The T3 Receptor β1 Isoform Regulates UCP1 and D2 Deiodinase in Rat Brown Adipocytes

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Brown adipose tissue (BAT) thermogenesis increases when uncoupling protein-1 (UCP1) is activated adrenergically and requires T3. In humans, UCP1 activation in BAT seems involved in body weight maintenance. BAT type 2 deiodinase (D2) increases in response to adrenergic agents, producing the T3 required for UCP1 expression. T3 actions are mediated by thyroid hormone nuclear T3 receptors (TR), TRα and TRβ. Studies in mice suggest that TRβ is required for UCP1 induction, whereas TRα regulates body temperature and adrenergic sensitivity. In the present study, we compare the effects of T3 vs. specific TRβ1 and TRα1 agonists [GC-1 and CO23] on the adrenergic induction of UCP1 and D2 in cultured rat brown adipocytes. T3 and GC-1 produced similar increases on UCP1, whereas CO23 increased UCP1 only at high doses (50 nM). GC-1 at low doses (0.2–10 nM) was less potent than T3, increasing the adrenergic stimulation of D2 activity and mRNA. At higher doses, GC-1 further stimulated whereas T3 inhibited D2 activity but not D2 mRNA, suggesting posttranscriptional effects. CO23 had no effect on D2 activity but increased D2 mRNA. T3, GC-1, or CO23 by themselves did not increase UCP1 or D2 mRNA. High T3 doses shortened D2 half-life and increased D2 turnover via proteasome, whereas GC-1 did not change D2 stability. The α1- and α2-adrenergic D2 responses increased using high T3 doses. In summary, T3 increases the adrenergic stimulation of UCP1 and D2 expression mostly via the TRβ1 isoform, and in brown adipocytes, D2 is protected from degradation by the action of T3 on TRβ1. (Endocrinology 151: 5074–5083, 2010)
Several regions of the rat UCP1 promoter have thyroid hormone response elements (14). There is a high correlation between the occupancy of nuclear T3 receptors (TRs) and increases in UCP1 expression (15).

During cold exposure, the type 2 deiodinase (D2) locally produces T3, which binds to and activates the nuclear TRs. T3 is required for a full thermogenic function (16, 17), for the adrenergic induction of UCP1, and for lipogenesis (18). T3 is also required for the differentiation of adipocytes (19). D2 is a selenoenzyme that generates T3, via 5' deiodination of T4 in several organs, including brain, pituitary, BAT, skin, placenta, and human heart, muscle, and thyroid. In BAT, D2 (and the production of T3) is activated by adrenergic stimulation and cold exposure, and a synergism between α1- and β-adrenergic pathways has been described (20). In cultured brown adipocytes, the presence of T3 is required for the adrenergic stimulation of D2 (21).

T3 acts through its nuclear TRs, ligand-dependent transcription factors, encoded by two genes: TRα and TRβ (22). Most actions of T3 are mediated by one of these TR isoforms, which are expressed at different levels in different tissues (23). TRβ1 is predominant in liver and TRα1 in heart. TRα1 regulates body temperature and gene expression in heart. TRβ1 is essential for TSH regulation, cochlear development, and hepatic cholesterol metabolism (24, 25). Both TR isoforms are present in adipose tissue, but TRα is more abundant than TRβ. TRβ1, TRα1, and c-erbA-α2 mRNAs are present in rat brown adipocytes in primary culture (26), in which exposure to T3 increased TRβ1 and decreased TRα1. It is of great importance to delineate which are the T3 actions mediated through each isoform, to optimize the therapeutic benefits, and to minimize the toxic side effects of thyromimetic therapeutic agents.

The development of T3 analogs that bind preferentially to TRβ could be a strategy to obtain beneficial T3 effects on lipids while avoiding undesirable side effects. The T3 analog GC-1 (also called sobetirome or QRX-431) has an affinity for TRβ equal to that of T3 and a 10-fold reduced affinity for TRα, and shows TRβ1 selective actions (27). GC-1 lowers serum cholesterol and triglycerides in mice treated with equal or greater potency than T3, without stimulation of heart rate (28–30). The effect of T3 on UCP1 is mediated by the TRβ isoform, because GC-1 increased UCP1 levels, but GC-1 produced an impaired NE response as measured by the lack of cAMP accumulation in isolated adipocytes from GC-1-treated hypothroid mice and failed to maintain BAT and core body temperature (30). The differential effects of GC-1 vs. T3 on the thermogenesis in BAT may be the result of the GC-1 TRβ selectivity. Therefore, the TRα1 isoform seems required to maintain the normal adrenergic responsiveness of the brown adipocyte and body temperature, whereas TRβ mediates T3-induced UCP1 gene expression.

The aim of the present study is to compare the effect of T3 and GC-1 (TRβ selective analog) and CO23 (TRα1 selective analog) (31), on the adrenergic stimulation of UCP1 mRNA and that of D2 activity and mRNA, using primary cultures of rat brown adipocytes.

Materials and Methods

Primary cultures of brown adipocytes

All the rats were housed under humane conditions, under veterinary control, according to the European Community Guidelines and after the approval of the protocol by the Ethics Committee of our institution. Precursor cells were obtained from the interscapular BAT of 3-wk-old rats (Sprague Dawley), using the method described by Néchad et al. (32) with modifications (21), using collagenase digestion (0.2%) in DMEM + 1.5% BSA at 37 C, and filtration through 250-μm pore size silk filters. Mature cells were allowed to float, and the infiltrant was filtered through 25-μm pore size silk filters and centrifuged. Precursor cells were seeded to get 1500–2000 cells/cm² on d 1 and grown in DMEM supplemented with 10% newborn calf serum (NCS), 3 nM insulin, 10 mM HEPES, 50 IU penicillin and 50 μg streptomycin/ml, and 15 μM ascorbic acid. Culture media were changed on d 1 and every 2nd day thereafter. Precursor cells proliferated actively in these conditions, reached confluence on days 4th–5th after seeding (40,000–80,000 cells/cm²), and were fully differentiated by day 8th into mature brown adipocytes. Studies were performed during the period of differentiation (8th culture day) using NCS or hypothroid serum in the presence of thyroid hormones or other treatments, as specified.

Both NCS and hypothroid serum were used for cell culture. The hypothroid serum was obtained by depleting NCS of thyroid hormones, using the anion exchange resin AG1X8 (Bio-Rad, Richmond, CA), as described (33). This treatment also depletes serum from some growth factors and other hormones. Hypothesis serum contained less than 10% of the original amount of thyroid hormones, as assessed by RIA (34). In NCS, concentrations of T4 and T3 were 77 and 0.7 nM T3, respectively. These levels were decreased to 2.2 nM T4 and 0.13 nM T3 in Hypo serum (21). These concentrations are below 10% dilution in the culture medium.

The free T4 and T3 concentrations were measured by ultrafiltration and RIA in the culture medium (DMEM + 10% NCS): 35 pm T4 and 2.5 pm T3 (0.45 and 4% of the total T4 and T3 concentrations, respectively) (21). We also measured the free T3 concentrations, when using DMEM + 10% hypothroid serum + T3. The free T3 concentration was 50 and 170 pm T3, when using 1 and 10 nM T3 in 10% hypothroid serum, respectively. In these conditions, the cellular T3 concentrations were 2.3 and 9 nM T3 for 1 and 10 nM T3, respectively. Cellular T3 concentrations were 45 nM T3 when using 50 nM T3 added to the culture medium (DMEM + 10% hypothroid serum).

D2 activity

Cells were scraped, collected in buffer A [0.32 mM sucrose, 10 mM HEPES, and 10 mM dithiothreitol (DTT) (pH 7.0)], and
D2 activities were determined in homogenates measuring the release of iodide, as described (35, 36), using as final concentrations: 2 nM T₄ (50,000 cpm [¹²⁵I]-T₄), 1 µM T₃, 50 mM DTT, 1 mM 6-n-propyl-2-thiouracil (PTU), 80–100 µg protein in 100 µl of total volume (pH 7.0) during 1 h at 37°C. In these conditions, more than 95% of D2 activity was insensitive to inhibition by 6-n-propyl-2-thiouracil (PTU). Each cell homogenate was tested in triplicate, using two culture flasks per treatment. The protein content was determined by the method of Lowry (37), after precipitation of the homogenates with trichloroacetic acid to avoid interference of DTT in the colorimetric reaction (35). Results were expressed in fmol/h/mg protein.

The specific activity [¹²⁵I]-T₄ used was obtained in our laboratory (>3000 µCi/µg) using chloramine T and T₄ as substrate (35, 36). Before each assay, [¹²⁵I]-T₄ was purified by paper electrophoresis to separate the contaminating iodide, using ammonium acetate 0.05 M (pH 6.8). The amount of iodide in the blanks assay was routinely less than 1% of the total radioactivity. Preliminary experiments were performed to validate the assay: 1) the production of equimolar amounts of iodide and T₃; 2) the linear production of iodide using increasing amounts of protein; and 3) the within-assay coefficient of variation that was less than 5%.

RNA preparation and analysis of UCP1 and D2 mRNA

Total cellular RNA was extracted using TRI reagent (Sigma, St Louis, MO) following the manufacturer’s protocol. For Northern blot analysis, 15 µg total RNA was denatured, electrophoresed, and transferred to nylon membranes to be hybridized with Ucp1 cDNA. A 1200-bp rat Ucp1 cDNA clone (38) was used as a probe by labeling with [α-³²P] deoxy-CTP using random primers (>10⁶ cpm/µg DNA). Filters were hybridized and washed (39). Autoradiograms were obtained from the filters and quantified by NIH Image software. The membranes were routinely dyed using methylene blue to visualize the rRNAs, and the 28S band was used to correct for differences between lanes.

D2 mRNA and Ucp1 mRNA were also measured by TaqMan quantitative RT-PCR (qRT-PCR), using specific TaqMan gene expression assays from Applied Biosystems (rat Dio2, Rn00581867-m1; rat Ucp1, Rn00562126-m1; Applied Biosystems, Foster City, CA). Results were normalized using rat ubiquitin as internal control (rat Ubc, Rn01789812-g1). For D2 and Ucp1 expression, results were expressed as fold change vs. controls and were calculated using the 2⁻ΔΔCt method. All experiments were repeated at least two to four times, and the figures show the mean of two to four experiments.

cAMP analysis

Brown adipocytes were incubated for 30 min with NE or BRL 37344 in hypothyroid serum supplemented with increasing doses of T₃, GC-1, or CO23 for 24 h. cAMP levels were measured using the PerkinElmer NEN Life Science Products kit (PerkinElmer Life Sciences, Boston, MA), according to the manufacturer instructions.

Statistical analysis

Results are expressed as means ± se. When required, one-way ANOVA was used. Statistical significance difference between groups was assessed using the protected least significant difference test. All the calculations were done as described in Snedecor and Cochran (40).

Results

Effects of T₃ and GC-1 on the adrenergic stimulation of UCP1 mRNA

We have previously shown that in primary cultures of rat brown adipocytes, the basal expression of Ucp1 mRNA is undetectable under standard culture conditions (10% NCS and insulin), and NE or T₃, separately, stimulate poorly Ucp1 mRNA. In rat brown adipocytes, the adrenergic stimulation of Ucp1 mRNA requires the presence of NE and T₃ (11).

Figure 1A shows the expression of Ucp1, as measured by Northern blot analysis, stimulated by NE and increasing doses of T₃ or GC-1 (0.2–50 nM). Ucp1 mRNA reached maximal mRNA expression around 2–10 nM T₃. The effect of GC-1 on Ucp1 mRNA was similar to that of

![Figure 1](image1.png)

**FIG. 1.** Effect of T₃, GC-1, and CO23 on the adrenergic stimulation of Ucp1 mRNA. Rat brown adipocytes were grown in standard conditions (10% NCS). Cells were treated during the last 24 h with 1% hypothyroid serum and different doses of T₃ and GC-1 (A) or different doses of T₃, GC-1, and CO23 (B). NE (5 µμ) was added during the last 15 h, and RNA was isolated. A, Ucp1 mRNA expression was measured by Northern blot analysis, and rRNA was shown to correct for differences between lanes. B, Ucp1 mRNA expression was measured by qRT-PCR, calculated by the 2⁻ΔΔCt method, and expressed as fold increase vs. cells treated only with NE (no T₃, GC-1, or CO23). Ubiquitin was used as reference gene. Results are mean ± SEM (n = 4 – 6 per point) from three experiments. * P < 0.05 vs. equimolar doses of T₃ or GC-1.
T₃ in the Northern blot analysis, but the lowest GC-1 concentration seemed to have a lower effect. To check the results found by Northern blot analysis, we further analyzed Ucp1 expression by qRT-PCR, using TaqMan probes and testing the TRα1 analog CO23 for comparison (Fig. 1B). T₃ and GC-1 both increased Ucp1 expression at all the doses tested (P < 0.05 vs. NE), except at 0.2 nM, producing a similar dose-dependent effect on Ucp1 (no difference between equimolar doses of T₃ vs. GC-1). The effect of CO23 was negligible at all except the highest concentration tested (50 nM; P < 0.05 vs. NE alone), and CO23 had always a lower effect than T₃ or GC-1. We conclude that the effect of T₃ on the adrenergic stimulation of Ucp1 is clearly via the TRβ1 isoform, as it was reproduced using the TRβ1 agonist, GC-1. Ucp1 was not detectable in the absence of NE. T₃, GC-1, or CO23 per se had no effect (data not shown).

**Effects of T₃ and GC-1 on the adrenergic stimulation of D2 activity and mRNA**

We have previously shown that the adrenergic stimulation of D2 activity and mRNA requires the presence of T₃ (21, 35). Figure 2 shows the effect of T₃, GC-1, and CO23 on D2 activity (Fig. 2, left) and D2 mRNA (Fig. 2, right). At low doses (2–10 nM), T₃ had a higher effect than GC-1, increasing the adrenergic stimulation of both D2 activity and mRNA, which reached a maximum at 10 nM T₃. At higher doses (>10 nM), T₃ inhibited D2 activity but not D2 mRNA. The high doses of GC-1 (up to 400 nM) further increased D2 activity, and an inhibition of D2 was never observed even at the highest GC-1 doses tested. We did time-course experiments (from 3 to 24 h) using 10 and 50 nM T₃ and 10 and 100 nM GC-1, to exclude changes in D2 responses at different times. Before 6 h, 50 nM T₃ inhibited D2 activity by only 10–30%, and 40–50% D2 inhibition was observed after 6 h (from 12 to 24 h). GC-1 increased D2 activity linear and steadily from 3 to 24 h (data not shown). The effect of GC-1 on D2 mRNA was also lower than that of T₃ up to 10 nM, but at higher doses, T₃ and GC-1 had the same effect on D2 mRNA. CO23 (0.5–400 nM) had little effect on D2 activity (about 8–15% of 10 nM T₃) but had some effect on D2 mRNA at high doses (10 and 50 nM), about 50% the effect of GC-1. NE, T₃, GC-1, or CO23 per se did not increase D2 activity. Therefore, the effect of T₃ on the adrenergic stimulation of D2 preferentially involves TRβ1 at D2 activity and mRNA levels. A TRα effect on D2 mRNA could not be excluded.

The inhibition by high T₃ doses of D2 activity, but not D2 mRNA, suggests posttranscriptional effects at the protein level (changes in D2 half-life and D2 degradation) or a possible modulation of the adrenergic pathways. The inhibition found using high doses of T₃ but not using GC-1 suggests differences between the action of T₃ through both TR isoforms on the stability of D2 activity.

**Effect of high doses of T₃ and GC-1 on the adrenergic pathways for induction of D2 activity**

Because we observed different patterns of stimulation of D2 activity and mRNA when using high doses of T₃ and GC-1, we tested whether changes in the adrenergic pathways were occurring. We have previously shown that the adrenergic responses of D2 activity and expression occur via β3-adrenergic pathways (21). In Fig. 3A, we confirmed that the adrenergic response of D2 activity is mostly β3-adrenergic, as shown using the β3 agonist BRL 37344 in the presence of both T₃ and GC-1. The inhibitory effect of high doses of T₃ (50 nM) was observed using both NE and BRL 37344, though the β3-adrenergic pathway seemed lower at the high T₃ doses (from 75% at 10 nM T₃ to 60% at 50 nM T₃), and again, no inhibition was observed using high GC-1 doses either using NE or BRL 37344.

We then analyzed whether the differences between T₃ and GC-1 could be due to changes in the stimulation of different
adrenergic pathways (β- and α-adrenergic pathways). For this, we used NE and 10 or 50 nM T₃ or 50 nM GC-1, in the presence of α₁, α₂- and β-adrenergic antagonists (prazosin, yohimbine, and alprenolol, respectively). Figure 3B shows that at 10 nM T₃, the adrenergic response was mostly β-adrenergic (60% of control), whereas the α₁-adrenergic pathway was 25% of control (+ prazosin). Blocking of β- and α₁-adrenergic receptors, we observed that both adrenergic pathways were additive, and the inhibition of D2 activity was about 80%. A small α₂-adrenergic component (not significant) was also detected using yohimbine (<10%).

At high doses (50 nM T₃), we found differences in the adrenergic responses of D2 activity; the α₁-adrenergic pathway increased up to 54%, and the α₂-adrenergic pathway increased up to 20%. At 50 nM GC-1, the results were similar to that of 10 nM T₃. The β-adrenergic pathway was 72%, and the α₁-adrenergic component was 30% of control, and no participation of the α₂-adrenergic pathway was found. In summary, high T₃ doses change the pattern of adrenergic responses increasing the α-adrenergic pathways.

**cAMP levels**

We then examined whether the differences found in the participation of the different adrenergic pathways using high doses of T₃, the lack of stimulation of D2 activity by CO23, and the inhibition of D2 activity at high doses of T₃ could be due to a change in cAMP production.

Brown adipocytes were incubated with increasing doses of T₃, GC-1, or CO23 for 24 h, NE or BRL 37344 were added for the last 30 min, and the cAMP produced was measured (Table 1). NE or BRL 37344 produced a 7-fold increase in cAMP production (from 0.74 to 5 pmol/ml). T₃ or GC-1 (10 nM and above) further increased cAMP (Table 1); the largest cAMP increases were found using the β3 agonist BRL 37344 and 200 nM GC-1.

T₃ and CO23 induced similar increases in cAMP, which were lower than using GC-1, indicating that CO23 is as effective as T₃ stimulating cAMP production. We did not observe inhibition in cAMP production in the presence of high doses of T₃ or using CO23. The levels of cAMP are higher using BRL 37344, increasing with high T₃ and GC-1 doses.

**Mechanisms of degradation of D2 activity with T₃ and GC-1**

We also investigated whether the half-life and the degradation of D2 activity were different in the presence of 10 and 100 nM T₃ and 200 nM GC-1.
Brown adipocytes were treated with 10 nM, 100 nM T3, or 200 nM GC-1 for 24 h, and NE was added for the last 15 h. Then, cycloheximide was added and cells collected at different times up to 2 h. Figure 4A shows that D2 half-life was similar when using 10 nM T3 or 200 nM GC-1, and both induced a stabilization of D2 activity, but the half-life in the presence of 100 nM T3 was shorter than using 10 nM T3 or 200 nM GC-1 (<1 h), indicating a higher turnover rate of D2 activity.

D2 degradation

We also tested whether high doses of T3 could be modulating the degradation of D2 via the proteasome. Previously, we confirmed the role of proteasome in D2 degradation in our cultures of brown adipocytes (41).

TABLE 1. cAMP levels in response to 5 μM NE or BRL37344. Effect of T3, GC-1, and CO23 addition

<table>
<thead>
<tr>
<th>Doses</th>
<th>NE (5 μM)</th>
<th>+T3</th>
<th>+GC-1</th>
<th>+CO23</th>
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<tbody>
<tr>
<td>0.2 nM</td>
<td>4.69 ± 0.32</td>
<td>7.53 ± 1.75</td>
<td>6.48 ± 0.85&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.60 ± 0.48</td>
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<tr>
<td>2 nM</td>
<td>5.57 ± 1.11</td>
<td>6.08 ± 0.31&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.85 ± 0.67</td>
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<tr>
<td>10 nM</td>
<td>11.55 ± 2.18&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>16.15 ± 0.61&lt;sup&gt;a,b,c,d&lt;/sup&gt;</td>
<td>7.16 ± 0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>50 nM</td>
<td>16.15 ± 0.61&lt;sup&gt;a,b,c,d&lt;/sup&gt;</td>
<td>7.64 ± 0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>100 nM</td>
<td>6.59 ± 0.11</td>
<td>16.35 ± 2.36&lt;sup&gt;a,b,c,d&lt;/sup&gt;</td>
<td></td>
<td></td>
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<tr>
<td>200 nM</td>
<td>5.65 ± 0.70</td>
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BRL 37344 (5 μM) | 6.10 ± 0.59<sup>d</sup> | 10.04 ± 1.70 |

<table>
<thead>
<tr>
<th>Doses</th>
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<th>+T3</th>
<th>+GC-1</th>
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</tr>
</thead>
<tbody>
<tr>
<td>0.2 nM</td>
<td>6.10 ± 0.59&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10.04 ± 1.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 nM</td>
<td>7.97 ± 1.86&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.49 ± 1.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>10 nM</td>
<td>14.04 ± 2.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.34 ± 1.96&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>50 nM</td>
<td>19.87 ± 1.90&lt;sup&gt;a,b,c,d&lt;/sup&gt;</td>
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<td>100 nM</td>
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Cells were incubated for 24 h with several doses of T3, GC-1, and CO23 in 10% hypothyroid serum. NE or BRL37344 (at 5 μM) were added for the last 30 min. Data are expressed as picomoles per ml (means ± SEM, n = 2–4). Basal levels (without NE) were 0.74 ± 0.13 pmol cAMP/ml.

<sup>a</sup> P < 0.05 vs. NE or BRL 37344.
<sup>b</sup> P < 0.05 vs. its corresponding lower dose (0.2 nM).
<sup>c</sup> P < 0.05 vs. the equimolar T<sub>4</sub> dose.
<sup>d</sup> P < 0.05 vs. its corresponding 10 nM dose of T3 or GC-1.

FIG. 4. A, Half-life of D2 using 10 or 100 nM T3 and 200 nM GC-1. Cells were treated for 24 h with 10 or 100 nM T3 or 200 nM GC-1 in 10% hypothyroid serum. NE (5 μM) was added during the last 15 h. Then, cycloheximide was added and cells collected at different times up to 2 h. Figure 4A shows that D2 half-life was similar when using 10 nM T3 or 200 nM GC-1, and both induced a stabilization of D2 activity, but the half-life in the presence of 100 nM T3 was shorter than using 10 nM T3 or 200 nM GC-1 (<1 h), indicating a higher turnover rate of D2 activity.

D2 degradation

We also tested whether high doses of T3 could be modulating the degradation of D2 via the proteasome. Previously, we confirmed the role of proteasome in D2 degradation in our cultures of brown adipocytes (41).
Now, we compare the effect of the proteasome inhibitor MG132 added during the last hour of adrenergic stimulation in cells treated with 100 nM T₃ or 200 nM GC-1. The use of MG132 (Fig. 4B) led to increases in D₂ activity in 100 nM T₃-treated adipocytes (140%), whereas no differences in D₂ activity were found in cells treated with or without MG132 in the presence of 200 nM GC-1. This indicates that high doses of T₃ increase the proteasome activity and higher degradation rates of D₂ than with GC-1 treatment.

Modulation of TR isoforms by GC-1 and CO23

We also examined whether T₃ or the analogs used (GC-1 and CO23) in the conditions and doses used in the present study could be modulating the expression of the TR isoforms (TRα and TRβ). The changes found were small and did not show dose-dependent changes (Martinez de Mena R and Obregon MJ, unpublished results). The changes do not justify the responses found in UCP1 and D₂. The expression of TRα was always higher than that of TRβ, and the expression of both isoforms was higher in cultured cells than in BAT tissue, although the proportion of both TR isoforms (TRα>TRβ) is maintained in BAT and cells (Martinez de Mena, R. and M. J. Obregon, unpublished results).

Finally, we have summarized the findings of this study and from other studies in Fig. 5.

Discussion

T₃ has a profound influence in adipose tissue, regulating the differentiation of the adipocyte, the process of lipogenesis and lipolysis, and many genes involved in lipid metabolism (19). T₃ is also involved in the regulation of energy balance by regulating body weight and basal and adaptive thermogenesis. Research is currently underway to identify the TR isoform responsible for each specific action of T₃, to enable and facilitate the development of selective thyromimetic therapeutic agents. The use of genetically modified mice for TRα, TRβ, or both isoforms has shed some light on the specific physiological functions regulated by each isoform (42), but due to redundancy of the isoforms, some effects are not yet clearly identified. Therefore, the use of specific analogs for TRα and TRβ may help to identify

FIG. 5. Role of TRβ and TRα isoforms in brown adipocyte thermogenesis. Binding of NE to the β-adrenergic receptors activates adenylate cyclase (AC), increasing cAMP levels. This event activates lipolysis and the expression of cAMP-dependent genes, namely, UCP1, D₂, and proteins involved in adrenergic responses. Intracellular T₃ increases through D₂ activation, and there is a synergism between T₃ and cAMP actions. T₃ increases cAMP, through both TR isoforms. T₃, via TRβ, increases UCP1 expression and D₂ activity and expression, although a TRα effect on D₂ expression is also present. High doses of T₃ change the pattern of D₂ adrenergic stimulation increasing α₁-adrenergic pathways. High T₃ doses shorten D₂ half-life and increase D₂ degradation, whereas the TRβ1 isoform stabilizes D₂ activity. CRE, cAMP response element; FFA, free fatty acids; AR, adrenergic receptors; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; PKA, protein kinase A; PIP2, phosphatidylinositol 4,5-bisphosphate.
selective effects of T₃ mediated predominantly by one of the TR isoforms (43).

In the present study, we examine the effect of T₃ action on TRα1 and TRβ1 in the adrenergic stimulation of UCP1 mRNA and D2 activity and mRNA, using specific agonists of the TRα1 and TRβ1 isoforms. The T₃ effect on both genes is mediated preferentially via the TRβ isoform, although the TRα isoform might participate in the regulation of D2 mRNA.

The adrenergic stimulation of UCP1 is clearly mediated by the TRβ isoform as shown by using GC-1, and GC-1 is as effective as T₃. This agrees with previous work using injections of GC-1 in hypothyroid mice, describing that the T₃ effect on UCP1 occurs via TRβ (30). In that report, GC-1 restored UCP1 levels in hypothyroid mice, but did not increase cAMP levels in floating brown adipocytes from GC-1-treated hypothyroid mice incubated with adrenergic agents (30). In contrast to these findings, we find that GC-1 + NE increases cAMP levels even better than T₃ + NE, and no defect is found to increase cAMP production in brown adipocytes. It should be considered that the systems used in the Ribeiro study (30) and the present ones are different (the species used, hypothyroid-treated mice vs. control rats, floating vs. cultured adipocytes, etc.), which could account for the differences found. A recent report described defective UCP1 levels in TRβ PV mutant mice, indicating the need of the TRβ isoform for UCP1 expression (44) and for cAMP production, although no clear-cut effects were found in a previous report using mice devoid of all TRs (45).

T₃ increases UCP1 transcription but also stabilizes its mRNA by increasing its half-life (11, 13). Our results indicate that GC-1 possibly acts on both effects of T₃ on UCP1, because the effect of CO23 on UCP1 is very small and only found at high doses.

We have also examined another key enzyme in BAT, D2. D2 is the main source of T₃ in BAT, especially under adrenergic stimulation, providing the T₃ required for thermogenesis. D2 is frequently used as a marker of thermogenesis and for the presence of BAT, because T₃ is highly increased under adrenergic stimulation together with the up-regulation of UCP1. In rat brown adipocytes, the adrenergic stimulation of D2 expression requires T₃ (21, 35). The specific TR isoforms involved in the regulation of D2 activity and mRNA have not been previously studied. Our data indicate that TRβ is the main pathway for the activation of the D2 adrenergic stimulation by T₃. However, we find several important differences between the effect of T₃ and GC-1 on D2 and UCP1. First, the TRα1 analog CO23, at high doses, has an effect on D2 mRNA but not on D2 activity. It is possible that CO23 at high doses might be interacting with TRβ, because the functional selectivity of this compound is about 10-fold (31). Second, the dose-response curves for GC-1 and T₃ are shaped differently: GC-1 displays a standard sigmoidal curve, whereas the T₃ curve is bell shaped, making it difficult to assess the dose dependence of T₃.

Another main difference is the inhibition of D2 activity by high T₃ doses, which was not found using large doses of GC-1. We have previously observed a similar inhibition of D2 activity when using triiodothyroacetic acid (Triac), a natural thermogenic compound, in brown adipocytes (46). Triac increased D2 activity at doses lower than T₃ with a maximum at 1 nM, and at higher doses, Triac inhibited D2 activity. The inhibition found using high T₃ doses might be a regulatory pathway down-regulating T₃ production by D2, against a T₃ excess. In hyperthyroidism, D2 is inhibited (47), and during chronic cold exposure, D2 activity decreases after some days, indicating that high T₃ concentrations in BAT switch off the mechanisms of T₃ production, via D2. T₃ concentrations in BAT are rather high (5–8 nM) in control rats and after 48 h cold exposure increase up to 25 nM T₃, as measured by RIA in BAT (48). The T₃ concentrations we used for cultures are rapidly inactivated in culture by D₃ (activated by serum), and after 24 h, we found only 40% of the initially added T₃ (49). Using 10 and 50 nM T₃, the T₃ concentrations in our cells are 9 and 45 nM T₃, as measured by RIA (see Materials and Methods). Therefore, using high T₃ doses, the T₃ concentration in our cells (45 nM) is double those found in BAT of cold-exposed rats for 2 d (25 nM), a physiological condition in BAT.

Because the inhibition observed on D2 activity was not found at the mRNA level, this suggested posttranscriptional effects of T₃ on D2 stability. D2 is inactivated by the proteasome; rT₃ reduces D2 activity by accelerating its degradation (50). The effect of MGI32 on the proteasome inhibits substrate-induced degradation, and the stabilization of D2 occurs (51). D2 is inactivated by conjugation to ubiquitin, and the protein deubiquitinating enzyme-1 reverses its inactivation prolonging the D2 half-life. Deubiquitinating enzyme-1 markedly increases in brown adipocytes by NE and cold exposure (52), suggesting stabilization of D2 in cold exposure. Ubiquitination is a regulator of D2 stability, a step critical for its catalytic activity and dimerization (53). It has not been described that T₃ could regulate the proteasome activity or increase D2 degradation, as this process was always associated to degradation by the sustrate, rT₃, but it is evident that high T₃ doses increase D2 degradation as shown using the proteasome inhibitor MG132 and measuring D2 half-life. The TRβ1 isoform could be participating in the stabilization of D2.
Another possibility is that T3 induces changes in the adrenergic pathways used to stimulate D2 activity. In our culture, the adrenergic stimulation of D2 is mostly β3-adrenergic (21), but the first studies on D2 activity in rat BAT identified the adrenergic response to cold as α1-adrenergic (54), because prazosin inhibited D2 increases and local T3 production. The changes we observed with high T3 doses point in the same direction: an increase in the α1-adrenergic pathway. TR6 is likely to play a role in adrenergic signaling at one of the multiple sites under thyroid hormone regulation as the regulation of the different adrenergic pathways (55).

The level of expression of both TR isoforms could be modulated by the treatments used, especially when using high doses of T3 or the analogs. But our preliminary studies on TRs expression does not support the hypothesis of an induction of the TRα and TRβ isoforms by the treatments used that could justify the changes found in UCP1 and D2.

Our studies support the view that TRβ agonists, used to control cholesterol in humans, might be useful tools to increase energy expenditure and facultative thermogenesis in humans (27). Other TRβ agonists, such as GC-24, increases energy expenditure, eliminating the increased adiposity without causing cardiac hypertrophy and normalized plasma triglycerides, BAT being the main target (56). A recent report confirms the effect of GC-24 on a battery of genes in brown adipocytes but not in myocytes, together with increases in energy expenditure and clear effects on BMI, and gene expression in control mice, although these changes were not observed in obese mice on high-fat diet (57). Metabolic diseases in addition to dyslipemia, such as obesity, metabolic syndrome, and diabetes, could be treated safely and effectively by TRβ analogs. GC-1 by increasing UCP1 and D2 activity and its stability could help to maintain an adequate T3 production in BAT and to prolong the thermogenic effects of T3, diminished in obesity, as well as in the low T3 syndrome induced during caloric restriction and fasting, situations in which thermogenesis is diminished.

**References**

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