment ($\Delta_{\text{insulinemia}}$) of each time from ivGTT was obtained by subtracting the fasting plasma glucose and insulin levels. Increases in the total Δ_{glycemia} and $\Delta_{\text{insulinemia}}$ were calculated by using the glycemia and/or insulinemia AUC at 45 min post ivGTT.

Electrical recording of nerves

When the rats were 150 days old, a batch of rats from each group was anesthetized with thiopental (45 mg/kg BW)

after a 12 h fast and a surgical longitudinal incision was made on the anterior cervical region. Under a dissecting microscope, the nerve bundle of the left vagus superior branch was severed from the carotid artery, close to the trachea. The nerve trunk was pulled with a fine cotton line, and a pair of recording silver electrodes (0.6 mm diameter) was placed under the nerve. The nerve was covered with silicone oil to avoid further dehydration. The electrode was then connected to an electronic device (Bio-Amplificator, Insight, Ribeirão Preto, SP, Brazil) that amplified the electrical signal up to 10 000 times. To exclude low and high frequencies, recordings of <1 and >80 kHz were discarded by a filter. The neural signal output was acquired by an Insight interface (Insight), viewed online and stored on a personal computer running software developed by Insight. During all data acquisition, animals remained in a Faraday cage to avoid any electromagnetic noise. Nerve activity was analyzed by the number of spikes over the course of 5 s. Spikes were characterized by depolarization that surpassed 0 mV. After stabilization of the signal over the course of 2 min, 20 record frames of 15 s were randomly chosen from each animal for spike counting. The average numbers of spikes were used to calculate the rate of nerve firing for each rat.

The sympathetic branch nerve from the superior cervical ganglia was dissected from another batch of anesthetized rats from both experimental groups, also after 12 h of fasting. The electrode was placed under the sympathetic branch nerve. Firing rates were obtained as described for the vagus nerve.

Pancreatic islets isolation

Pancreatic islets were isolated by the collagenase technique as previously described (Gravena *et al.* 2002). At 150 days of age, rats were anesthetized with thiopental (45 mg/kg BW) and immediately decapitated, and the abdominal wall was cut and open. After that, 8 ml Hanks buffered saline solution (HBSS (mmol/l): NaCl, 136.9; KCl, 5.4; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.81; Na_2HPO_4 , 0.34; KH_2PO_4 , 0.44; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.26; NaHCO_3 , 4.16; glucose, 0.06; BSA 15, and (v/v; O_2 , 95% + CO_2 , 5% mixed)/10 min, pH 7.4) containing (w/v) 0.1% collagenase type XI, 5% BSA, and 0.6% HEPES (*N*-(2-hydroxyethyl-piperazine)-*N'*-(2-ethanesulphonic acid)) (Sigma–Aldrich) were injected into each rats' common bile duct.

The pancreas, swollen with the collagenase solution, was quickly excised and incubated in a glass beaker for 17–18 min at 37°C . The suspension was then discarded, and the precipitate was washed three times with HBSS.

Islets were easy to find in the final precipitate and were collected with the aid of a stereomicroscope. At least three rats from three different litters were used for each experimental procedure from each group.

Stimulation of insulin secretion

To adapt isolated islets to a baseline glucose concentration (5.6 mmol/l), the islets were pre-incubated for 60 min in 1 ml of Krebs–Ringer solution ((mmol/l): NaCl, 115; NaHCO₃, 24; KCl, 1.6; MgCl₆H₂O, 1; CaCl₂2H₂O, 1; BSA, 15) at pH 7.4 containing 5.6 mmol/l glucose. This solution was gassed with (v/v) 95% O₂ and 5% CO₂ (mixture) to maintain pH 7.4.

After the pre-incubation, islets were incubated with different glucose concentrations for another 60 min. The supernatants from the incubations were collected and stored for further insulin measurements. To study muscarinic acetylcholine receptor (mAChR) function, some islets from other batches were incubated after pre-incubation for a further 60 min in Krebs–Ringer solution containing 8.3 mmol/l glucose and/or 8.3 mmol/l glucose + 10 µmol/l acetylcholine in the presence of 10 µmol/l neostigmine to avoid the acetylcholinesterase action found in the islets. In addition, a non-selective mAChR antagonist, atropine (10 µmol/l), was also used. To block the function of the mAChR subtypes M₂ and M₃ the following antagonists were used: 1 µmol/l methoctramine and 100 µmol/l 4-diphenylacetoxy-N-methylpiperidine methiodide, also known as 4-DAMP.

Doses of the antagonists had been tested previously, and the concentrations that induced at least 20% inhibition or potentiation of the insulinotropic effect of 10 µmol/l acetylcholine in islets incubated with 8.3 mmol/l glucose were chosen.

To study the function of adrenoceptors in another batch of islets from both groups, islets, after pre-incubation with 5.6 mmol/l glucose, were stimulated with a high glucose concentration (16.7 mmol/l) either in the presence of 1 µmol/l epinephrine and an α₂-adrenoceptor antagonist, yohimbine, at 10 µmol/l or in the presence of epinephrine and a β₂-adrenoceptor antagonist, propranolol, at 1 µmol/l.

As with the muscarinic receptor studies, doses of antagonists had been tested previously, and concentrations that induced at least 50% inhibition or potentiation of insulin secretion stimulated with 16.7 mmol/l glucose were chosen. All drugs used to study the muscarinic and adrenergic function were purchased from Sigma–Aldrich.

Fat pad weight evaluation

At 150 days old, the rats were anesthetized with thiopental (45 mg/kg BW) and killed by decapitation. Fat pad stores (retroperitoneal, periepididymal, and visceral) were removed and weighed to assess the state of obesity.

Statistical analyses

Results are given as the mean ± S.E.M., and were subjected to Student's *t*-test or one-way ANOVA followed by Bonferroni's post-test analysis. *P* < 0.05 was considered statistically significant. Tests were carried out using GraphPad Prism, version 5.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Results

Effects of a LP diet treatment during adulthood on BW gain, food intake, fat pad store, and plasma levels of glucose and insulin

As shown in Table 1, the LP diet given over 30 days did not change the final BW or body length of the rats when they reached 150 days old. While a modest increase of 8% in the fasting glycemia was observed in the LP rats compared with the NP rats (*P* < 0.05, *n* = 15–16), the fasting insulinemia did not present any significant difference between the two groups. Tissue weight of the fat pads showed that treatment with the LP diet induced a higher increase in the retroperitoneal (29%; *P* < 0.0001, *n* = 20–25),

Table 1 Effects of LP diet treatment started at the beginning of adulthood on adult rats later in life. Data are presented as mean ± S.E.M. obtained from 6–25 rats of each experimental group

Biometric parameters	NP	LP
Body weight (g)	460.9 ± 6.31	448.2 ± 7 (NS)
Body length (cm)	24.22 ± 0.12	23.85 ± 0.2 (NS)
Retroperitoneal fat (g/100 g BW)	1.63 ± 0.07	2.11 ± 0.08*
Periepididymal fat (g/100 g BW)	1.57 ± 0.08	1.80 ± 0.04 [†]
Visceral fat (g/100 g BW)	0.95 ± 0.04	1.07 ± 0.04 [†]
Fasting glycemia (mmol/l)	5.31 ± 0.09	5.73 ± 0.13 [†]
Fasting insulin (pmol/l)	40.48 ± 3.64	44.33 ± 3.24 (NS)
ISI	130.0 ± 9.57	99.42 ± 8.36 [†]

**P* < 0.0001 and [†]*P* < 0.05 by Student's *t*-test. NP, normal-protein; BW, body weight; LP, low-protein; NS, no statistical difference.

Figure 1

Body weight (BW) gain and food intake. Data are presented as mean $\pm$ S.E.M. of 20–24 rats from four to five different litters for each group. Progression of BW (A) from 60 to 90 days of age; and food intake (B) from 60 to 90 days of age, (during the LP diet). The inset to the two figures, shows the area under the curve (AUC). *** $P < 0.0001$, ** $P < 0.01$, and * $P < 0.05$ by Student's *t*-test. NP, normal-protein; LP, low-protein.

periepididymal (12.4%), and visceral (14.6%) fat pads of the LP rats compared with the NP rats ($P < 0.05$, $n = 20$ –25). Finally, as shown in Table 1, the LP rats presented a 23% increase in ISI compared with the NP animals ($P < 0.05$, $n = 6$). Severe growth retardation was observed during the LP diet treatment, resulting in a smaller BW in the LP rats compared with the NP rats, as shown in Fig. 1A ($P < 0.0001$, $n = 20$ –24). During the same period, the LP rats ate less than the NP rats, as shown in Fig. 1B. The AUC for food intake also decreased by 18% ($P < 0.01$, $n = 4$ –5 litters).

After the protein restriction treatment had ended, BW growth increased significantly in the LP group compared with the NP group. The LP rats exhibited catch-up growth

between 90 and 150 days, with higher AUC values compared with the NP animals ($P < 0.0001$, $n = 20$ –24; Fig. 2A). When the food intake was evaluated during the same period, the LP rats ate 10% more than the NP rats ($P < 0.05$, $n = 4$ –5 l; Fig. 2B).

Plasma glycemia and insulinemia during the ivGTT

Data from the ivGTT are shown in Fig. 3; a modest increase of 8% in the total glycemia of the LP rats was observed compared with the NP rats, as indicated by the AUC in Fig. 3A ($P < 0.01$, $n = 15$ –16). The insulin levels of the LP rats were 15% higher than those of the NP rats during the ivGTT, as shown by the AUC in Fig. 3B ($P < 0.05$, $n = 15$ –16).

Figure 2

Body weight (BW) gain and food intake. Data are presented as mean $\pm$ S.E.M. of 20–24 rats from four to five different litters for each group. Progression of BW (A) from 90 to 150 days of age; and food intake (B) from 90 to 150 days of age (after the LP diet was replaced by an NP one). The inset to the two figures, shows the area under the curve (AUC). *** $P < 0.0001$, ** $P < 0.01$, and * $P < 0.05$ by Student's *t*-test. NP, normal-protein; LP, low-protein.

Figure 3

Plasma glycemia (A) and insulinemia (B) during the intravenous glucose tolerance test (ivGTT). Data are presented as mean $\pm$ s.e.m. of 13–17 rats at least from four to five different litters. The inset for each figure shows the area under the curve (AUC). ** $P < 0.01$ and * $P < 0.05$ by Student's *t*-test. NP, normal-protein; LP, low-protein.

Glucose-induced insulin secretion and muscarinic/adrenergic regulation on this process in isolated pancreatic islets

Isolated pancreatic islets from both experimental groups, when exposed to increasing glucose concentrations, increased insulin secretion in a dose-dependent manner, and the secretory response did not show any significant magnitude differences between the two groups (Fig. 4; $P = 0.588$, $n = 16$).

Figure 5 shows the effects of the mAChR agonist and antagonists on the insulinotropic effects of acetylcholine. Glucose (8.3 mmol/l) stimulated insulin secretion in the islets of both groups. In addition, the insulinotropic effect of acetylcholine on glucose-induced insulin secretion was increased by twofold in islets of both groups compared with their respective controls (Fig. 5A; $P < 0.01$, $n = 16$); however, no significant differences were observed between

the LP and the NP islets groups ($P = 0.455$, $n = 16$). The use of a non-selective mAChR antagonist, atropine, inhibited the insulinotropic action of acetylcholine by 43 and 51% in isolated islets from the LP and NP rats respectively ($P < 0.001$, $n = 16$). A similar effect was observed with the use of the mAChR M_3 subtype-selective antagonist, 4-DAMP, but there were no significant differences between the LP and NP islets groups ($P = 0.073$, $n = 16$). On the other hand, islets incubated with methoctramine, a mAChR M_2 subtype-selective antagonist, exhibited an increased insulinotropic effect of acetylcholine of $\sim 20\%$ in both the LP and the NP islet groups ($P < 0.05$, $n = 16$), but without any significant differences in the magnitude (Fig. 5B; $P = 0.6$, $n = 16$).

As expected, 16.7 mmol/l glucose stimulated insulin secretion in the islets of both groups in a similar way (Fig. 6A); however, when isolated islets were incubated with epinephrine, a 40% inhibition of insulin secretion was observed in the islets of both groups (Fig. 6A; $P < 0.0001$, $n = 16$). The use of yohimbine, a selective α_2 -adrenoceptor antagonist, blocked the insulinostatic effect of epinephrine, causing potentiation of 250 and 155% for the glucose-induced insulin secretion in islets from the NP and LP rats respectively (Fig. 6B; $P < 0.0001$, $n = 16$). On the other hand, the use of a selective β_2 -adrenoceptor antagonist, propranolol, provoked an inhibition of 34 and 33% for the glucose-induced insulin secretion in the presence of epinephrine in islets from the NP and LP groups respectively ($P < 0.05$, $n = 16$).

Effect of an LP diet treatment on ANS activity

Compared with the NP rats, the LP rats exhibited an increase of 21% in the superior vagus nerve activity ($P < 0.05$, $n = 17$ –23; Fig. 7A and C), and the sympathetic nerve firing rate was decreased by 28% ($P < 0.01$, $n = 17$ –23; Fig. 7B and C).

Figure 4

Insulinotropic effect of different glucose concentrations. Data are presented as mean $\pm$ s.e.m. of insulin secreted by the pancreatic islets obtained from four to eight rats from at least four or five different litters. Statistical analyses between NP and LP groups for each different glucose concentrations were carried out by Student's *t*-test. NP, normal-protein; LP, low-protein; NS, no significant difference.

Figure 5

Effect of muscarinic receptor agonist and antagonists on glucose-induced insulin secretion. Data represent mean $\pm$ S.E.M. of insulin secreted by pancreatic islets from six rats from four to five different litters. (A) Insulin secretion stimulated by 8.3 mmol/l Glu and potentiated by 10 μ mol/l ACh. Symbols above the bars indicate significance levels: [#] $P < 0.001$ for the NP and ^Ω $P < 0.01$ for the LP group treated with 10 μ mol/l ACh compared with those treated with 8.3 mmol/l Glu by one-way ANOVA. (B) The zero value represents 100% glucose-induced insulin release potentiated by 10 μ mol/l ACh in both the NP and LP groups. Bars above or below to the zero level represent the percentage increase or decrease provoked by the indicated concentrations of nonselective antagonist (Atr) and agonists selective to M₂mAChR (MTT), and M₃mAChR (4-DAMP) on glucose-induced insulin release potentiated by 10 μ mol/l ACh in both NP and LP groups. Symbols above or below the bars indicate significance levels: *** $P < 0.001$ between the NP and LP groups, [#] $P < 0.01$ for each indicated muscarinic antagonist compared with 10 μ mol/l ACh treatment for the NP, or ^Ω $P < 0.01$ for the LP group by one-way ANOVA. NP, normal-protein; LP, low-protein; Glu, glucose; ACh, acetylcholine; Atr, atropine; MTT, methoctramine; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide.

Discussion

In addition to DOHaD concept, which links stressful stimulus early in life to metabolic changes later in life, we have shown for the first time, to our knowledge, that a LP diet treatment starting during early adulthood can promote changes in metabolism later in adulthood, as indicated by increased food intake, fat tissue accretion, glucose intolerance, and peripheral tissue insulin

resistance. Although these effects are not as robust as those found in adult rats that were protein-malnourished during maternal pregnancy and/or the lactation period (Roseboom *et al.* 2001), metabolism impairment is evident in adult rats that were protein-restricted at the beginning of adulthood.

In rats treated with a LP diet (8% protein starting at 60 days old for 6 months), the total number of neurons in the hippocampus were reduced without changes in the hippocampal synapses even after 2 months of nutritional rehabilitation (Lukoyanov & Andrade 2000). Our data indicate that protein restriction during the beginning of adulthood changes glucose and energy metabolism, which might persist in the later stage of life even when the LP diet intervention had been removed. Protein malnourishment during pregnancy causes evident obesity when the rodents reach adulthood (Anguita *et al.* 1993, Fernandez-Twinn & Ozanne 2006, Howie *et al.* 2012); however, the current study does not provide strong enough evidence to indicate that a LP diet during young adulthood provokes obesity later in life. The current results indicate the induction of a slight increase in fat tissue, which is supported by a modest increase in insulin blood levels after a glucose bolus infusion. It has been shown that insulin and leptin are hormones associated with tissue adiposity. Increasing insulin levels indicate an increase in lipogenesis (Schwartz *et al.* 2000). Adolescent rats that received a LP diet from 30 to 60 days of age exhibited hyperinsulinemia, severe hyperleptinemia, insulin resistance, high-fat-tissue accretion, and disruption of β -cell function later in life (de Oliveira *et al.* 2012b). The end of adolescence occurs very close to protein deprivation period used in the current protocol, indicating that during early adulthood animals may have a propensity to undergo metabolic changes that can persist as a long-term consequence, similar to the perinatal and pubertal developmental stages. Although the present data indicate that protein restriction during early adulthood causes significant changes in metabolism, the magnitude of these events is minor compared with the metabolic disruptions observed when the nutritional insult occurs during the perinatal phase (Barbosa *et al.* 1999, Vickers *et al.* 2000). In addition, a LP maternal diet provokes metabolism dysfunction in the offspring of these rats when they reach adulthood that correlates with the severity of the protein deprivation (Barbosa *et al.* 1999). Therefore, with less severe protein restriction, such as the 8% protein diet or higher-protein diets, differences such as increased fat tissue storage and insulin resistance might not have been observable.

Figure 6

Effect of adrenoceptors agonist and antagonists on glucose-induced insulin secretion. Data represent mean $\pm$ S.E.M. of insulin secreted by pancreatic islets from six rats from four or five different litters. (A) Insulin secretion stimulated by 16.7 mmol/l Glu and blocked by 1 μ mol/l Epi. Symbols above the bars indicate significance levels: [#] $P < 0.0001$ for the NP and ^Ω $P < 0.0001$ for the LP group treated with 1 μ mol/l Epi compared with the values for treatment with 16.7 mmol/l Glu by one-way ANOVA. (B) The zero value represents the insulin secretion stimulated by 16.7 mmol/l Glu in the presence of 1 μ mol/l Epi for both NP and LP groups. Bars represent the percentage increase triggered by the indicated concentrations of selective α_2 -adrenoceptor (Yoh), and β_2 -adrenoceptor (Pro) antagonists on insulin secretion stimulated by (16.7 mmol/l Glu in the presence of 1 μ mol/l Epi) in both NP and LP groups. Symbols above the bars indicate significance levels: ^{***} $P < 0.0001$ among NP and LP groups; [#] $P < 0.05$ for the NP, and ^Ω $P < 0.0001$ for the LP islets treated with 10 μ mol/l Yoh or 10 μ mol/l Pro in relation to 10 μ mol/l Epi treatment by one-way ANOVA. NP, normal-protein; LP, low-protein; Glu, glucose; Epi, epinephrine; Yoh, yohimbine; Pro, propranolol.

In the current study, unchanged BW gain associated with decreased food intake is evident in the LP rats from 60 to 90 days of age, implying that energy metabolism is drastically affected by the LP diet treatment. Protein-calorie deprivation has been reported to be a key factor involved in alterations in the energy-controlling hypothalamic neurons (Plagemann *et al.* 2000b, Attig *et al.* 2008, Delahaye *et al.* 2008). According to the thrifty phenotype hypothesis, protein-calorie deprivation

programs the metabolism for survival, in this case starvation status, which may endure long-term; however, in the opposite situation in which the nutritional content is higher, disturbances may appear because of the metabolic programming imprinted previously (Vickers *et al.* 2000, Hales & Barker 2001). Indeed, we observed that the LP rats presented high catch-up growth associated with high food intake immediately after replacement of the LP diet with the 2-month diet rehabilitation. Also, the unbalanced activity of the ANS was a remarkable finding of the current study. The high vagus tonus associated with low sympathetic activity may induce LP rats to be obesity-prone. In agreement with the autonomic hypothesis (Inoue *et al.* 1991), recognized as a key factor involved in obesity onset and/or maintenance; our data might be important for highlighting that undernourishment, even in adulthood, could disturb autonomous functions controlling energy metabolism.

Metabolism homeostasis is highly dependent on the quantity and activity of insulin. Pancreatic β -cells, which are one of four cell types of pancreatic islets, produce, store, and release insulin into the blood stream. Therefore, β -cells play a pivotal role in the regulation of metabolism (Gautam *et al.* 2006, Chang-Chen *et al.* 2008). β -cells are very sensitive to insults during any phase of life, especially during early and late life, which can result in malfunctions with limited capacity for regeneration or neogenesis (Dumortier *et al.* 2007, Frantz *et al.* 2012, Supale *et al.* 2012). In contrast with other periods of LP intervention such as pregnancy, lactation, and adolescence (Hoet *et al.* 1992, Reusens & Remacle 2006, de Oliveira *et al.* 2011, 2012b), a LP diet during early young adult life does not have a significant effect on the function of the pancreatic islets. Islets isolated from the LP rats exhibit the same glucose-induced insulin secretion magnitude as the NP rats, suggesting that pancreatic β -cells are resistant to LP diet insult early in adulthood, at least up to 150 days of age.

In spite of the known long-lasting disturbances in glucose-induced insulin secretion due to the nutritional injuries imprinted early in life (Ozanne *et al.* 2006, Zambrano *et al.* 2006, Fagundes *et al.* 2007), acute effects of food deprivation, such as weak insulin secretion of pancreatic islets, have been reported in adult rats after a 48-h fast (Carpinelli *et al.* 1986) or even as a result of protein or calorie restriction, which, interestingly, have induced hypoinsulinemia (Leon-Quinto *et al.* 1998) or hyperinsulinemia (He *et al.* 2012), respectively, immediately after the diet-restriction treatment. However, these metabolic changes have been observed immediately after the stressor effects, implying that even during later developmental

Figure 7

Sympathetic and parasympathetic electrical activity from the cervical superior nerves. Bars represent mean $\pm$ S.E.M. of the firing rates of the vagus (A) and cervical sympathetic (B) nerves from 17–23 rats from five to seven

different litters. Representative records of nerve tonus are shown in the lower panel. ** $P < 0.01$ and * $P < 0.05$ by Student's *t*-test. NP, normal-protein; LP, low-protein.

stages, physiological machinery adjustments are used to maintain the glucose–insulin homeostasis. In contrast, the stressor effects of protein-deprivation were spread over a long time, as the current work used a short period of stress followed by nutritional rehabilitation. Although the ability of pancreatic islets to secrete insulin in response to either glucose or muscarinic agents was not affected, metabolic impairments commonly diagnosed in obese and/or pre-diabetic individuals are present, indicating that phenotype changes similar to the those induced early in life by intrauterine undernutrition were imprinted in the LP rats even during a later stage of life (with low plasticity in organ/tissue development). It has been previously reported that humans who experienced the Dutch hunger winter between 1944 and 1945 over a short time during childhood, adolescence and/or young adulthood exhibited a strong tendency for the development of type 2 diabetes later in life (van Abeelen *et al.* 2012), which supports the results of this study.

Those results and the data presented in the current study indicate that pancreatic β -cells have a great capacity to resist nutritional stress insults or have a potential functional recovery rate in the later developmental stages of life, even under the unbalanced action of the ANS which exhibits a pivotal role in controlling insulin secretion.

Changes in the epinephrine secretory response of β -cells were observed in which glucose-induced insulin secretion was potentiated by an α_2 -adrenoceptor antagonist with a greater magnitude in the isolated islets from the LP rats. These results were associated with high electrical firing of the vagus nerve and low sympathetic tonus, indicating the influence of an ANS imbalance on the number and function of adrenergic receptors in the pancreatic β -cells. A close relationship has been observed between pancreatic islet malfunction and impairment of the ANS in adult rats and mice that were metabolically programmed early in life (Balbo *et al.* 2007, Grassioli *et al.* 2007, Gravena *et al.* 2007, de Oliveira *et al.* 2011); Scomparin *et al.* (2009) however, muscarinic and adrenergic receptor of pancreatic β -cells did not show substantial functional differences in the current study.

In conclusion, protein restriction performed during early adulthood induces weak hallmarks of metabolic malfunctions in rats later in life; however, pancreatic β -cell function is not affected, indicating that during young adulthood, the central architecture and peripheral control of metabolism are already close to completion of their development. The data presented here provide a better understanding of the concept of DOHaD by highlighting the importance of the early phases of life in programming lifelong metabolism patterns, even though

some impairment could be imprinted by stressful nutritional insults after the main critical developmental periods had passed.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A M and P C F M designed the study and revised the manuscript; A M, J C O, T A S R, L P T, K V P, R A M, G E, C C S F, A R A, A B T, and A P conducted the research; A M, J C O, L F B, C G, and L A N analyzed the data; A M and P C F M wrote the manuscript; and P C F M acted as the principal investigator, provided support, and reviewed the data and manuscript.

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