Low replacement doses of thyroxine during food restriction restores type 1 deiodinase activity in rats and promotes body protein loss

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Abstract

During food restriction, decreased basal metabolic rate secondary to reduced serum thyroid hormones levels contributes to weight loss resistance. Thyroxine (T4) and 3,3',5-tri-iodothyronine (T3) administration during caloric restriction produce deleterious side effects; however, the administration of physiological doses of T4 during food restriction has never been evaluated. The aim of this study was to analyze the effects of low replacement doses of T4 in Wistar rats subjected to 40% food restriction. Food restriction for 30 days led to significantly reduced liver type 1 deiodinase activity, serum TSH, leptin, T4, T3, metabolic rate, and body mass. The significant reduction in hepatic deiodinase activity found during food restriction was normalized in a dose-dependent manner by T4 replacement, showing that decreased type 1 deiodinase (D1) activity is secondary to decreased serum thyroid hormone levels during caloric restriction. The lowest replacement dose of T4 did not normalize resting metabolic rate, but was able to potentiate the effects of food restriction on carcass fat loss and did not spare body protein. The highest dose of T4 produced a normalization of daily oxygen consumption and determined a significant reduction in both carcass fat and protein content. Our results show that serum T4 normalization during food restriction restores serum T3 and liver D1 activity, while body protein is not spared. Thus, decreased serum T4 during caloric restriction corresponds to a protective mechanism to avoid body protein loss, highlighting the importance of other strategies to reduce body mass without lean mass loss.


Introduction

Moderate reduction in caloric intake has well-known systemic consequences, including weight loss and a decrease in fat mass; however, homeostatic mechanisms impair further weight loss after long periods of food restriction, such as the decrease in basal metabolic rate (Ravucin et al. 1985, Rosenbaum et al. 2002). The responses to food restriction have been well documented in a number of species, including humans (Kelley et al. 1993), monkeys (Kemnitz et al. 1994), rats (Dean et al. 1998), and mice (Harris et al. 1994). Thyroid hormones are the predominant regulators of basal metabolic rate and their serum levels are reduced during caloric deprivation (Vagenakis et al. 1977, Siba et al. 1979, Kinlaw et al. 1985, Douyon & Schteingart 2002). Since thyroid hormones seem to play a crucial role in energy homeostasis by regulating both energy intake and expenditure, analogs of these hormones have long been considered to be potential drugs for controlling body mass (Oh & Kaplan 1994, Moreno et al. 2003, Lanni et al. 2005, Villicev et al. 2007).

Decreased thyroid function seems to be related to impaired thyrotropin-releasing hormone (TRH) and thyroid-stimulating hormone (TSH) secretion (Ahima 2000, Krotkiewski 2000), and to changes in the peripheral deiodination of thyroid hormones (Bianco et al. 2002). Three iodothyronine deiodinases have been identified and characterized in different tissues. Type 1 deiodinase (D1) is found in liver, kidney, and thyroid gland and catalyzes both the outer and inner ring deiodination of iodothyronines, while types 2 (D2) and 3 (D3) deiodinases act exclusively as outer and inner ring deiodinases respectively (Bianco et al. 2002). Previous studies have shown that liver and pituitary D1 activities are significantly decreased during fasting in rats. However, since the main positive regulator of liver and kidney D1 activity is T3 (Bianco et al. 2002, Aceves et al. 2003), which is also reduced under caloric restriction, it is difficult to establish whether decreased D1 activity is related to a direct effect of fasting, per se, or secondary to serum thyroid hormone reduction (St Germain & Galton 1985, O’Mara et al. 1993, Aceves et al. 2003).

Although it has long been recognized that thyroid hormones modify body mass composition, these previous studies have used high T4 doses (25–250 µg/100 g body mass; Aranda et al. 1972, Okajima & Ui 1979, Müller & Seitz 1980), which lead to thyrotoxicosis and several deleterious effects on the organism such as protein loss, heart hypertrophy, and arrhythmias (Klein & Ojamaa 2001).
However, the replacement of a low physiological dose of T\(_4\) during food restriction in normal rats has not been reported so far. The lowest T\(_4\) dose found in the literature has been tested by Burini et al. (1981) in a previous report using thyroidectomized rats under food restriction (2 \(\mu\)g T\(_4\)/100 g b.w.), but apart from using thyroidectomized rats, no oxygen consumption or deiodinase activities have been evaluated. Some other previous reports describe the effects of T\(_3\) administration on food-restricted (FR) obese patients, and the great majority uses a high T\(_3\) dose (Bray et al. 1973, Wilson & Lamberts 1981). On the other hand, the effects of low replacement doses of T\(_4\) during a period of food restriction have not been studied so far, although an early report of leptin replacement and serum T\(_4\) restoration indicates beneficial actions in humans under caloric restriction (Rosenbaum et al. 2002).

In the present paper, our aim was to restore serum T\(_4\) levels during food restriction in male Wistar rats in order to determine whether physiological T\(_4\) replacement restores liver and kidney type 1 deiodinase activities, resting metabolic rate (RMR), and body mass composition.

Materials and Methods

**Animals**

Adult male Wistar rats were housed at controlled temperature (23 °C) with daily exposure to a 12 h light:12 h darkness cycle and free access to water and standard rat chow. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and was approved by the institutional animal welfare committee. All animals were individually housed for a 1-week acclimation period and baseline control food intake was assessed.

**30 days food restriction**

Food intake was assessed over 7 days for each rat by offering food ad libitum and measuring the quantity consumed. After this period, the ad libitum (control – C) group had free access to food, and the FR group received 60% of their individual baseline intake for 30 days, so that the food was 40% restricted. During the food restriction period, all rats were weighed every 2 days.

**T\(_4\) treatment**

After 15 days of the beginning of food restriction, control (C) and FR rats were randomly assigned to start chronic treatment by daily single s.c. injections of (t-T\(_4\) Sigma) or saline (0.9-9% NaCl). The use of T\(_4\) instead of T\(_3\) is of physiological relevance, since T\(_4\) is the major product of the thyroid gland and its secretion is decreased during food restriction. T\(_4\) treatment protocol was done as follows: 1.0 \(\mu\)g/100 g b.w. (FR T\(_4\) (1)), which has been used to restore the euthyroid status in hypothyroid animals (Ortiga-Carvalho et al. 1996), and a dose of 5.0 \(\mu\)g/100 g b.w. (FR T\(_4\) (5)). T\(_4\) administration was conducted for the last 15 days of experiment, every 1000 h for all groups of animals. The animals were killed 24 h after the last T\(_4\) administration by decapitation, and blood was collected from the trunk. Serum was obtained after centrifugation of blood at 1000 g for 20 min and stored at \(-20^\circ\)C until specific RIA (TSH, T\(_3\), T\(_4\) and leptin). Liver and kidney tissue samples were dissected out and stored at \(-70^\circ\)C until processing for D1 activity.

**Body composition**

Body composition (fat and protein) was determined by carcass analysis, as previously described (Toste et al. 2006). The retroperitoneal and epididymal fats were completely removed, weighed for evaluation of central adiposity and discarded. The total carcass protein concentrations were determined by the method of Lowry et al. (1951). The carcass results were expressed as g/100 g carcass. The retroperitoneal and epididymal fats were weighted and expressed as g/100 g b.w.

**Resting metabolic rate**

RMR was measured using open-circuit indirect calorimetry during 24 h, after 30 days of experimental time. The rats were individually placed in a respiration chamber (25×25×15 cm), air flow was maintained constant at 500 ml/min by a mass flow controller (Sable System International, Las Vegas, NV, USA). Oxygen was measured using an O\(_2\) analyzer (Sable System International). Oxygen consumption was recorded at 15 min intervals and the results were expressed in LO2/kg per h. To avoid disruption caused by an adaptation to the chamber, the first 6 h were excluded from the analyses.

**Serum TSH, leptin, and total T\(_3\) and T\(_4\)**

Serum TSH levels were evaluated by a specific RIA obtained from the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA), and expressed in terms of the reference preparation 2 (R.P.-2). Intra- and inter-assay coefficients of variation were 7.7 and 7.4% respectively and the sensitivity was 0.50 ng/ml.

Serum total T\(_3\) and T\(_4\) concentrations were measured using commercial RIA kits (T\(_3\): DSL – 3100 Active, sensitivity of 4.3 ng/dl, inter- and intra-assay coefficients of variation varied from 4.2 to 6.0 and 5 to 6.5% respectively; T\(_4\): DSL – 3200 Active, sensitivity of 0.4 ng/dl, inter- and intra-assay coefficients of variation varied from 7.1 to 7.4 and 2.9 to 5.1% respectively; DSL, TX, USA), based on the presence of specific antibodies adhered to the internal surface of propylene tubes. Rat hormone-stripped serum was used for standard curves of total T\(_4\), T\(_3\), and TSH.

Serum leptin concentrations were measured using a specific RIA for rat leptin obtained from the Linco Research Company (St Charles, MO 63304, USA). The kit uses
Table 1 Body weight and composition of control (C) and food-restricted (FR) rats with or without thyroxine (T4) replacement. Values are expressed as mean±S.E.M. of at least ten animals per group from three to five experiments.

<table>
<thead>
<tr>
<th></th>
<th>BWi</th>
<th>BWf</th>
<th>Carcass protein</th>
<th>Retroperitoneal fat</th>
<th>Epididymal fat</th>
<th>Carcass fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C)</td>
<td>255±8·87</td>
<td>325±8·54</td>
<td>4·4±0·60</td>
<td>1·5±0·15</td>
<td>1·3±0·05</td>
<td>15·0±1·27</td>
</tr>
<tr>
<td>C + T4 (1)</td>
<td>256±7·95</td>
<td>316±7·37</td>
<td>3·3±0·35</td>
<td>1·6±0·27</td>
<td>1·5±0·20</td>
<td>12·9±1·32</td>
</tr>
<tr>
<td>C + T4 (5)</td>
<td>287±19·64</td>
<td>342±13·43</td>
<td>2·6±0·17*</td>
<td>1·6±0·10</td>
<td>1·8±0·08</td>
<td>15·4±1·31</td>
</tr>
<tr>
<td>Food restriction (FR)</td>
<td>266±6·96</td>
<td>236±5·19*</td>
<td>5·3±0·46</td>
<td>0·7±0·09*</td>
<td>0·9±0·09*</td>
<td>13·1±0·84</td>
</tr>
<tr>
<td>FR + T4 (1)</td>
<td>271±6·76</td>
<td>236±5·81*</td>
<td>2·8±0·47*</td>
<td>0·6±0·22*</td>
<td>1·1±0·27*</td>
<td>8·8±1·48‡</td>
</tr>
<tr>
<td>FR + T4 (5)</td>
<td>284±10·17</td>
<td>220±8·65*</td>
<td>2·6±0·53*</td>
<td>0·3±0·09*</td>
<td>0·6±0·16*</td>
<td>8·3±0·55‡</td>
</tr>
</tbody>
</table>

All fat compartments are expressed as g/100 g of b.w. BWi, initial body weight; BWf, final body weight. Control with T4 replacement (1 μg/100 g body weight) – C + T4 (1), and control with thyroxine replacement (5 μg/100 g body weight) – C + T4 (5). Food restriction with thyroxine replacement (1 μg/100 g body weight) – FR + T4 (1), and food restriction with T4 replacement (5 μg/100 g body weight) – FR + T4 (5). Carcass parameters are expressed as g/100 g carcass; retroperitoneal and epididymal fats as g/100 g body weight. *P<0·05 versus controls treated or not with T4; †P<0·05 versus FR and control; ‡P<0·05 versus controls treated or not with T4 and FR.

Thyroxine replacement during food restriction

Results

Body composition – mass, fat, and protein contents

Final body mass was lower in FR compared with C groups (Table 1, P<0·001), and the two doses of T4 did not produce any further body mass change (Table 1).

Retroperitoneal and epididymal fat masses were significantly lower in FR compared with C groups (Table 1, P<0·001), and no further reduction in these fat compartments were observed with both T4 doses (Table 1). On the other hand, carcass fat was not affected by FR per se, while the two doses of T4 significantly decreased carcass fat mass (P<0·01). These results show that T4 effects on adipose tissue depends on the fat compartment analyzed.

Carcass protein content was not significantly different between C and FR groups, demonstrating that 40% food deprivation for 30 days did not affect body protein content. However, exogenous T4 administration during food restriction produced a significant decrease in body protein content with the use of either the lowest (FR T4 (1)) or the highest (FR T4 (5)) T4 doses (Table 1, P<0·001). We have also observed a significant decrease in carcass protein content in control rats fed ad libitum and treated with the highest dose of T4 (Table 1).

Serum TSH, leptin, and total T3 and T4

Serum TSH and leptin concentrations were significantly reduced (P<0·001) after 30 days of food restriction, and remained low in the T4 replaced groups (FR T4 (1) and (5); Table 2).

Deiodinase analyses

Type 1 iodothyronine deiodinase activity was evaluated as previously described by our group (Fortunato et al. 2006) and based on Berry et al. (1991). In short, tissue (liver or kidney) samples of 25 mg were homogenized in 1 ml of 0·1 M sodium phosphate buffer containing 1 mM EDTA, 0·25 M sucrose, and 10 mM dithiothreitol (pH 6·9). Homogenates (30 μg protein from liver or kidney) were incubated in a water bath in duplicate for 1 h at 37 °C with 1 μM rT3 (Sigma, equal volumes of 125I rT3 (Perkin-Elmer Life Sciences, Boston, MA, USA) previously purified using sephadex LH-20, and 10 mM dithiothreitol in 100 mM potassium phosphate buffer containing 1 mM EDTA (pH 6·9) in a reaction volume of 300 μl. Blank incubations were carried out in the absence of protein. The reaction was stopped after incubation at 4 °C followed by the addition of 100 μl fetal bovine serum (Cultilab, Campinas, Brazil) and 200 μl trichloroacetic acid (50%, v/v). The samples were centrifuged at 8000 g for 3 min, and the supernatant was collected for measurement of 125I liberated during the deiodination reaction.

Protein concentration in the homogenates was measured by the Bradford method (Bradford 1976), after incubation of homogenates with NaOH (2·5 M). The specific enzyme activity was expressed as picomoles of rT3 deiodinated/min/mg protein.

Statistical analyses

Results were expressed as mean±S.E.M. Body mass, fat and protein content, total serum T3, T4, TSH and leptin concentrations, deiodinase activity, and oxygen consumption were analyzed by two-way ANOVA, followed by Bonferroni multiple comparison tests. Statistical analyses were done using the software Graphpad Prism (version 4, Graphpad Software, Inc., San Diego, CA, USA). The differences were considered significant when P<0·05.
Serum concentrations in control (C), control with T4 replacement of 1 μg/100 g body weight – C + T4 (1) or 5 μg/100 g body weight – C + T4 (5); food restriction (FR), food restriction with T4 replacement 1 μg/100 g body weight – FR + T4 (1) or 5 μg/100 g body weight – FR + T4 (5). *P<0.01 or †P<0.001 versus control; §P<0.001 versus control treated or not with T4. 

In five to six animals serum TSH or leptin were below the limit of detection.

Serum T4 and T3 concentrations were significantly reduced after 30 days of food restriction (Table 2), and increased in FR rats treated with the lowest dose of T4 (Table 2). Serum T4 levels significantly increased in FR T4 (5) in relation to FR T4 (1) and FR (Table 2); however, serum T3 did not increase significantly in the FR T4 (5) (Table 2).

**Deiodinase activity**

Liver type 1 iodothyronine deiodinase activity was significantly reduced after 30 days of food restriction (Fig. 2A, P<0.05). When the groups submitted to food restriction were treated with exogenous T4, D1 activity increased in a dose-dependent manner (Fig. 2A, P<0.001).

Kidney D1 activity was not significantly different after 30 days of food restriction (Fig. 2B). Treatment with the lowest dose of T4 in FR rats did not change the enzyme activity and only the highest dose of T4 led to a significant increase in kidney D1 activity when compared with the FR group (Fig. 2B, P<0.01).

**Discussion**

We show herein that 30 days of food restriction leads to reduction in serum TSH, T4, T3, and leptin, which parallel the reduction in body mass, retroperitoneal, and epididymal fat compartments; however, no changes were observed on carcass fat and protein contents. Interestingly, the replacement of T4 to FR groups with a dose just able to normalize serum T4 did not affect body mass and retroperitoneal and epididymal fats, although significant body protein and fat carcass loss were detected. These results differ from studies that described significant modifications in body mass when animals and humans were treated with higher doses of T4 or triiodothyronine (25–250 μg/100 g body mass). Bray et al. (1973) evaluated the effect of triiodothyronine (150 μg/day) in combination with a liquid formula diet (900 kcal/day) and showed that T3 produces a higher body weight loss. Wilson & Lamberts (1981) also showed a significant reduction on body mass using T3 (25 μg/day) in obese patients during drastic

![Figure 1](https://example.com/figure1.png)

**Figure 1** Resting metabolic rate in control (C) and food-restricted (FR) Wistar rats with saline (dark gray bar) or T4 replacement (1 μg/100 g b.w., gray bar and 5 μg/100 g b.w., light gray bar). (A) Oxygen consumption during 18 h (C; n = 20 FR; n = 7 FR 1; n = 4 FR 5; n = 5) and (B) Area under the curve. *P<0.05 versus control.

Table 2 Serum thyroid-stimulating hormone (TSH), leptin, total tri-iodothyronine (T3) and thyroxine (T4) of control (C) and food-restricted (FR) Wistar rats with or without T4 replacement. Values are expressed as mean ± S.E.M. of at least ten animals per group from three to five experiments.

<table>
<thead>
<tr>
<th>Group</th>
<th>T3 (ng/ml)</th>
<th>T4 (μg/ml)</th>
<th>TSH (ng/ml)</th>
<th>Leptin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C)</td>
<td>190.7 ± 46.3</td>
<td>12.0 ± 2.8</td>
<td>1.2 ± 0.7</td>
<td>4.6 ± 2.3</td>
</tr>
<tr>
<td>C + T4 (1)</td>
<td>190.3 ± 46.3</td>
<td>12.0 ± 2.8</td>
<td>1.2 ± 0.7</td>
<td>4.6 ± 2.3</td>
</tr>
<tr>
<td>C + T4 (5)</td>
<td>190.3 ± 46.3</td>
<td>12.0 ± 2.8</td>
<td>1.2 ± 0.7</td>
<td>4.6 ± 2.3</td>
</tr>
<tr>
<td>Food restriction (FR)</td>
<td>49.1 ± 25.9</td>
<td>3.5 ± 1.1</td>
<td>1.2 ± 0.7</td>
<td>4.6 ± 2.3</td>
</tr>
<tr>
<td>FR + T4 (1)</td>
<td>71.4 ± 38.5</td>
<td>7.4 ± 2.6</td>
<td>1.2 ± 0.7</td>
<td>4.6 ± 2.3</td>
</tr>
<tr>
<td>FR + T4 (5)</td>
<td>56.3 ± 39.4</td>
<td>10.0 ± 5.3</td>
<td>1.2 ± 0.7</td>
<td>4.6 ± 2.3</td>
</tr>
</tbody>
</table>


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calorie restriction. These differences could be related to the fact that in these studies a large dose of T₃ was used, while we used a low dose of T₄. Our results also show the concomitant reduction of serum T₄ and RMR during food restriction. Also, Moreno et al. (2003) showed that a single dose of T₃ (25 µg/day) to fasted rats restores uncoupling protein-3, uncoupling by regulating the levels of coenzyme Q. The changes in metabolism created by the association of two inverse energy conditions: food deprivation (decreased energy) and T₄ administration (increased energy) led to a significant decrease in body protein compartment, showing that the reduction in RMR during FR could represent a mechanism of physiological protection against body protein loss, as previously suggested (Gardner et al. 1979).

The effects of food restriction on serum TSH, leptin, and thyroid hormones were strikingly similar to those previously described by other authors (Säih et al. 1993, Gazdag et al. 1999, Davidson et al. 2002). The mechanisms that explain T₄ reduction during calorific restriction have been partially defined. Serum leptin decreases together with increased serum corticosterone during fasting, what leads to reduced pro-TRH in the hypothalamic paraventricular nucleus, with a consequent decrease of serum TSH (Legradi et al. 1997, Ahima et al. 2000, Coppola et al. 2005). However, decreased D₁ activity could also contribute to the lower serum T₃ found during caloric deprivation. It has not been established to what extent the peripheral reduction of T₃ production could also be relevant for the decreased RMR and increased resistance to further weight loss. Conjugation of thyroid hormones involves glucuronidation or sulfation of the phenolic hydroxyl group, which increases its water solubility and facilitates urinary and biliary clearance (De Herder et al. 1988, Burchell & Coughtrie 1989). Sulfation also promotes the inactivation of thyroid hormones, because the inner ring deiodination of sulfated T₄ and T₃ by D₁ is accelerated 40– to 200-fold, whereas the outer ring deiodination of sulfated T₄ is completely blocked (Visser 1994). This mechanism might be implicated in the drop of serum T₃ levels by as much as 33% in subjects submitted to weight loss programs (Rosenbaum et al. 2000).

During FR, the use of both T₄ doses increased serum T₄ concentrations in a dose-dependent manner. However, serum T₃ did not increase accordingly, despite the fact that type 1 deiodinase activity was increased. Considering the fact that T₄ might be deiodinated in the outer or inner rings, sulfated or glucuronidated in the liver, the respective products to be formed are: T₃, reverse T₃, sulfated T₃ or sulfated rT₃, and others. During food restriction T₄ sulfation seems to increase. Maglich et al. (2004), have shown that during energy deprivation the main product of type 1 deiodinase is not T₃ as the outer ring deiodination of sulfated T₄ is almost undetectable, whereas the rate of inner ring deiodination increases over 130-fold increasing the production of sulfated rT₃. Recently, Enmi et al. (2007) have demonstrated that the transcription and activity of rat uridine diphosphate–glucuronosyltransferase 1A7 is positively regulated by T₄, and the enzyme in turn metabolizes and inactivates T₄. To date, the regulation of uridine diphosphate–glucuronosyltransferase 1A7 during food restriction has not been determined, but it is tempting to speculate whether under calorific restriction it could be more sensitive to serum T₄ variations. Thus, injected T₄ could be converted into metabolites other than T₃ during food restriction, as the same dose of T₄ administered to normal rats had indeed increased serum T₃ as expected, different from what was found in FR animals. We can only hypothesize that during calorific restriction, liver metabolization of T₄ and T₃ is largely regulated and might be responsible for the lower serum T₃ due to increased clearance of these hormones.

Our findings show that decreased liver D₁ activity is a consequence rather than the cause of decreased serum T₃ during food restriction. Moreover, normalization of serum T₄ and T₃ in FR rats led to a significant decrease in protein and carcass fat contents, showing unequivocally the deleterious effects of any strategy to normalize these hormones during calorie deprivation with the aim of body weight control.

We conclude that decreased serum T₄ during calorie restriction is a protective mechanism to avoid body protein loss. Other strategies such as the use of thyroid hormone analogs that might spare body protein are of great importance.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Figure 2 Type 1 iodothyronine deiodinase (D₁) activity in control (C) and 40% food-restricted (FR) rats with saline (white bar) or T₄ replacement (1 µg/100 g b.w., light gray bar and 5 µg/100 g b.w., dark gray bar). (A) D₁ Hepatic (C, n=8; FR, n=13; FR T₄ (1), n=12; FR T₄ (5), n=15) and (B) D₁ Kidney (C, n=9; FR, n=15; FR T₄ (1), n=12; FR T₄ (5), n=13). Results are shown as mean±S.E.M. *P<0.05 versus control; **P<0.01 versus control and FR; ***P<0.001 versus all the other groups.
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