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-triiodothyronine (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.

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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, Scriba *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 ($2 \mu\text{g } T_4/100 \text{ g b.w.}$), 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 (L - T_4 Sigma) or saline (0.9% 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: $1.0 \mu\text{g}/100 \text{ g b.w.}$ (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 $5.0 \mu\text{g}/100 \text{ g b.w.}$ (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°C until specific RIA (TSH, T_3 , T_4 and leptin). Liver and kidney tissue samples were dissected out and stored at -70°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 $\text{g}/100 \text{ g carcass}$. The retroperitoneal and epididymal fats were weighted and expressed as $\text{g}/100 \text{ g b.w.}$

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 ($25 \times 25 \times 15 \text{ cm}$), 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 $\text{LO}_2/\text{kg per h}$. 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 (RP-2). Intra- and inter-assay coefficients of variation were 7.7 and 6.5% 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 $0.4 \mu\text{g/dl}$, 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

	BW _i	BW _f	Carcass protein	Retroperitoneal fat	Epididymal fat	Carcass fat
Control (C)	255 ± 8.87	325 ± 8.54	4.4 ± 0.60	1.5 ± 0.15	1.3 ± 0.05	15.0 ± 1.27
C + T ₄ (1)	256 ± 7.95	316 ± 7.37	3.3 ± 0.35	1.6 ± 0.27	1.5 ± 0.20	12.9 ± 1.32
C + T ₄ (5)	287 ± 19.64	342 ± 13.43	2.6 ± 0.17 [†]	1.6 ± 0.10	1.8 ± 0.08	15.4 ± 1.31
Food restriction (FR)	266 ± 6.96	236 ± 5.19*	5.3 ± 0.46	0.7 ± 0.09*	0.9 ± 0.09*	13.1 ± 0.84
FR + T ₄ (1)	271 ± 6.76	236 ± 5.81*	2.8 ± 0.47 [†]	0.6 ± 0.22*	1.1 ± 0.27*	8.8 ± 1.48 [‡]
FR + T ₄ (5)	284 ± 10.17	220 ± 8.65*	2.6 ± 0.53 [†]	0.3 ± 0.09*	0.6 ± 0.16*	8.3 ± 0.55 [‡]

All fat compartments are expressed as g/100 g of b.w. BW_i, initial body weight; BW_f, 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.

¹²⁵I-labeled rat leptin and a rat leptin antiserum to determine the level of serum leptin by the double antibody/polyethylene glycol (PEG) technique. Intra- and inter-assay coefficients of variation were 3.3–6.8 and 4.1–2.0% respectively and the sensitivity was 0.50 ng/ml using 100 µl sample size.

All the procedures were carried out following the recommendations as given in the kits.

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.

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 mass 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).

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

	T ₃ (ng/dl)	T ₄ (µg/dl)	TSH (ng/ml)	Leptin (ng/ml)
Control (C)	87.1±22.84	4.7±1.3	1.5±0.78	4.6±2.38
C+T ₄ (1)	130.4±40.46*	7.2±1.9*	1.0±0.20 ^{†,a}	4.1±2.18
C+T ₄ (5)	190.7±46.53 [†]	12.0±2.8 [†]	<0.5	4.9±2.40
Food restriction (FR)	49.1±25.93 [†]	3.5±1.1*	<0.5	0.99±0.09*
FR+T ₄ (1)	71.4±38.52	7.4±2.6 [†]	<0.5	0.94±0.14 ^{†,§}
FR+T ₄ (5)	56.3±39.42*	10.0±5.3 [†]	<0.5	1.48±0.65 ^{†,§}

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.
^aIn 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).

Resting metabolic rate

Figure 1 summarizes the results collected over a 24-h period of time. The results were plotted as mean oxygen consumption and were analyzed as the area under the curve. There was a 45% reduction in total oxygen consumption when FR was compared with the control group (Fig. 1, *P*<0.05). The lowest dose of T₄ did not normalize RMR, but treatment with the highest T₄ dose restored RMR to control values, suggesting that the reduction in basal metabolism observed during food deprivation is, in part, dependent on the decreased serum thyroid hormone concentrations.

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 tri-iodothyronine (25–250 µg/100 g body mass). Bray *et al.* (1973) evaluated the effect of tri-iodothyronine (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 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.

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.

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 ([Salih 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, [Emi 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 metabolism 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 caloric deprivation with the aim of body weight control.

We conclude that decreased serum T₄ during caloric 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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