How the thyroid controls metabolism in the rat: different roles for triiodothyronine and diiodothyronines

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1. Although the first evidence of a relationship between the thyroid and metabolism was reported in 1895, the mechanism by which thyroid hormones influence resting metabolic rate in whole animals is still poorly understood. This paper reports an attempt to test whether diiodothyronines (T_2s) and triiodothyronine (T_3) have different roles in the control of resting metabolism (RM).

2. Changes in resting metabolic rate were measured in hypothyroid rats treated acutely (25 μg (100 g body weight)$^{-1}$) either with one of the T_2s or with T_3. Injection of T_3 induced an increase of about 35% in RM that started 25–30 h after the injection and lasted until 5–6 days after the injection, the maximal value being observed at 50–75 h. The injection of T_2s evoked a temporally different pattern of response. The increases in RM started 6–12 h after the injection, had almost disappeared after 48 h, and the maximal stimulation was observed at 28–30 h.

3. When actinomycin D (an inhibitor of protein synthesis) and T_3 were given together, the stimulation of RM was almost completely abolished. The simultaneous injection of actinomycin D and either of the T_2s, on the other hand, did not cause any attenuation of the stimulation seen with the T_2s alone.

4. Following chronic treatment (3 weeks) with either T_3 or T_2s there was a stimulation of organ growth only after the administration of T_3.

5. Chronic administration of either T_2s or T_3 to hypothyroid rats significantly enhanced the oxidative capacity of each of the tissues considered. In the case of T_2s the stimulation was almost the same whether it was expressed as an increase in specific activity or total tissue activity. In the case of T_3 the increases were, in the main, secondary to the hypertrophic or hyperplastic effect.

6. These results indicate that T_2s and T_3 exert different effects on RM. The effects of T_2s are rapid and possibly mediated by their direct interaction with mitochondria. Those of T_3 are slower and more prolonged, and at least partly attributable to a modulation of the cellularity of tissues that are metabolically very active.

Among the most pronounced physiological effects attributed to thyroid hormones in many adult endothermic vertebrates is their control over metabolism. Although the first evidence of an increase in metabolic rate in subjects given thyroid extract was reported as long ago as 1895 by Magnus-Levy, the literature contains very few papers dealing with the action of thyroxine (T_4) and T_3 on metabolic rate of whole animals. Furthermore, although more than 30 years have elapsed since the appearance of the articles by Tata (Tata, Ernster & Lindberg, 1962; Tata, 1963) on the control of basal metabolic rate by thyroid hormones, the mechanism by which thyroid hormones influence basal or resting metabolic rate in whole animals (also called the calorigenic effect) is very poorly understood.

Several hypotheses have been proposed to explain the calorigenic effect of thyroid hormones, but none has received universal acceptance (Sestoft, 1980). Indeed, conflicting results are to be found in the literature and a debate is still in progress as to whether the cellular target for the early action of thyroid hormones on energy metabolism is the nucleus or the mitochondria (for review, see Soboll, 1993). To some extent, the controversies may be a consequence of the use of a wide variety of animal models in the various

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investigations of the actions of thyroid hormones. Indeed, the use of acute, as opposed to chronic, hormonal treatment and the use of different methods to induce hypothyroidism could have led different authors towards different interpretations (Lanni, Moreno, Lombardi & Goglia, 1996). In fact, studies on the characteristics of deiodinase enzymes (Köhrle, 1994) have revealed that very different animal models are produced depending on whether the hypothyroidism is induced by surgical or chemical thyroidectomy (Lanni *et al.* 1996).

In the past, the iodothyronines other than T_4 and T_3 that are present in biological fluids have been regarded as inactive. Consequently, studies of the calorigenic effect of thyroid hormones have focused almost exclusively on T_4 and T_3. Indeed, of the thyroid hormones, T_3 has been universally considered to be the active form. Recently, however, a growing number of studies have indicated that two diiodothyronines, 3,3′-diiodo-L-thyronine (3,3′-T_2) and 3,5-diiodo-L-thyronine (3,5-T_2) (together referred to as T_2s) could be of biological relevance. These iodothyronines are able rapidly to stimulate the oxidative capacity and respiration rate of rat liver mitochondria (Lanni, Moreno, Cioffi & Goglia, 1992, 1993; O'Reilly & Murphy, 1992; Lanni, Moreno, Lombardi & Goglia, 1994*b*) and these effects could be mediated by the binding of T_2s to specific mitochondrial sites (Goglia, Lanni, Horst, Moreno & Thoma, 1994; Lanni, Moreno, Horst, Lombardi & Goglia, 1994*a*). Recently, we reported that T_2s induce a dose-dependent calorigenic effect when chronically injected into hypothyroid rats (Lanni *et al.* 1996). In such animals, following the administration of T_2s, we found a good correspondence between the increase in resting metabolism and the increase in the specific oxidative capacity (expressed as cytochrome oxidase (COX) activity in (ng atoms O) min^{-1} (mg protein)$^{-1}$) of tissues that are metabolically very active, such as liver, gastrocnemius muscle, heart and brown adipose tissue (BAT). However, a similar close correspondence could not be shown in hypothyroid rats injected with T_3. In fact, while the administration of T_3 increased their resting metabolism to euthyroid values or above, a relevant stimulatory effect on specific oxidative capacity at the tissue level was observed only in the liver. This could not account for the observed increase in RM. This apparent discrepancy could be explained if we assume that the effects of T_2s and T_3 on RM are mediated by different mechanisms at the cellular level. Thus, the effect of T_3 could be mediated via a nuclear pathway, resulting in a hypertrophic or hyperplastic effect on tissues that are metabolically very active. In contrast, if T_2s have mitochondria as their target, this could result in a direct and more rapid stimulation of RM, without an effect on organ mass.

We have tested these hypotheses by: (a) treating rats acutely either with T_2s or with T_3 and measuring the time of onset and following the time course of the calorigenic effect (measured as the effect on resting metabolism in the whole animal); and (b) treating rats chronically with the same substances and following the development of the hypertrophic or hyperplastic effect (in the liver, gastrocnemius muscle, brown adipose tissue and heart) by determining the mass of the tissues as well as their total protein and DNA content.

As a model for both acute and chronic studies, we used hypothyroid animals in which hypothyroidism was induced by combined treatment with propylthiouracil (PTU) and iopanoic acid (IOP). This treatment, which induces a severe hypothyroidism and, at the same time, inhibits all the three known types of deiodinase enzyme, permits us to attribute the observed effects to the iodothyronines injected, rather than to any of their deiodinated products. Moreover, in hypothyroid rats the presence of endogenous iodothyronines is strongly reduced and the endogenous occupation of the binding sites should consequently be lowered.

A preliminary version of these results has been published in abstract form (Moreno, Lanni, Lombardi & Goglia, 1996).

METHODS

Animals and treatments

Male Wistar rats (250–300 g) living in a temperature-controlled room at 28 °C were kept one per cage under an artificial lighting regime of 12 h light:12 h darkness. A commercial mash (Charles-Rivers, Lecco, Italy) was available *ad libitum* and the animals had free access to water. All experiments were performed in accordance with local and national guidelines covering animal experiments. At the end of the experiment, the rats were anaesthetized by I.P. administration of chloral hydrate (40 mg (100 g body weight (BW))$^{-1}$) and killed by decapitation. Hypothyroidism was induced in some rats by the administration of PTU (0·1% w/v in drinking water for 3 weeks) together with IOP (6 mg (100 g BW)$^{-1}$) (Lanni *et al.* 1996). These rats are referred as the 'P + I' group.

To enable determination of the time course of the calorigenic effect of iodothyronines, hypothyroid animals were acutely injected I.P. with either (i) a single dose of 25 μg (100 g BW)$^{-1}$ of either T_3 or 3,5-T_2, or (ii) in the case of 3,3′-T_2, three doses of 25 μg (100 g BW)$^{-1}$ at 12 h intervals. As a control for the possible effects of the injection itself, saline was injected I.P. into hypothyroid control rats. The dose of 25 μg (100 g BW)$^{-1}$ was used because it produces a clear-cut effect on RM and indeed restores it to the level observed in euthyroid control rats (Tata, Ernster, Lindberg, Arrhenius, Pedersen & Hedman, 1963). This dose given acutely is not a large dose; in fact, it has been established that 200 μg (100 g BW)$^{-1}$, given I.V., is the acute dose needed to obtain $\geqslant$95% nuclear receptor saturation for 24 h (Jump, Narayan, Towle & Oppenheimer, 1984).

It is conceivable that the administration of PTU and IOP and the consequent alteration in iodothyronine metabolism could induce non-physiological rather than physiological metabolic effects. Actually, it is known that the substances we used to induce hypothyroidism have no effect on metabolism in man (Acheson & Burger, 1980). Nevertheless, to be sure that these two substances do not induce any non-physiological effects in rats, we also measured the effect of the acute administration of 25 μg iodothyronines (100 g BW)$^{-1}$ on RM in two other control groups of rats in which hypothyroidism was induced either by surgical thyroidectomy

without administration of P + I (referred to as Tx) or by thyroidectomy and concomitant P + I administration (referred to as Tx + P + I). A similar effect on RM in all the hypothyroid groups, regardless of the way hypothyroidism was induced, would indicate that non-physiological effects were not induced in animals treated with P + I.

To enable evaluation of the possible involvement of changes in protein synthesis in the action of the iodothyronines tested, actinomycin D (8 μg (100 g BW)$^{-1}$) was injected in combination with 25 μg (100 g BW)$^{-1}$ of 3,5-T_2 or T_3. At the extremely low doses used here, actinomycin D only inhibits the synthesis of messenger RNA (Goldberg & Rabinowitz, 1962).

To enable the hypertrophic or hyperplastic effect of iodothyronines on the tissues to be followed, P + I rats received, by a once-daily I.P. injection over a 3 week period, one of three different doses (2·5, 5 or 10 μg (100 g BW)$^{-1}$) of either T_3, 3,5-T_2 or 3,3′-T_2. This method also allowed us to plot a dose–response relationship. The above doses fall within the range of doses used in a previous study of dose dependency (Oppenheimer, Schwartz, Lane & Thompson, 1991). At the end of the treatment, each rat was killed, the trunk blood collected and the serum separated and stored at −20 °C for later measurement of the concentration of T_3 and T_4. Interscapular BAT, heart, gastrocnemius muscle and liver were dissected out, cleaned, immediately weighed (wet weight) and processed for the determination of COX activity, protein content and DNA content. As an index of cellularity, we adopted the protein/DNA ratio which, following an increase in organ weight, indicates a hypertrophy if it increases, but a hyperplasy if it decreases or remains unchanged.

Resting metabolism

The resting metabolism was measured using open-circuit indirect calorimetry. The rat was placed in a respiration chamber (~32 cm × 20 cm × 19 cm) with airflow being measured using an O_2-ECO mass flow controller (Columbus Instruments International Corporation, Columbus, OH, USA). Details of this set-up and of the way that measurements are made have been given by Lanni *et al.* (1996). The measurements for the calculation of RM (the lowest metabolic rate of a resting animal when it is not in a post-absorptive or fasting state and is not sleeping) were taken at 28 °C between 11.00 h and 16.00 h, when the energy expenditure was at a lower level than in any other period of the day. Measurements were taken before and at various intervals after the rat was injected with T_3 or with one of the T_2s (see above).

Analytical procedures

Cytochrome oxidase (COX) activity was determined polarographically at 25 °C, using a Clark oxygen electrode and a modification (Barrè, Bailly & Rouanet, 1987) of the procedure of Aulie & Grav (1979). This required 1·5 ml of reaction medium containing 30 μM cytochrome *c*, 4 μM rotenone, 0·5 mM dinitrophenol (DNP), 10 mM sodium malonate and 75 mM Hepes buffer, at pH 7·4. Samples of liver, skeletal muscle, brown adipose tissue and heart were finely minced, diluted 1/10 (w/v) and homogenized in modified Chappel-Perry medium (mM): 1 ATP, 50 Hepes buffer adjusted to pH 7·4, 100 KCl, 5 $MgCl_2$, 1 EDTA and 5 EGTA. The homogenate was then diluted 1:2 (v/v) in the same medium, with lubrol (100 mg (g tissue)$^{-1}$) being added to unmask the enzyme activity of the tissue. It was then left standing in ice for 30 min.

Cytochrome oxidase activity was measured as the difference between (i) the rate of oxygen consumption observed after the addition of substrate (4 mM sodium ascorbate with 0·3 mM *N,N,N′,N′*-tetramethyl-*p*-phenylene-diamine (TMPD)) and homogenate and (ii) the rate of oxygen consumption observed after the addition of substrate alone. This method took into account the auto-oxidation of ascorbate.

For the measurement of the activities of the deiodinases, tissue samples were individually homogenized on ice in 0·25 M sucrose and 10 mM Hepes (pH 7·0) containing 10 mM DTT (dithiothreitol). Liver and brain microsomes for type I deiodinase (ID-I) activity and type III deiodinase (ID-III) activity, respectively, were obtained as described by Visser, Kaptein, Terpstra & Krenning, (1988). BAT infranatants, for type II deiodinase (ID-II) activity, were obtained as described by Leonard, Mellen & Larsen (1983). They were all immediately frozen in a dry-ice–acetone bath and stored at −80 °C until assay.

Samples of reverse [3′,5′-^{125}I]T_3 ([3′,5′-^{125}I]rT_3) and [5′-^{125}I]T_3 were prepared by radioiodination of 3,3′-T_2 or 3,5-T_2 (Henning, Berlin, Germany), respectively, using the chloramine-T method as described by others (Visser, Docter & Hennemann, 1977; Visser, Krieger-Quist, Docter & Hennemann, 1978). Labelled products were purified by Sephadex LH-20 chromatography (Pharmacia Uppsala, Sweden) and purity was checked by HPLC analysis using unlabelled compounds as references. Free ^{125}I was eliminated from [3′,5′-^{125}I]rT_3 and [5′-^{125}I]T_3 on Sephadex LH-20 immediately before each experiment. Iodothyronines were obtained from MMDRI, Henning Berlin R&D (Berlin, Germany). [^{125}I]-Na for radioiodination was purchased from Amersham.

The essential step in the assay of type I deiodinase activity was the production of radioiodide from [3′,5′-^{125}I]rT_3 by ID-I deiodinase. A 2 μg sample of liver microsomal protein was incubated for 30 min at 37 °C with 0·1 μM rT_3 and ~100 000 c.p.m. [3′,5′-^{125}I]rT_3 in 200 μl of 0·2 M phosphate buffer (pH 7·2) with 4 mM EDTA and 5 mM DTT. Incubations were performed in triplicate in a shaking waterbath and the reactions were stopped by the addition of 100 μl 5% bovine serum albumin at 0 °C. Protein-bound iodothyronines were precipitated by the addition of 500 μl of 10% trichloroacetic acid. After incubation of the mixtures at 0 °C, they were centrifuged and the radioactivity in the supernatant was then determined. Enzymatic deiodination was corrected for non-enzymatic ^{125}I production (as determined in blank incubations without microsomes) and multiplied by 2 to account for random labelling and the deiodination of the 3′ and 5′ positions in [3′,5′-^{125}I]rT_3.

The activity of the type II deiodinase enzyme was measured in BAT according to the method of Leonard *et al.* (1983); this involves measuring the release of radioiodide from [3′,5′-^{125}I]rT_3. A 20 μg sample of BAT infranatant proteins (obtained by centrifugation of BAT homogenate at 500 × *g* for 10 min) was incubated in triplicate for 60 min at 37 °C in a shaking waterbath with 2 nM rT_3 and ~100 000 c.p.m. of [3′,5′-^{125}I]rT_3 in 200 μl 0·1 M phosphate buffer (pH 7·2) with 2 mM EDTA and 20 mM DTT. The reactions were stopped by the addition of 100 μl 5% BSA and the ^{125}I produced was isolated and analysed as described above. Random labelling and deiodination of the 3′ and 5′ positions of [3′,5′-^{125}I]rT_3 was taken into account in the calculation of ID-II activity.

Type III deiodinase activity in brain microsomes was determined by measuring the formation of 3[3′-^{125}I]T_2 from [5′-^{125}I]T_3 by HPLC analysis as reported by Schoenmakers, Pigmans & Visser (1995). A 100 μg sample of brain microsomal protein was incubated in triplicate for 60 min at 37 °C in a shaking waterbath with 1 nM T_3 and ~100 000 c.p.m. of [5′-^{125}I]T_3 in 200 μl of 0·1 M phosphate buffer (pH 7·2) with 4 mM EDTA and 10 mM DTT. The reactions were stopped by the addition of 300 μl methanol on ice. After centrifugation of precipitated proteins, the supernatants were

Table 1. Total and free T_3 and total T_4 serum levels in euthyroid (N), hypothyroid (P + I) and hypothyroid rats chronically treated with T_3 (P + I + T_3)

Group	Total serum T_3 (nmol l^{-1})	Free serum T_3 (pmol l^{-1})	Total serum T_4 (nmol l^{-1})
N	$0{\cdot}88 \pm 0{\cdot}06^{b}$	$4{\cdot}1 \pm 0{\cdot}4^{b}$	$62{\cdot}0 \pm 3{\cdot}0^{b}$
P + I	$0{\cdot}17 \pm 0{\cdot}02^{a}$	$0{\cdot}5 \pm 0{\cdot}05^{a}$	$7{\cdot}3 \pm 0{\cdot}8^{a}$
P + I + T_3 (2·5 μg T_3 (100 g BW)$^{-1}$)	$1{\cdot}11 \pm 0{\cdot}11^{b}$	$3{\cdot}6 \pm 0{\cdot}3^{b}$	$6{\cdot}8 \pm 0{\cdot}6^{a}$
P + I + T_3 (5 μg T_3 (100 g BW)$^{-1}$)	$1{\cdot}28 \pm 0{\cdot}14^{b}$	$4{\cdot}9 \pm 0{\cdot}5^{bc}$	$6{\cdot}5 \pm 0{\cdot}5^{a}$
P + I + T_3 (10 μg T_3 (100 g BW)$^{-1}$)	$1{\cdot}76 \pm 0{\cdot}16^{c}$	$5{\cdot}8 \pm 0{\cdot}7^{c}$	$6{\cdot}1 \pm 0{\cdot}5^{a}$

Results are the means ± S.E.M. of 5 experiments for each group, each experiment being performed in triplicate. Values labelled with different letters are significantly different ($P < 0{\cdot}05$) from each other.

analysed for 3[3′-^{125}I]T_2 formation by HPLC analysis involving elution with a 45:50 (v/v) mixture of methanol and 20 mM ammonium acetate (pH 4·0) at a flow of 0·8 ml min^{-1}.

Serum total T_4 and T_3 levels and free T_3 were determined in samples of serum using reagents and protocol supplied by Becton-Dickinson (Orangeburg, NJ, USA).

The protein concentration was determined by the method of Hartree (1972) using bovine serum albumin as standard. The DNA content was measured by a colorimetric method (Burton, 1956).

Results are expressed as means ± S.E.M. The statistical significance of differences between groups was determined by a one-way analysis of variance followed by a Student–Newman–Keuls test. Comparison between independent means was performed using a Student's t test.

RESULTS

Total and free T_3 and total T_4 concentrations

The combined administration of PTU and IOP produces rats with severe hypothyroidism. Indeed, total and free T_3 and total T_4 levels were significantly lower in such hypothyroid rats than in euthyroid ones (by about 80, 92 and 88%, respectively; Table 1). In Tx and in Tx + P + I rats, the reduction in all three levels was of the same order as that seen in P + I animals (data not shown).

Following the chronic injection of various doses of T_3 to P + I rats, the total and free T_3 levels were (i) within the range of values observed in euthyroid animals (at a dose of 2·5 μg (100 g BW)$^{-1}$), (ii) slightly higher (at a dose of 5 μg (100 g BW)$^{-1}$) or (iii) significantly higher (at a dose of 10 μg (100 g BW)$^{-1}$) (see Table 1). Our values for these circulating levels in euthyroid, hypothyroid and hypothyroid + T_3-treated animals are in agreement with values previously reported in rats under similar conditions (Francavilla *et al.* 1991).

Deiodinase activity in hypothyroid rats

The activities of the deiodinase enzymes in P + I animals were either completely blocked or, in the case of the type II deiodinase, greatly reduced (by 66%) (Table 2).

Similar results were obtained in Tx + P + I animals, but in TX animals the activities were differently affected. In the latter animals, type I and type III deiodinases were inhibited by about 63% and 67%, respectively, while type II activity was increased nearly 4-fold (Table 2).

Effect of acute injection of iodothyronines on the RM of hypothyroid rats

RM was considerably lower in all the hypothyroid groups than in euthyroid controls (0·94 ± 0·02, 0·90 ± 0·02, 0·89 ± 0·03 and 1·45 ± 0·03 l O_2 $(kg^{0.75})^{-1}$ h^{-1}, in P + I, Tx, Tx + P + I and euthyroid control rats, respectively), thus confirming the hypothyroid status of the animals. Figure 1 illustrates the effect of the acute administration of either 3,5-T_2 or T_3 on the RM of P + I rats. The injection of T_3 caused an increase of about 35% in resting metabolic rate; the increase started 25–30 h after the injection, reached maximal values at 50–75 h and lasted until 5–6 days after the injection (Fig. 1, ●). This trend was in accordance with that previously observed by Tata in his early studies on thyroidectomized rats (Tata *et al.* 1962; Tata, 1963).

The injection of T_2s, on the other hand, evoked a temporally different pattern of response. The injection of 3,5-T_2 caused an increase of about 40% in RM (Fig. 1, ◆). However, in this case the increase started between 6 and 12 h after the injection, peaked at about 30 h and had almost disappeared at 48 h. A similar trend was observed in the response to 3,3′-T_2 (data not shown), but in that case we needed to give at least three injections 12 h apart to produce the effect. With 3,3′-T_2, the increase in RM started 6–12 h after the last injection and the response had almost disappeared by 30 h after the last injection (not shown). As the RM actually decreased during the first few hours of treatment in the case of animals injected with 3,3′-T_2, the increase was by about 17% if referred to the initial RM value (before the beginning of the treatment), but by about 27% if referred to the RM value measured immediately before the last injection.

The simultaneous injection of actinomycin D and either of the T_2s did not cause any attenuation of the stimulation

Table 2. Activity of liver ID-I, BAT ID-II and brain ID-III in hypothyroid (P + I, Tx + P + I and Tx) and euthyroid (N) rats

Group	ID-I activity (pmol I (min (mg protein))$^{-1}$)	ID-II activity (fmol I (h (mg protein))$^{-1}$)	ID-III activity (fmol 3,3′-T_2 (min (mg protein))$^{-1}$)
P + I	Undetectable	14 ± 1 *	Undetectable
Tx + P + I	Undetectable	13 ± 1	Undetectable
Tx	75 ± 5 *	153 ± 14 *	9 ± 1 *
N	202 ± 12	41 ± 3	27 ± 1

Results are the means ± S.E.M. of 5 experiments for each group, each experiment being performed in triplicate. * Values significantly different ($P < 0.05$) from N rats.

seen with the T_2s alone (Fig. 1, ◇). In contrast, when actinomycin D and T_3 were given together, the stimulation of RM by T_3 was almost completely abolished (Fig. 1, ○).

The effects of the T_2s and T_3 on RM were almost the same in Tx animals as in Tx + P + I animals and none of these effects was substantially different from the corresponding effect in the P + I group. However, one difference was observed between Tx animals and the others and lay essentially in the steepness of the curves showing changes in RM as a function of time. Anyhow, in Tx rats: (i) the peak increase in RM induced by T_2s preceded that induced by T_3 by 26–36 h and (ii) the maximal percentage changes following iodothyronines administration were of the same order (+27–35% in the case of T_2 and +36% in the case of T_3).

It should be emphasized that the deiodinase activities were markedly altered in Tx animals as well as in P + I and Tx + P + I animals, but in a different way. In fact, in Tx rats ID-I and ID-III were inhibited, while I-DII was activated (Table 2). Further, in P + I and in Tx + P + I rats these activities (due to the inhibition exerted by PTU and IOP) remain unchanged by administration of iodothyronines. By contrast, it is known that in Tx rats the deiodinase activities are further altered following T_3 administration, and in a way that tends to reverse the effect of thyroidectomy (ID-I and ID-III are stimulated (Kaplan & Utiger, 1978; Esfandiari, Courtin, Lennon, Gavaret & Pierre, 1992) while ID-II is inhibited (Leonard, Kaplan, Visser, Silva & Larsen, 1981)). Consequently, using a Tx model we have a pattern of deiodination that is different before and after T_3 administration (this alteration may underlie the aforementioned slight differences in the steepness of the curves observed in Tx animals). This being so, and as stated in the Introduction, it becomes problematic to attribute an observed effect to a given iodothyronine rather than to its possible metabolic product (i.e. T_3 rather than T_2). Having considered all the above, we decided that our chronic studies with different doses of iodothyronines should be performed only on P + I rats. Our main reasons were: (a) the acute effect of iodothyronines were almost the same in the various hypothyroid conditions, (b) in P + I rats the deiodinase activities are always at the same level and (c) we could thus avoid imposing another form of stress (surgical thyroidectomy).

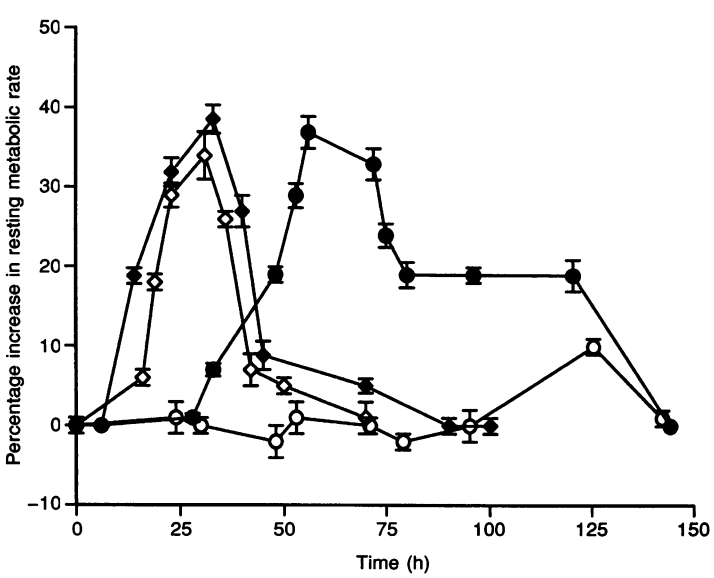

Figure 1. Changes in the resting metabolic rate of hypothyroid rats following administration of iodothyronines with and without actimomycin D

Hypothyroidism was induced by combined treatment with PTU and IOP (P + I). The dose was 25 μg (100 g BW)$^{-1}$ for both triiodothyronine (T_3) and 3,5-diiodo-L-thyronine (3,5-T_2). ◆, 3,5-T_2 alone; ◇, 3,5-T_2 + actinomycin D; ●, T_3 alone; ○, T_3 + actinomycin D. Each data point shows the mean ± S.E.M. from 5 rats. The values are expressed as the percentage change from the value at time 0 (i.e. immediately before the injection).

Table 3. Specific (SCOX) and total (TCOX) cytochrome oxidase activity from hypothyroid rats and from hypothyroid rats treated with various iodothyronines

	Liver		Gastrocnemius		Heart		BAT	
Group	SCOX	TCOX	SCOX	TCOX	SCOX	TCOX	SCOX	TCOX
P+I	353±30	404±32	230±13	35±2·8	1734±168	84±6·7	1442±109	10·0±0·9
P+I+3,5-T_2								
2·5 μg	402±39(14)	504±40(25)	302±22*(32)	42±4·2(20)	1790±171(3)	83±8·2(0)	1779±121(23)	10·7±0·9(7)
5 μg	477±6*(24)	696±60*(72)	304±21*(32)	54±4·3*(54)	2419±212(40)	119±12·2(42)	1989±159*(38)	14·8±1·5*(48)
10 μg	570±53*(61)	651±40*(61)	334±33*(45)	54±5·0*(54)	2425±221(40)	135±15·3*(61)	2307±187*(60)	20·2±2·1*(100)
P+I+3,3′-T_2								
2·5 μg	416±33(17)	488±20(21)	303±30*(32)	46±4·2(31)	1954±160(13)	88±7·9(5)	1476±122(2)	11·5±1·1(15)
5 μg	423±19(20)	537±22*(32)	359±10*(56)	63±5·0*(80)	2433±197(40)	126±13·0*(50)	1869±169(30)	17·3±1·4*(70)
10 μg	469±13*(32)	510±20*(23)	376±35*(63)	51±4·8*(46)	2421±213(40)	116±12·1(38)	2173±132*(51)	14·7±1·2*(47)
P+I+T_3								
2·5 μg	473±45(34)	789±80*(95)	265±30(15)	52±4·8*(49)	1800±177(4)	153±13·2*(82)	1668±103(16)	22·8±2·3*(128)
5 μg	508±48*(44)	749±75*(85)	280±25(22)	62±5·0*(77)	1850±200(7)	134±12·7*(60)	1586±152(10)	18·4±1·5*(84)
10 μg	614±58*(74)	1060±100*(162)	290±10(26)	67±7·0*(91)	2048±183(18)	165±14·2*(96)	1658±127(15)	17·3±1·4*(73)

SCOX is expressed as (ng atoms O) min^{-1} (mg protein)$^{-1}$; TCOX is expressed as (μg atoms O) min^{-1}. Results are presented as means±S.E.M. Values in parentheses represent the percentage increase over P + I value. * Values significantly different ($P < 0·05$) from P + I rats. $n = 9$ for P + I group, $n = 5$ for each hypothyroid iodothyronine-treated group, where n is no of experiments. Groups given different doses of 3,5-T_2, 3,3′-T_2 and T_3 are distinguished by the dose which is given per 100 g BW under the group names in the left column.

Effect of chronic treatment with iodothyronines on specific and total tissue COX activity of liver, gastrocnemius muscle, BAT and heart

The values for the specific (SCOX) and total oxidative (TCOX) capacity of the various tissues are reported in Table 3. An analysis of variance revealed that, in the case of 3,3′-T_2 and 3,5-T_2, hormonal treatment was effective in stimulating both specific and total COX activity ($P < 0·05$; $F > 3·72$). The effect of the administration of T_2s was dose dependent, the maximal effect occurring at a dose of 10 μg (100 g BW)$^{-1}$. Administration of 3,5-T_2 to hypothyroid rats at a dose of 10 μg (100 g BW)$^{-1}$ stimulated the specific oxidative capacity of the tissues by 40–61%, the effects on heart and muscle (40 and 45%, respectively) being weaker than those on BAT and liver (60 and 61%, respectively). Administration of 3,3′-T_2 to hypothyroid rats at a dose of 10 μg (100 g BW)$^{-1}$ stimulated the specific oxidative capacity of the tissues by 32–63%, the effects on liver and heart (32 and 40%, respectively) being weaker than those on BAT and muscle (51 and 63%, respectively). When the oxidative capacity was expressed as total activity, a similar pattern was observed. In percentage terms, in fact, the stimulation of COX activity by T_2s was almost the same whether the activity was expressed as specific or total activity.

In the case of T_3, an analysis of variance revealed that hormonal treatment was effective in stimulating specific activity only in the liver ($P < 0·02$; $F = 7·12$). Actually, the lack of an effect of T_3 on specific COX activity in BAT is not surprising since it has been shown that BAT preferentially utilizes plasma T_4 and that it only poorly utilizes T_3 derived from the plasma and extracellular space (Bianco & Silva, 1987). However, when the oxidative capacity was expressed as total activity, the stimulation was evident and significant in all organs and tissues tested. In percentage terms (Table 3; values in parentheses), the changes from P + I values were much greater when expressed in terms of total activity than when expressed in terms of specific activity. At a dose of 10 μg (100 g BW)$^{-1}$, in fact, expressing the increases in COX activity in terms of total activity (rather than in terms of specific activity) caused the size of the increase to be more than doubled in the liver (162% *vs.* 74%), 3·5 times greater in muscle (91% *vs.* 26%), 5·5 times greater in the heart (96% *vs.* 18%) and about 5 times greater in BAT (73% *vs.* 15%).

Effect of chronic treatment with iodothyronines on body weight, organ mass, tissue protein content and tissue DNA content in hypothyroid rats

Body mass and the mass of individual organs are significantly lower in hypothyroid rats than in euthyroid ones (Table 4). Following the chronic administration of iodothyronines, all the organs examined were significantly heavier in T_3-treated animals than in hypothyroid ones (see Table 4), the greatest effects being observed in BAT and heart. However, no

Table 4. Body and organ masses from euthyroid (N), hypothyroid (P + I) and triiodothyronine-treated hypothyroid (P + I + T_3) rats

Group	*n*	Body mass (g)	Liver (g)	Gastrocnemius (g)	BAT (mg)	Heart (mg)
P + I	9	263 ± 8†	9·2 ± 0·6† (35·1 ± 1·9)	1·56 ± 0·08† (5·94 ± 0·29)	176 ± 11† (0·68 ± 0·05)	644 ± 33† (2·44 ± 0·09)
N	7	312 ± 10	12·5 ± 0·7 (40·0 ± 2·0)	1·86 ± 0·08 (5·9 ± 0·04)	263 ± 14 (0·84 ± 0·05)	870 ± 32 (2·80 ± 0·50)
P + I + T_3						
2·5 μg	5	305 ± 15*	11·4 ± 0·1 (37·7 ± 1·4)	1·82 ± 0·12 (5·96 ± 0·01)	281 ± 46* (0·91 ± 0·04)*	1125 ± 15* (3·74 ± 0·51)*
5 μg	5	282 ± 15	11·1 ± 0·6 (39·5 ± 0·5)	1·95 ± 0·02* (6·93 ± 0·33)	359 ± 24* (1·29 ± 0·16)*	973 ± 28* (3·45 ± 0·21)*
10 μg	5	306 ± 6*	12·8 ± 1·8* (41·7 ± 2·0)	2·13 ± 0·13* (6·94 ± 0·53)	446 ± 63* (1·41 ± 0·21)*	1137 ± 33* (3·7 ± 0·08)*

Values in parentheses represent tissue/body weight ratio × 10^3. Results are presented as means ± S.E.M.; *n*, no. of experiments. * Values significantly different ($P < 0·05$) from P + I rats; † values significantly different ($P < 0·05$) from N rats. Because neither 3,3′-T_2 nor 3,5-T_2 treatment had a significant effect on the above parameters (treated *vs.* hypothyroid rats), the data are not reported. Different P + I + T_3 groups are distinguished by the doses of T_3 which are given per 100 g BW under the group name in the left column.

Table 5. Total protein content (mg), total DNA content (mg) and protein/DNA ratio in liver, gastrocnemius muscle, BAT and heart homogenates from euthyroid (N), hypothyroid (P + I) and triiodothyronine-treated hypothyroid (P + I + T_3) rats

		P + I	P + I + T_3			N
			2·5 μg	5 μg	10 μg	
		(*n* = 9)	(*n* = 5)	(*n* = 5)	(*n* = 5)	(*n* = 7)
Liver	Protein	1138 ± 73·1†	1457 ± 70*	1553 ± 68*	1745 ± 153*	1529 ± 116
	DNA	36 ± 3·5	38 ± 0·5	36 ± 2·8	35 ± 2·9	36 ± 1·9
	Protein/DNA	31 ± 2·7†	38 ± 1·3	43 ± 3·4*	50 ± 4·5*	42 ± 4·00
Gastrocnemius	Protein	170 ± 15	192 ± 2	196 ± 16	204 ± 19	195 ± 2*
	DNA	3·6 ± 0·3	4·7 ± 0·1*	5·2 ± 0·4*	6·2 ± 0·6*	4·8 ± 0·3
	Protein/DNA	47 ± 4·0	41 ± 1·3	38 ± 2·7	33 ± 2·7*	41 ± 3·9
BAT	Protein	6·7 ± 0·6	13·7 ± 0·7*	11·6 ± 1·2*	10·3 ± 2·2*	9·4 ± 0·1*
	DNA	0·38 ± 0·03	0·84 ± 0·01*	0·98 ± 0·03*	1·28 ± 0·16*	0·51 ± 0·05
	Protein/DNA	18 ± 1·0	16 ± 0·8	12 ± 0·9*	8 ± 1·5*	18 ± 1·6
Heart	Protein	54 ± 3·8	65 ± 7·5	78 ± 6·8*	106 ± 10·1*	56·4 ± 4·3
	DNA	2·1 ± 0·2	2·5 ± 0·2	2·7 ± 0·3	3·2 ± 0·4	2·6 ± 0·2
	Protein/DNA	25 ± 2·2	27 ± 3·4	29 ± 2·7	33 ± 3·4	22 ± 2·0

Because neither 3,3′-T_2 nor 3,5-T_2 treatment had a significant effect on the above parameters (treated *vs.* hypothyroid rats), the data are not reported. Results are presented as means ± S.E.M.; *n*, no. of experiments. * Values significantly different ($P < 0·05$) from P + I rats; † values significantly different ($P < 0·05$) from N rats. Different P + I + T_3 groups are distinguished by the doses of T_3 which are given per 100 g BW under the group name.

differences were observed between hypothyroid rats and 3,3′-T_2- or 3,5-T_2-treated animals, whatever the dose (data not shown).

Calculation of the protein/DNA ratio (see Table 5) revealed that chronic T_3 administration induced a hypertrophic effect in the liver and heart and a hyperplastic effect in muscle and BAT. The evidence for this was as follows. In liver and heart, T_3 exerted a strong effect on protein content and a weaker effect (heart) or no effect (liver) on DNA content. The opposite was true for gastrocnemius muscle

and BAT (i.e. T_3 exerted a stronger effect on DNA content than on protein content).

DISCUSSION

T_3 is generally accepted to be the active form of the various thyroid hormones and some data seem to indicate that T_3 may exert both long- and short-term effects on the metabolism of most tissues. However, the question of whether T_3 has a direct action on mitochondria is still unresolved. In fact, in spite of several reports that purport to show rapid effects of T_3 on mitochondria, the very occurrence, and the physiological significance, of these effects has long been controversial. As mentioned in Introduction, this could be due to the great variety of experimental conditions used in different laboratories; in particular, the status of deiodinase enzymes has not always been adequately taken into account. This could have led to an effect being attributed to T_4 or T_3 when it was actually attributable to iodothyronines other than T_4 and T_3.

We have recently shown that isomers of T_2 (3,3′-T_2 and 3,5-T_2) induce a dose-dependent calorigenic effect when injected into hypothyroid rats (Lanni *et al.* 1996). In this present paper, we tried to differentiate the effects of T_3 and T_2. The most noteworthy result of this study is the finding that T_2s have a similar, but more rapid, effect on RM than T_3. In fact, the effect of T_2s starts rapidly, reaching a roughly 20% stimulatory effect in 12–15 h, while the effect of T_3 starts more slowly and reaches a roughly 20% stimulatory effect only after about 50 h.

The need to use a different method of treatment for 3,3′-T_2 than for 3,5-T_2 in order to show up its effect may be related to the different behaviour of the metabolic pathways involved in the degradation (metabolism) of 3,3′-T_2 and 3,5-T_2 (deiodination, glucuronidation and sulphation). Under our conditions, deiodination is almost completely blocked (see Table 2) and, that being so, sulphation and glucuronidation will be responsible for metabolizing these T_2s. In rat liver, the rate of glucuronidation is about 20 times faster for 3,3′-T_2 than for 3,5-T_2 (Visser *et al.* 1993), and the sulphation rate is immensely faster for 3,3′-T_2 than for 3,5-T_2 (700 nmol min^{-1} (mg protein)$^{-1}$ for 3,3′-T_2 and about zero for 3,5-T_2; Sekura, Sato, Cahnmann, Robbins & Jakoby, 1981).

The different time course of the effects exerted by T_2s and T_3 on RM may be an indication that they play different roles at a cellular level. The effect exerted by T_2s is too rapid to involve protein synthesis, as confirmed by experiments in the present study in which we used actinomycin D to inhibit protein synthesis. The simultaneous injection of actinomycin D with T_2s failed to cause any attenuation of the stimulation seen with T_2s alone. In contrast, when actinomycin D and T_3 were given together, the stimulation of RM by T_3 was almost completely abolished (except for a small effect after 5 days).

While the very rapid effect of T_2s (and the lack of any inhibition of their effect by actinomycin D) could be explained by their direct interaction with some components of the energy releasing apparatus – and the presence of specific binding sites for T_2s on cell organelles is in accordance with this notion – the effect of T_3 on RM could be mediated by a slower action on protein synthesis. This effect of T_3 is widely accepted. Indeed, it is well established that the 'long-term' effects of T_3 are exerted via actions on transcription and translation that are triggered by its binding to specific nuclear c-erb-A-related receptor proteins (for reviews see Oppenheimer, Schwartz, Mariash, Kinlaw, Wong & Freke, 1987; Lazar, 1993). However, it has still to be clarified which proteins are 'controlled' by T_3.

We wondered whether, in addition to the effect it exerts on the synthesis of particular mitochondrial components (Scarpulla, Kilar & Scarpulla, 1986; Luciakova & Nelson, 1992; Dumnler, Muller & Seitz, 1996), T_3 might regulate the cellularity (hypertrophy or hyperplasy) of those organs that are metabolically very active (such as liver, BAT, muscle and heart). To clarify this point, we chronically treated some groups of animals with either T_3 or T_2s. Following such treatment, we observed a stimulation of organ growth only after the administration of T_3. Moreover, a major part of the increase in oxidative capacity in a given tissue (total COX) appeared to be secondary to the hypertrophic or hyperplastic effect exerted by T_3. In fact, T_3 administration induced a hypertrophic effect in the liver and heart, but a hyperplastic effect in muscle and in BAT. As a consequence, and in contrast to the situation seen with T_2s, the percentage increase in COX activity in T_3-treated rats was considerably greater when expressed in terms of total tissue activity than when expressed in terms of specific activity. This analysis of the effects of T_3 also supplies an explanation for the apparent discrepancy between its sustained effect on RM and its weak stimulating effect on specific COX activity at tissue level when given chronically (as seen in a previous work (Lanni *et al.* 1996) and as mentioned in Introduction).

In conclusion, we propose that the data presented in this paper provide an answer to the problem of how iodothyronines are involved in the regulation of metabolic activity by the thyroid. According to our hypothesis, T_3 exerts an important part of its effect on metabolic rate by modulating the cellularity of those organs that are metabolically very active. This effect, inducing an enhancement of the percentage contribution by metabolically very active tissues to the total body weight (see Table 4), would increase the animal's oxygen consumption at rest. Owing to the involvement of the machinery of protein synthesis, this effect would take some days to start and some days to stop. On the other hand, T_2s would exert their effect more rapidly than T_3 and, as previously hypothesized (Lanni *et al.* 1992, 1993, 1994*a*,*b*, 1996), this effect would be mediated by a direct interaction between diiodothyronines and mitochondria, with 3,5-T_2 seeming to show a clearer effect. In normal animals, these

actions would probably not be mutually exclusive, instead they might co-operate in determining the final metabolic state of the organs and, consequently, of the whole animal. In other words, they might co-operate in establishing the 'normal level' of the animal's metabolic rate.

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