Increased 5'-iodothyronine deiodinase activity is a maternal adaptive mechanism in response to protein restriction during lactation

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Abstract

We have shown that protein restriction during lactation is associated with higher levels of serum and milk tri-iodothyronine (T3) with lower serum thyroxine (T4), suggesting an increased T4 to T3 conversion. To investigate this hypothesis, the activity of type 1 (D1) and/or type 2 (D2) iodothyronine deiodinases was evaluated on days 4, 12 and 21 of lactation in several tissues of dams fed an 8% protein-restricted (PR) diet and controls fed a 23% protein diet. Serum TSH, T3 and T4 were measured by radioimmunoassay. Deiodinase activity was determined by the release of 125I from 125I-reverse T3, under specific conditions for D1 or D2. PR dams had a transitory reduction in liver D1 activity ($P < 0.05$) on day 12, and a small increase in thyroid D1 on day 12 followed by a small decrease on day 21. However, thyroid D2 activity was higher than controls ($P < 0.05$) during the whole of the lactation period. Mammary gland D1 and D2 activities were lower on day 4 of lactation in PR dams ($P < 0.05$), and D2 was higher on day 21 ($P < 0.05$). Potentially, a lower conversion of T3 to di-iodothyronine in the mammary glands of PR dams at the beginning of lactation may serve to provide more T3 through the milk. Brown adipose tissue (BAT) D2 activity was higher ($P < 0.05$) in PR dams during all periods of lactation. PR dams showed higher skeletal muscle D1 activity only at the end of lactation, but no changes in D2 activity. Higher pituitary D1 and D2 activities in the PR group ($P < 0.05$) at the end of lactation could have contributed to the lower serum TSH. These data suggest that the higher thyroid and BAT D2 activity during the whole of lactation and skeletal muscle D1 activity at the end of lactation may contribute to the higher serum T3 in PR dams.

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Introduction

More than a third of the world’s children are affected by protein-energy malnutrition. For all the indicators (wasting, stunting and underweight), the most favourable situation of low or moderate prevalence occurs in Latin America, in Asia most countries have a high or very high prevalence, and in Africa a combination of these circumstances is found. A total 80% of the children affected live in Asia, 15% in Africa and 5% in Latin America (de Onis et al. 1993).

The prevalence of stunting has fallen in developing countries from 47% in 1980 to 33% in 2000, although progress has been uneven according to the region. Stunting has increased in Eastern Africa, but decreased in Asia and South America, Northern Africa and the Caribbean show modest improvement, and Western Africa and Central America show very little progress. Despite an overall decrease in stunting in developing countries, child malnutrition still remains a major public health problem in these countries (de Onis et al. 2000).

The effect of malnutrition upon thyroid function in humans (LoPresti et al. 1991) and other adult animals is well documented (Harris et al. 1978, Moura et al. 1987). Our previous studies showed short- and long-term effects of malnutrition during lactation on the thyroid function of mothers and their offspring (Ramos et al. 2000, Passos et al. 2001a, k, 2002). Dams submitted to a protein-restricted (PR) diet during lactation had, at weaning, a significant decrease in 131I thyroid uptake and an increase in 131I uptake by the mammary gland and a higher transfer of 131I to the milk when compared with controls (Ramos et al. 2000). These animals had higher tri-iodothyronine (T3) levels in serum and milk, and lower serum levels of...
Deiodinase activity in protein-restricted lactating rats

P C LISBOA and others

Materials and Methods

Wistar rats were kept in a room with controlled temperature (25 ± 1 °C) and with artificial light:darkness cycles (lights on from 0700 to 1900 h). Three-month-old, nubile female rats were housed with a male rat and, after mating, each female was placed in an individual cage with free access to water and food until parturition. The use of the animals in our experimental design was approved by the Animal Care and Use Committee of the Biology Institute of State University of Rio de Janeiro, based on the principles described in the Guide for the Care and Use of Laboratory Animals (Bayne 1996).

The dams were randomly assigned to one of two groups: a control group, with free access to a standard laboratory diet containing 23% protein and a PR group, with free access to an isoenergy and PR diet containing 8% protein. Table 1 shows the composition of the diets.

The PR diet was prepared in our laboratory using the control diet and replacing part of its protein with cornstarch. The amounts of starch, mineral and vitamin mixtures were calculated so as to make up for the decrease in energy, vitamin and mineral content due to protein reduction. Vitamin and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93 G recommendation for rodent diet and contain the recommended amount of iodine (Reeves et al. 1993).

Excess pups were removed within 24 h of birth, so that only six pups were kept per dam because it has been shown that this procedure maximizes lactation performance (Fishbeck & Rasmussen 1987). Malnutrition started at birth, which was defined as day 0 of lactation, and continued to the day on which the animal was killed.

The lactating dams received 0·6 µCi125I i.p. on days 4, 12 and 21 of lactation, and 2 h later they were killed with a lethal dose of ether (Ramos et al. 1997). Thyroid glands were excised and weighed and thyroidal 125I uptake was individually determined with a gamma-counter (Cobra Auto-gamma; Packard Instrument Co., Downers Grove, IL, USA).

To study D1 and D2 deiodinase activities during three different periods of lactation we used four to six dams in each period. On days 4, 12 and 21 of lactation the dams from each group were killed with a lethal dose of

Table 1 Composition of the control and low-protein diets

<table>
<thead>
<tr>
<th>Ingredients (g/kg)</th>
<th>Control*</th>
<th>Low protein†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean + wheat</td>
<td>230·0</td>
<td>80·0</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>676·0</td>
<td>826·0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>50·0</td>
<td>50·0</td>
</tr>
<tr>
<td>Vitamin mix‡</td>
<td>4·0</td>
<td>4·0</td>
</tr>
<tr>
<td>Mineral mix‡</td>
<td>40·0</td>
<td>40·0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Macronutrient composition (%)</th>
<th>Control*</th>
<th>Low protein†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>23·0</td>
<td>8·0</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>66·0</td>
<td>81·0</td>
</tr>
<tr>
<td>Fat</td>
<td>11·0</td>
<td>11·0</td>
</tr>
<tr>
<td>Total energy (kJ/kg)</td>
<td>17 038·7</td>
<td>17 038·7</td>
</tr>
</tbody>
</table>

*Standard diet for rats (Nuvilab-NUVITAL Nutrientes LTDA, Paraná, Brazil). †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.

ff‡ff

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pentobarbital and blood was obtained by cardiac puncture. The thyroid, liver, pituitary, mammary gland, skeletal muscle and BAT were excised and they were kept individually at −70 °C until assay. Blood samples were centrifuged to obtain serum, which was kept at −20 °C until assay.

**Deiodinase activity measurement**

Thyroid, pituitary, liver, muscle and BAT deiodinase activities were measured based on methods previously described (Pazos-Moura et al. 1991, Curty et al. 2000, Lisboa et al. 2001) and, for mammary deiodinase activities, we used protocols described previously (Aceves & Valverde 1989, Song et al. 2000), with slight modifications. D1 and D2 assays were performed in phosphate buffer containing 1 mM EDTA, pH 6·9. To evaluate D1 activity in thyroidal and hepatic microsomal fractions, we added 1·5 µM reverse T3 (rT3) and 10 mM dithiothreitol (DTT). In other tissues, deiodinases were accessed using 2 nM rT3 and 40 mM DTT. In order to distinguish D1 and D2 activity, assays were carried out in the presence of 1 mM propylthiouracil (PTU) (to inhibit D1) or 100 nM T4 (to suppress D2). Equal aliquots of 125I-rT3 (1·07 mCi/µg; New England Nuclear-Dupont, Boston, MA, USA), which was purified by paper electrophoresis, were placed in each assay tube. The reaction was started by the addition of the samples, corresponding to the following amount of protein (µg): 4–18 thyroid, 15–30 pituitary, 25–117 liver, 10–30 BAT, 110–230 skeletal muscle and 100–660 mammary gland. A blank tube was run in parallel with each set of assays; this contained 50 µl of the substrate solution and 50 µl of buffer, and its value was subtracted from that of enzyme samples. Reactions were performed in a shaking bath at 37 °C, and stopped after 30 min (thyroid and liver D1), 1 h (thyroid D2; pituitary), 2 h (BAT D2; muscle) or 4 h (mammary gland) by the addition of a mixture of 8% bovine serum albumin and 10 mM PTU, followed by cold 20% trichloroacetic acid. Samples were centrifuged (2000 g, 4 °C, 5 min) and 200 µl of the supernatants were applied to Dowex 50 W-X2 columns (100–200 mesh hydrogen form; BioRad, Richmond, CA, USA). Free 125I, eluted from the column with 10% acetic acid, was measured in a gamma-counter. Deiodination percentage in the presence of the enzyme was around 10–20%, except in the mammary gland which was 2–4% of the total released iodide. The amount of free 125I in the blank was generally less than 1–2% of the total radioactivity in the reaction mixture. The specific enzyme activity was expressed in fmol, pmol or nmol rT3 deiodinated/h per mg protein. Protein was measured by the method described by Bradford (1976).

**Figure 1** Liver D1 (A), thyroid D1 (B) and thyroid D2 (C) activities in lactating rats fed a control (solid bars) and PR diet (open bars). Values represent the means ± s.e.m. of four to six rats per group. *P<0·05, significant difference between groups.
Serum thyrotropin (TSH), T3 and T4 quantification

Serum TSH was determined by specific radioimmunoassay (RIA), using a kit for rat TSH supplied by the NIDDKD (Bethesda, MD, USA) and data are expressed in terms of the reference preparation provided (RP-3).

Total serum T3 (TT3) and T4 (TT4) were measured by RIA, using commercial kits (Coat-A-Coat; DPC, Los Angeles, CA, USA), in which we used control standard curves diluted in iodothyronine-free rat serum (charcoal-treated).

Statistical analysis

Values are given as means ± S.E.M. Statistical significance of experimental observations was determined by two-way ANOVA followed by Student’s t-test (non-paired). The level of significance was set at P<0.05.

Results

The effects of a PR diet on hepatic D1 activity, and thyroid D1 and D2 activities of lactating rats at different stages of lactation are demonstrated in Fig. 1. PR dams had a lower liver D1 activity on day 12 compared with controls (74%, P<0.05), but not at days 4 and 21. The PR group had a significantly higher thyroid D2 activity on days 4 (sevenfold), 12 (+55%) and 21 (+83%) compared with controls. With regard to thyroid D1, the PR group had a higher activity on day 12 (+50%, P<0.05) and a lower activity on day 21 of lactation (−20%, P<0.05), as shown in Fig. 1.

We detected lower mammary gland D1 (−58%, P<0.05) and D2 (−67%, P<0.05) activities on day 4 in PR dams, compared with controls. At weaning, there was a higher mammary D2 activity (+48%) in PR animals compared with controls (Fig. 2).

BAT D2 and skeletal muscle D2 and D1 activities are presented in Fig. 3. BAT D2 activity was higher in the PR group at all periods of lactation investigated; however, it was significant only on days 4 and 12. The PR group did not show any alteration in skeletal muscle D2 activity, although they showed higher skeletal muscle D1 activity at the end of lactation (2.5-fold, P<0.05).

As verified in Fig. 4, the PR group showed an important increase in both pituitary D1 (five times) and D2 (3.8 times) activity at the end of lactation.

Table 2 shows serum TSH, T3 and T4 concentrations and 2-h thyroid radioiodine uptake during lactation. From the middle to the end of the lactation period, the PR group had lower serum TSH compared with controls. Serum T3 was higher in PR mothers during all stages of lactation compared with controls (46%, 75% and 46%, days 4, 12 and 21 respectively, P<0.01). On the contrary, serum T4 was lower in PR mothers at the beginning (day 4, 19%, P<0.05) and at the end (day 21, 34%, P<0.05) of lactation. Thyroid radioiodine uptake was lower in PR mothers during all stages of lactation compared with the controls (85%, 79% and 67%, days 4, 12 and 21 respectively, P<0.001).

Discussion

The higher serum T3 of dams fed a low-protein diet cannot be explained by alterations in liver D1 activity, which showed a transitory decrease at mid lactation. Also, thyroid D1 seems not to be contributing to the rise in the circulating T3 of PR dams observed during all lactation periods, since the increase in the enzyme activity at mid lactation was followed by a decrease in late lactation. On the other hand, thyroid D2 activity was higher during all periods of lactation investigated, suggesting that thyroidal T4-to-T3 conversion may be contributing to the higher serum T3 in PR dams. In favour of this possibility is the fact that in rats, contrary to humans, the thyroid contributes to a significant proportion of total body T3 production, around 50% (Chanoine et al. 1993).
Until recently, the D1 pathway was considered to be the major one for extrathyroidal T₃ production in euthyroid rats. The D2 pathway was considered to contribute to extrathyroidal T₃ production only when its activity was sufficiently elevated and/or liver and kidney D1 activity was depressed (Silva & Larsen 1985). However, in both rats and humans, evidence has been presented that D2 contributes significantly to the generation of the serum T₃ pool. Nguyen et al. (1998) showed that, in rats, the D2 activity is responsible for the generation of approximately 50% of T₃ from T₄. D2 is present in BAT, brain, pituitary and recently it has been detected in skeletal muscle (Salvatore et al. 1996a) and in human and rat thyroid glands (Salvatore et al. 1996b, Bates et al. 1999, Dutra et al. 2003).

The D2 activity of BAT was increased in PR dams during all periods of lactation, especially from the beginning to mid lactation and, therefore, BAT exportation of T₃ is another important mechanism that may explain the rise in serum T₃ in the PR dams. Evidence that BAT is a source of serum T₃ was produced by studies on cold-exposed rats, whose BAT 5’-D2 activities were elevated (Silva & Larsen 1985).

Despite the relevance of D2 activity in human skeletal and cardiac muscle for T₃ peripheral production (Salvatore et al. 1996a, Hosoi et al. 1999, Sabatino et al. 2000), we did not find important changes in skeletal muscle D2 activity at any time evaluated. On the other hand, the rise in skeletal muscle D1 activity at the end of lactation potentially contributes to higher serum T₃ in PR dams (Fig. 3C), but the large mass of this tissue in the body must be considered.

Our initial hypothesis was that one of the important sites for T₃ production for serum and milk in PR dams was the mammary gland, since some authors (Valverde & Aceves 1989, Jack et al. 1994) reported increased mammary D1 activity in lactating rats. However, contrary to this initial hypothesis, we observed lower mammary D1 and D2 activities at the beginning of lactation. It is possible that this change could work as an adaptive mechanism, aiming to prevent T₃ inactivation, since those deiodinases are also capable of catalysing T₃ deiodination to di-iodothyronine (Körhle 1999). Probably, the T₃ that reaches the mammary tissue in this critical period must be further transferred to the pups through the milk, in order to minimize the effects of neonatal hypothyroidism, especially during CNS development. However, at the end of lactation, the high mammary D2 activity present in the PR group may potentially contribute to elevate serum T₃ concentrations.

At the end of the lactation period, there was an increment in both pituitary D1 and D2 activities in PR dams. These changes could contribute to the TSH suppression observed in these rats. On the other hand, the low serum T₄ may be the cause of the increase in thyroid,

![Figure 3 BAT D2 (A), and skeletal muscle D2 (B) and D1 (C) activities in lactating rats fed a control (solid bars) and PR diet (open bars). Values represent the means ± s.e.m. of four to six rats per group. *P<0.05, significant difference between groups.](image-url)
pituitary and BAT D2 activity. A possible mechanism is an increase in the enzyme half-life, since T4 promotes higher rates of D2 proteolysis (Steinsapir et al. 1998, 2000).

Another interesting observation from the present study is the evidence that in special situations, such as malnutrition, other factors may be more important than TSH in stimulating thyroid deiodinases (Erickson et al. 1982, Wu et al. 1985, Salvatore et al. 1996b, Murakami et al. 2001), since the D2 activity was increased despite the lower serum TSH in PR dams.

It is unlikely that the higher T3 serum concentration is caused by an increase in T3/T4 production by the thyroid, since serum TSH and thyroid radioiodine uptake are lower (Table 2). The fact that thyroid iodine uptake was not higher makes it unlikely that an iodine deficiency caused by the reduced diet or protein malnutrition, affecting either ingestion or iodine intestinal absorption, could be responsible for the changes observed here. On the other hand, the content of iodine in the low protein diet was compensated by the addition of a mineral mix with an adequate amount of iodine.

In conclusion, the present study has shown that nutritional factors modulate both 5'-deiodinase isoforms differently in several tissues. Moreover, this study has suggested that the higher thyroid and BAT D2 activities are the major contributors for the higher serum T3 in PR dams, although skeletal muscle D1 may be important at late stages of lactation. Therefore, alterations in 5'-deiodinase represent a maternal protective mechanism against the drop in serum T4 induced by protein restriction during lactation.

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