

Neonatal Low-Protein Diet Changes Deiodinase Activities and Pituitary TSH Response to TRH in Adult Rats

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Protein malnutrition during neonatal programs for a lower body weight and hyperthyroidism in the adult offspring were analyzed. Liver deiodinase is increased in such animals, contributing to the high serum triiodothyronine (T3) levels. The level of deiodinase activities in other tissues is unknown. We analyzed the effect of maternal protein restriction during lactation on thyroid, skeletal muscle, and pituitary deiodinase activities in the adult offspring. For pituitary evaluation, we studied the *in vitro*, thyrotropin-releasing hormone (TRH)-stimulated thyroid-stimulating hormone (TSH) secretion. Lactating Wistar rats and their pups were divided into a control (C) group, fed a normal diet (23% protein), and a protein-restricted (PR) group, fed a diet containing 8% protein. At weaning, pups in both groups were fed a normal diet until 180 days old. The pituitary gland was incubated before and after TRH stimulation, and released TSH was measured by radioimmunoassay. Deiodinase activities (D1 and D2) were determined by release of ¹²⁵I from [¹²⁵I]reverse triiodothyronine (rT3). Maternal protein malnutrition during lactation programs the adult offspring for lower muscle D2 (–43%, $P < 0.05$) and higher muscle D1 (+83%, $P < 0.05$) activities without changes in thyroidal deiodinase activities, higher pituitary D2 activity (1.5 times, $P < 0.05$), and lower TSH

response to *in vitro* TRH (–56%, $P < 0.05$). The evaluations showed that the lower *in vivo* TSH detected in adult PR hyperthyroid offspring, programmed by neonatal undernutrition, may be caused by an increment of pituitary deiodination. As described for liver, higher skeletal muscle D1 activity suggests a hyperthyroid status. Our data broaden the knowledge about the adaptive changes to malnutrition during lactation and reinforce the concept of neonatal programming of the thyroid function. Exp Biol Med 233:57–63, 2008

Key words: protein-restricted diet, lactation, programming, deiodinase, TSH, TRH

Introduction

Protein-energy malnutrition is the most prevalent form of nutritional disorder among children in developing countries. It is estimated that more than 3.7 million deaths in 2000 could be attributed to being underweight (1). Protein malnutrition often occurs during gestation, lactation, and the first 2 years of life (2). Despite an overall decrease of stunting in developing countries, childhood malnutrition remains a major public health problem (3).

Malnutrition effects upon thyroid function in humans (4) and animals (5, 6) have been well reported. Previously, we have shown that malnutrition during lactation has long-term effects on the thyroid function of adult offspring (7).

The enzyme 5'-iodothyronine deiodinase is responsible for the metabolism of thyroxine (T4) to triiodothyronine (T3), the bioactive hormone. Based on several functional criteria, tissue distribution, and different protein sequences, 5'-deiodinases are classified into two isoenzymes: type 1 (D1) and type 2 (D2). In rats, D1 is predominantly found in liver, kidney, and thyroid and generates most of the serum T3. D2 is predominantly expressed in brain, pituitary, and brown adipose tissue (BAT), where the local T₄-to-T₃ conversion is catalyzed. However, according to current concepts, D1 and D2 make similar contributions in generating plasma T3. In peripheral tissues, including liver,

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D1 is stimulated by circulating thyroid hormones. In hyperthyroidism, an increase in D1 activity and a reduction in D2 activity occur, and conversely, in hypothyroidism, the opposite effect upon the deiodination profile has been observed (8).

Barker et al. (9) associated low birth weight with diseases related to Metabolic Syndrome (diabetes, obesity, and hypertension) in the adulthood. That association has been denominated *programming*, which is defined as the basic biological phenomena that putatively underlie relationships among nutritional experiences of early life and adult diseases (10, 11).

Kajantie et al. (12) recently showed that small body size at birth and during childhood are predictors for development of spontaneous hypothyroidism in adult women, suggesting an association of early life events with thyroid disorders and autoimmunity. Adult rats whose mothers were protein-restricted during lactation had higher thyroid iodine uptake, higher serum thyroid hormones, and lower serum thyroid-stimulating hormones (TSH; 7, 13). We also showed that the high T3 level is due to a higher T4 conversion by liver D1 (13).

Here, we tested whether other sites, such as thyroid, skeletal muscle, and pituitary, may contribute to maintaining the high T3 level (serum and local) observed in the adult offspring whose mothers were protein-restricted during lactation. In addition, we intended to investigate the intrinsic pituitary response to thyrotropin-releasing hormone (TRH) of adult PR hyperthyroid animals programmed by neonatal undernutrition, which depends, at least in part, on the local T4 to T3 conversion in the pituitary gland.

Materials and Methods

Wistar rats were kept in a room with a controlled temperature ($25^{\circ} \pm 1^{\circ}\text{C}$) and a dark/light cycle (lights on from 0700–1900 hrs). Three-month-old, virgin, female rats were caged with a male rat at a proportion of 3:1. After mating, each female was placed in an individual cage with free access to water and food until delivery. Our experimental design was approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro, which based their analysis on the principles described in the *Guide for the Care and Use of Laboratory Animals* (14).

At birth, 12 mothers were randomly separated into (i) a control group (C; $n = 6$) with free access to a standard laboratory diet (23% protein); and (ii) a protein-restricted group (PR; $n = 6$) with free access to an isoenergy and a low-protein diet (8% protein).

The PR diet was made in our laboratory using the control diet and replacing part of its protein with cornstarch (Table 1). The amount of starch was calculated to make up for the decrease in energy content because of protein reduction (15–21).

To avoid the influence of litter size on the programming

effect, only mothers whose litter size was 10–12 offspring were used. At 24 hours after birth, litter adjustment was performed, and 6 male pups were kept per C or PR dam because it has been shown that this procedure maximizes lactation performance (22).

Neonatal malnutrition was started at birth and ended at weaning (21 days), when pups received a normal diet until 180 days of age (7, 13, 15–20). Food intake and body weight of the control and malnourished mothers were measured during throughout lactation (15, 21).

During lactation, a pup's body weight was monitored daily. From weaning until the 180th day, body weight and relative food intake (g/100 g body wt) were monitored every 4 days.

Two adult animals from each litter were killed by decapitation, to collect pituitary, thyroid, and skeletal muscle (solum). Visceral fat mass (VFM) was excised and weighed for evaluation of the central adiposity, mesenteric, epididymal, and retroperitoneal (23).

In Vitro TRH-Stimulated TSH Release. Pituitaries of C and RP groups were dissected quickly. The anterior pituitary was separated from the posterior pituitary and transected with a longitudinal midline cut. Each anterior hemipituitary was immediately transferred to a tube containing 1 ml of Krebs-Ringer-bicarbonate medium, at pH 7.4, and incubated at 37°C in an atmosphere of 95% O_2 and 5% CO_2 in a Dubnoff metabolic shaker (50 cycles/min). After a 20-min preincubation period, the medium was removed, and the hemipituitaries were kept in 1 ml of fresh medium. After 1 hr of incubation, an aliquot was collected for basal TSH measurement, and then, TRH (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 50 nM. Glands were incubated for 30 mins to determine TSH release in response to TRH. Each hemipituitary was homogenized in phosphate-buffered saline (PBS), at pH 7.6, for intrapituitary TSH-content measurement (24, 25).

TSH levels were measured by murine-specific radioimmunoassay (RIA; National Institutes of Health, Bethesda, MD) and reported in terms of reference preparation (RP3). All measurements were performed in only one assay. Intraassay variation was 2%.

Deiodinase Activity Determination. D1 and D2 activities were measured based on methods previously described (13, 26, 27) by the release of ^{125}I from [^{125}I]-reverse T3 (rT3) in thyroid microsomes, pituitary total homogenate, and skeletal muscle homogenate. Assays were performed in phosphate buffer containing 1 mM of EDTA at pH 6.9. The D1 assay was performed in the presence of 1.5 μM of rT3, 10 mM of dithiothreitol (DTT), and 100 nM of T4 (to inhibit D2). The D2 assay was performed with 2 nM rT3, 40 mM DTT, and 1 mM propylthiouracil (PTU; to inhibit D1). Equal aliquots of [^{125}I]rT3 (1.07 mCi/ μg ; New England Nuclear-Dupont, Boston, MA) were purified by paper electrophoresis and were placed in each tube assay. Reaction was started by adding samples with the following

Table 1. Composition of the Diets

	Control ^a	Low protein ^b
Ingredients (g/kg)		
Soybean + wheat	230.0	80.0
Corn starch	676.0	826.0
Soybean oil	50.0	50.0
Vitamin mix ^c	4.0	4.0
Mineral mix ^c	40.0	40.0
Macronutrient composition (%)		
Protein	23.0	8.0
Carbohydrate	66.0	81.0
Fat	11.0	11.0
Total energy (kJ/kg)	17,038.7	17,038.7
Mean energy intake (kJ/day)	512.2	311.8

^a Standard diet for rats (Nuvilab-NUVITAL Nutrientes LTDA, Paraná, Brazil).

^b The low-protein diet was prepared in our laboratory using the control diet and replacing part of its protein with cornstarch. The amount of the latter was calculated so as to make up for the decrease in energy content due to protein reduction.

^c Vitamin and mineral mixtures were formulated according to the AIN-93G recommendation for rodent diets (35).

amounts of protein (μg): 25–150 thyroid, 40–65 pituitary, and 70–250 skeletal muscle. A blank tube containing 50 μl of the substrate solution and 50 μl of buffer was run in parallel to each assay, which had its values subtracted from the enzyme samples. Reactions were performed in a shaking bath at 37°C and stopped after 30 (thyroid D1), 60 (thyroid and pituitary D2), or 120 mins (muscle) by addition of a mixture of 8% bovine serum albumin (BSA) and 10 mM of PTU, followed by cold 20% trichloroacetic acid. Samples were centrifuged (1000 g, 4°C, 5 mins), and 200 μl of the supernatants were applied to Dowex 50WX2 columns (100–200 mesh, hydrogen form; BioRad, Richmond, CA). Free ^{125}I , eluted from the column with 10% acetic acid, was measured in a gamma counter. The percentage of deiodination in the presence of the enzyme was about 10%–20%. The amount of free ^{125}I in the blank sample was generally less than 1%–2% of the total radioactivity in the reaction mixture. The specific enzyme activity was expressed in femtomoles, picomoles, or nanomoles of rT3 deiodinated per hour per milligram of protein. Protein was measured by the method described by Bradford (28).

Liver Mitochondrial α -Glycerol-3-Phosphate Dehydrogenase (GPD) Activity Determination. To confirm the hyperthyroid state of adult PR offspring programmed by neonatal undernutrition, the liver GPD was measured in the mitochondrial fraction using phenazinemethosulfate (PMS) as an electron transporter between the reduced enzyme and iodonitrotetrazolium chloride violet (INT; 29, 30). The assay was performed in presence of 0.1 M of DL- α -glycerophosphate, diluted in potassium cyanide/potassium phosphate buffer (KCN/KPB) and a solution of 7.9 mM INT and 0.12 mM PMS. Samples were analyzed at 500 nm, and the values were expressed as absorbance (optical density [OD]/milligram) of mitochondrial protein.

Protein was measured using the method described by Bradford (28).

Serum Hormones Determination. All measurements were performed in only one assay. Total T4 and T3 were measured by RIA using commercial kits (ICN Pharmaceuticals, Inc, Costa Mesa, CA). The intraassay variations were 4% (T3) and 3% (T4), respectively. TSH was measured by specific RIA, using a kit for rat TSH supplied by the National Institutes of Health and expressed in terms of the reference preparation (RP-3) provided. The intraassay variation was 2%.

Statistical Analysis. Data are reported as means $\pm$ standard errors of the mean (SEM). Body weight and food intake evolution were analyzed by two-way analysis of variance (ANOVA) and Newman-Keuls multiple comparison tests. The other experimental data were analyzed by Student's unpaired *t* test, and differences were considered significant at $P < 0.05$.

Results

Nutritional Evaluation. According to previous studies (15, 21), protein malnutrition caused a lower food intake (–34%; $P < 0.05$) and body weight (–29%; $P < 0.05$) in lactating rats. During lactation, the PR pup's body weight was about 40% lower, as already published (15, 19, 31).

Food intake and body weight evolution of rats whose mothers who were PR during lactation are shown in Figure 1. PR offspring showed no change in food intake from weaning until adulthood (Fig. 1A), despite the lower body weight since weaning (Fig. 1B, $F_{61,3308} = 13.17$; $P < 0.0001$), as previously reported in several studies by our group (13, 15, 16, 18). At 180 days old, PR offspring showed lower visceral fat mass (–47%; $P < 0.05$; Fig. 1C).

Mitochondrial GPD (mGPD) Activity and Serum Hormones. Adult PR animals presented high levels of liver mGPD activity, confirming their hyperthyroid state (C: 0.044 ± 0.006 vs. PR: 0.060 ± 0.003 OD/min/mg protein [ptn], $n = 12$, $P < 0.05$). These animals had higher serum total triiodothyronine (TT3; C = 21.1 ± 1.9 vs. PR = 59.3 ± 3.8 nmol/L; $P < 0.001$) and total thyroxine (TT4; C = 11.8 ± 0.6 vs. PR = 13.4 ± 0.4 nmol/L; $P < 0.05$) and lower serum TSH (C = 3.0 ± 0.4 vs. PR = 2.2 ± 0.1 ng/ml; $P < 0.05$) compared with controls.

In vitro TSH Post-TRH Secretion of Adult Offspring Whose Mothers Were PR During Lactation. The *in vitro* TSH release in adult offspring whose mothers were PR during lactation is shown in Figure 2. The *in vitro* basal TSH release was not different from controls (Fig. 2A). However, PR offspring showed lower TSH post-TRH secretion (–56%; $P < 0.05$; Fig. 2B), but no significant changes in intrapituitary TSH content (Fig. 2C).

Deiodinase Activities of Adult Offspring Whose Mothers Were PR During Lactation. As shown in Figure 3, pituitary D2 activity was higher (+1.5 times; $P < 0.05$) as was skeletal muscle D1 activity (+83%; $P < 0.05$).

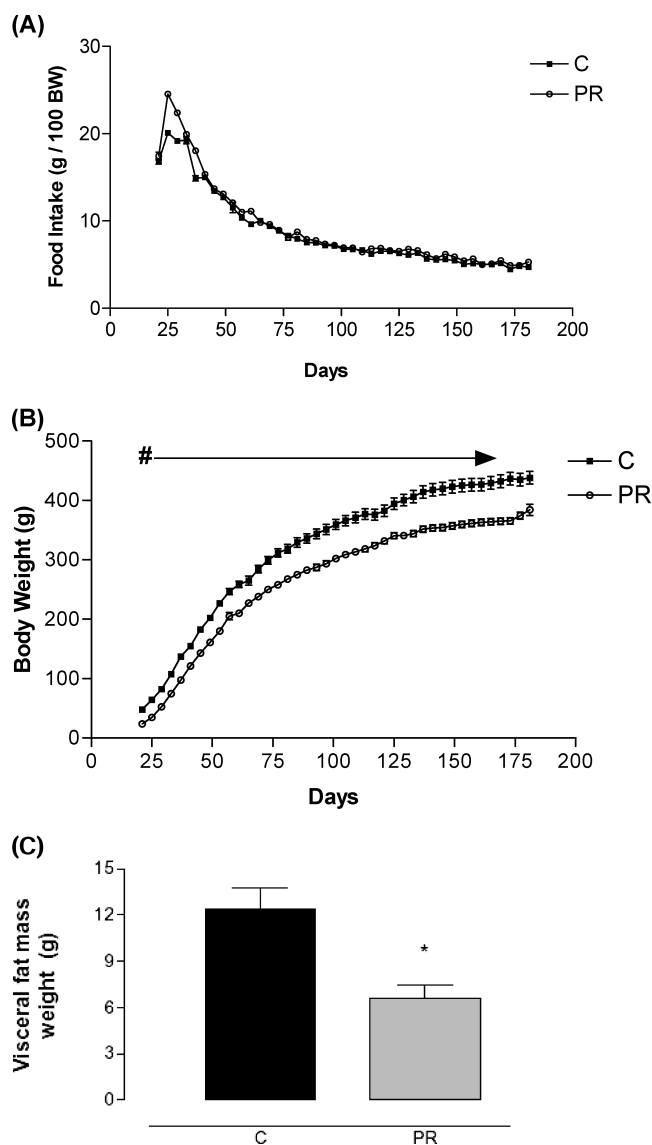


Figure 1. Nutritional evaluation of adult animals. (A) Food intake per body weight, (B) body weight evolution, and (C) visceral fat mass weight in 180-day-old rats whose mothers were fed a normal (C) or protein-restricted (PR) diet during lactation. C group had free access to standard laboratory diet (23% protein); PR group had free access to isoenergy and low-protein diet (8% protein). Data are reported as mean $\pm$ SEM # vs. C at $P < 0.001$, * vs. C at $P < 0.05$, $n = 12$ animals/group.

Muscle D2 activity was lower (-43% ; $P < 0.05$) in the PR group, as depicted in Figure 4, and no changes were detected in thyroidal D1 and D2 activities (Fig. 5).

Discussion

We found that during lactation, maternal nutrition can program body weight (15–21, 31) and thyroid function (7, 13) of their offspring in adult life. So, reinforcing our previous report, the present study provides evidence that maternal protein restriction, during lactation, programs others parameters of the thyroid function in the adult offspring, which helps in understanding observed hyper-

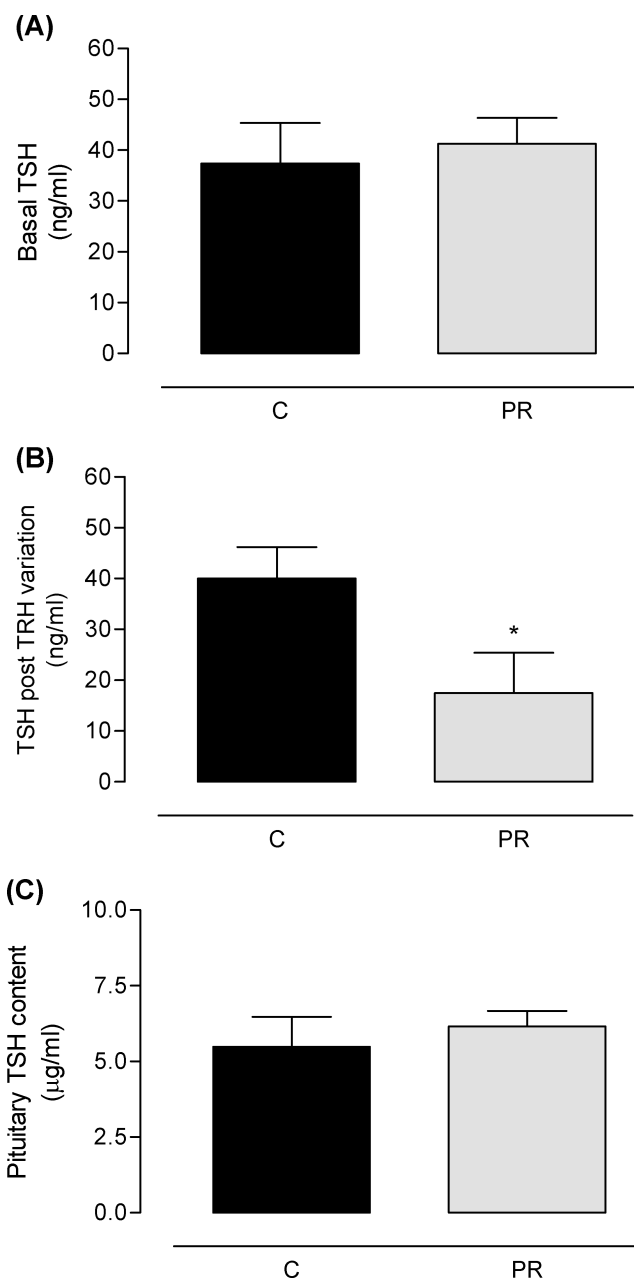


Figure 2. *In vitro* TSH secretion (A) at basal conditions, (B) TSH secretion variation after 30 minute of 50 nM TRH stimulus, and (C) intrapituitary TSH content of hemipituitary of adult rats whose mothers were fed a normal (C) or protein-restricted (PR) diet during lactation. C group had free access to standard laboratory diet (23% protein); PR group had free access to isoenergy and low protein diet (8% protein). Data are reported as mean $\pm$ SEM. * $P < 0.05$ vs. C, $n = 12$ animals/group.

thyroidism. These animals presented higher muscle D1 and lower D2 activities, without changes in thyroidal deiodinases, and higher pituitary D2 activity and lower *in vitro* TSH response to TRH. In fact, we confirmed the thyroid hormone dysfunction of programmed rats through increased liver mGPD because its activity is induced by thyroid hormones (29, 30).

Adult rats whose dams were submitted to PR during

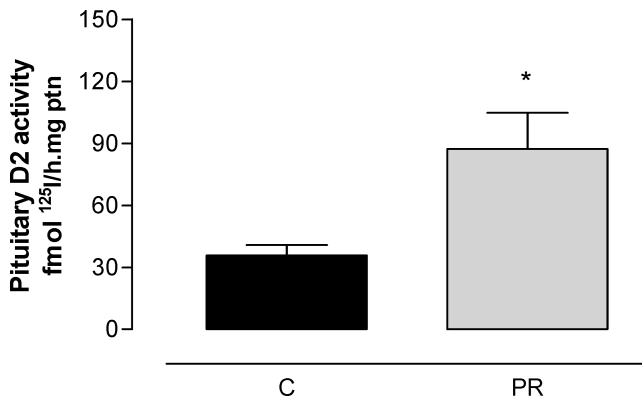


Figure 3. Pituitary D2 activity of adult rats whose mothers were fed a normal (C) or protein-restricted (PR) diet during lactation. C group had free access to standard laboratory diet (23% protein); PR group had free access to isoenergy and low protein diet (8% protein). Data are reported as mean $\pm$ SEM. * $P < 0.05$ vs. C, $n = 12$ animals/group.

lactation presented lower visceral fat mass. Thus, a reduction of the adipose tissue may be the main explanation for their lower body weight. It is possible that the lower body weight without changes in food intake was due to a hypermetabolic status associated with growth impairment. In fact, both hypotheses are possible because programmed PR animals presented hyperthyroidism (7, 13) and lower pituitary expression of growth hormone (GH) mRNA (19), despite of normal length.

We demonstrated that liver D1 activity in adult offspring was programmed by maternal nutrition during lactation (13). These rats showed higher liver D1 activity that could be important for the maintenance of higher serum T3 (7, 13). In the present study, increased muscle D1 activity in the PR rats (83%) is potentially contributing, together with the higher liver D1 activity, to enhanced circulating T3 in this programming model.

Regarding the skeletal muscle D2, programmed PR offspring presented lower activity compared with controls. In the PR group, changes of muscle deiodinase activities are correlated to the predominant regulation exerted by the serum thyroid hormone because, in hyperthyroidism, D1 is increased and D2 is decreased (8).

Adult animals whose mothers had PR diets during lactation showed normal thyroid D1 and D2 activity. These data were unexpected because TSH, which is the supposed main regulator of thyroid deiodination, is lower in our model. So, other, potentially unknown, regulators of thyroidal deiodinase activity may be acting in this situation.

Paradoxically, in our model, hyperthyroidism was associated with a higher pituitary D2 (8). Thus, other factors may be increasing D2 activity in this situation. Furthermore, the higher pituitary D2 activity in PR adult rats can explain the TSH suppression (7, 13, 16) by higher local T3 generation.

Previously, we have shown that adult rats whose mothers were malnourished during lactation presented

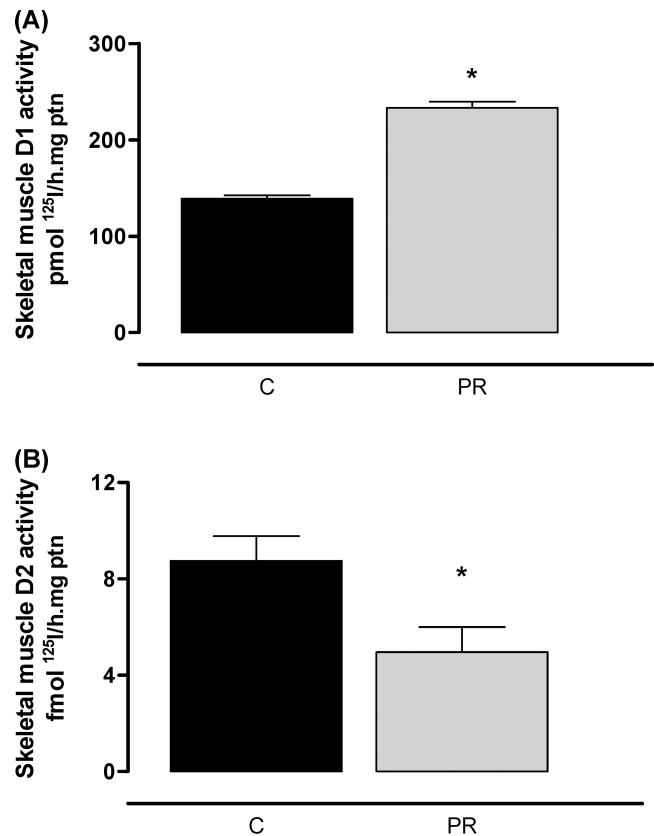


Figure 4. Muscle deiodinase activities. Skeletal muscle (A) D1, and (B) D2 activities of adult rats whose mothers were fed a normal (C) or protein-restricted (PR) diet during lactation. C group had free access to standard laboratory diet (23% protein); PR group had free access to isoenergy and low protein diet (8% protein). Data are reported as mean $\pm$ SEM. * $P < 0.05$ vs. C, $n = 12$ animals/group.

central leptin resistance (20). It is well known that leptin up-regulates TRH production (32, 33). In addition, it was recently described that leptin decreases hypothalamic D2 activity in fed, euthyroid rats (34). Therefore, if hypothalamic D2 behaves as pituitary D2, leptin central resistance could contribute to the higher activity of that enzyme. So, TRH could be lower in PR-programmed rats because of the absence of leptin stimulation as well as because of the higher hypothalamic D2 contributing to the local TRH inhibition.

An interesting observation from the present study is the evidence that, in the model of programming by neonatal malnutrition, other factors may be more important than thyroid hormones or TSH in controlling deiodinase activities. Because another deiodinase may be altered in this situation, such as renal D1, brown and white adipose tissue D2, or brain D3 activities, more studies should be done to better characterize the deiodination profile and its regulation in PR-programmed rats. Also, the measurement of intracellular T3 and T4 concentrations in the pituitary could help in understanding the paradoxical D2 activity. In addition, determining the cellular uptake of the thyroid hormone (monocarboxylate transporter 8 [MCT8] brain and

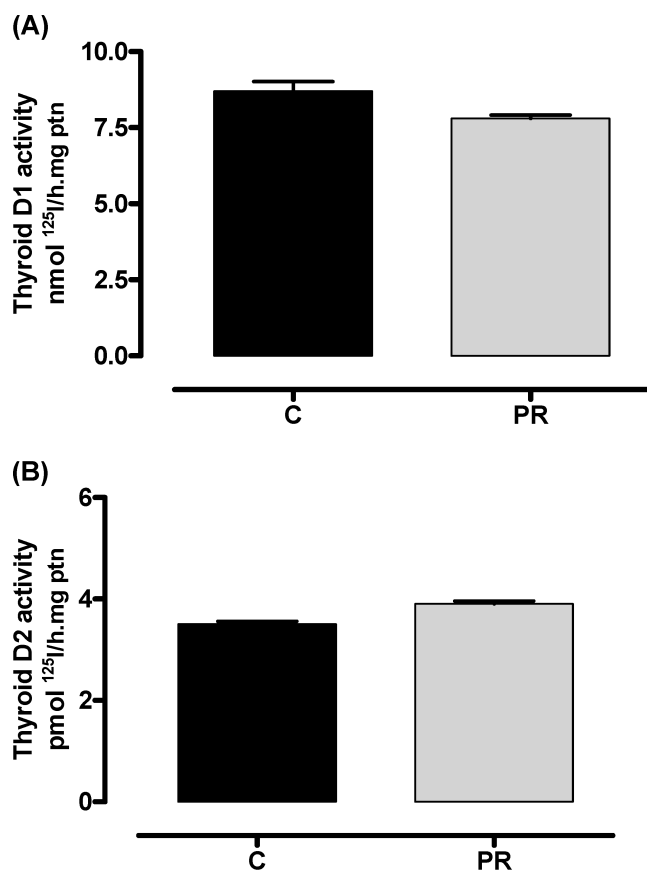


Figure 5. Thyroid deiodinase activities. Thyroid (A) D1, and (B) D2 activities of adult rats whose mothers were fed a normal (C) or protein-restricted (PR) diet during lactation. C group had free access to standard laboratory diet (23% protein); PR group had free access to isoenergy and low protein diet (8% protein). Data are reported as mean $\pm$ SEM. * $P < 0.05$ vs. C, $n = 12$ animals/group.

pituitary expression; liver taurocholate cotransporting polypeptide [NCTP] expression) could contribute to knowledge about intracellular T3 and T4 levels.

The PR group presented lower levels of *in vitro* TSH release in response to TRH. According to previous data, PR adult rats showed lower serum TSH concentration (7, 13, 16). Beyond the negative feedback exerted by thyroid hormones, it is possible that the lower TSH was also due to a decrease in TRH secretion because the same higher D2 activity could be found at the hypothalamic level. The pituitary TSH content did not change, indicating that TSH suppression by the thyroid hormone could be restricted to the inhibition of the secretory pool.

In general terms, epigenetic mechanisms, such as DNA methylation, induced by neonatal nutritional, environmental, or hormonal factors, may lead to an increased risk of metabolic diseases in the adult offspring (11). Thus, this explanation may help in understanding the mechanism by which protein restriction during lactation permanently changes the thyroid function. Whether that alteration can make undernourished children more susceptible to thyroid

disorders in adult life deserves epidemiological and prospective studies.

Our results reinforce the idea that peripheral conversion of T4 to T3 could be programmed by maternal nutritional status during lactation and may be an important mechanism in maintaining a higher T3 action, making the animal more able to mobilize fatty acid depots. That could be of evolutionary advantage in sparing protein depots in situations of protein deficiency.

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