The T_3 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 T_3. 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 T_3 required for UCP1 expression. T_3 actions are mediated by thyroid hormone nuclear T_3 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 T_3 *vs.* specific TRβ1 and TRα1 agonists [GC-1 and CO23] on the adrenergic induction of UCP1 and D2 in cultured rat brown adipocytes. T_3 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 T_3, increasing the adrenergic stimulation of D2 activity and mRNA. At higher doses, GC-1 further stimulated whereas T_3 inhibited D2 activity but not D2 mRNA, suggesting posttranscriptional effects. CO23 had no effect on D2 activity but increased D2 mRNA. T_3, GC-1, or CO23 by themselves did not increase UCP1 or D2 mRNA. High T_3 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 T_3 doses. In summary, T_3 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 T_3 on TRβ1. **(*Endocrinology* 151: 5074–5083, 2010)**

The brown adipose tissue (BAT) is the main site of adaptative thermogenesis, providing extraheat in hibernating animals, newborns, and cold-exposed mammals (cold-induced thermogenesis). Adaptative thermogenesis provides heat by uncoupling the oxidative phosphorylation, a function accomplished by the uncoupling protein-1 (UCP1) present in the inner mitochondrial membrane of brown adipocytes (1). BAT is also important in the maintenance of energy balance, because it is activated in response to diet (diet-induced thermogenesis) (2). Recent data showed that BAT is present in humans, and its activity correlates inversely with the percentage of body fat (3–5). BAT thermogenesis is activated in response to norepinephrine (NE), and the adrenergic stimulation induces UCP1 synthesis. The stimulation is β-adrenergic, acting through the β1- and β3-adrenergic receptors, although with a participation of α1-adrenergic pathways (6, 7).

Thyroid hormones play a key role in obligatory and adaptative thermogenesis. Hypothyroid rats are intolerant to cold and die when exposed to cold after several hours (8), due to a deficient basal and facultative thermogenesis. This deficiency is restored by replacement with thyroid hormones. It has been shown that thyroid hormones are necessary for the full expression of UCP1 *in vivo* (8, 9). Moreover, T_3 amplifies the adrenergic stimulation of UCP1 mRNA expression in cold-exposed rats (10, 11). T_3 also has a direct effect on UCP1 transcription and on the stabilization of UCP1 mRNA in brown adipocytes (12,

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Abbreviations: BAT, Brown adipose tissue; CO23, TRα1 agonist; D2, type 2 deiodinase; DTT, dithiothreitol; GC-1, TRβ1 agonist; NCS, newborn calf serum; NE, norepinephrine; qRT-PCR, quantitative RT-PCR; TR, T_3 receptor; Triac, triiodothyroacetic acid; UCP1, uncoupling protein-1.

13). Several regions of the rat UCP1 promoter have thyroid hormone response elements (14). There is a high correlation between the occupancy of nuclear T_3 receptors (TRs) and increases in UCP1 expression (15).

During cold exposure, the type 2 deiodinase (D2) locally produces T_3, which binds to and activates the nuclear TRs. T_3 is required for a full thermogenic function (16, 17), for the adrenergic induction of UCP1, and for lipogenesis (18). T_3 is also required for the differentiation of adipocytes (19). D2 is a selenoenzyme that generates T_3, via 5′ deiodination of T_4 in several organs, including brain, pituitary, BAT, skin, placenta, and human heart, muscle, and thyroid. In BAT, D2 (and the production of T_3) 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 T_3 is required for the adrenergic stimulation of D2 (21).

T_3 acts through its nuclear TRs, ligand-dependent transcription factors, encoded by two genes: *TR*α and *TR*β (22). Most actions of T_3 are mediated by one of these TR isoforms, which are expressed at different levels in different tissues (23). *TR*β1 is predominant in liver and *TR*α1 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 *TR*α is more abundant than *TR*ß. TRβ1, TRα1, and c-erbA-α2 mRNAs are present in rat brown adipocytes in primary culture (26), in which exposure to T_3 increased TRß1 and decreased TRα1. It is of great importance to delineate which are the T_3 actions mediated through each isoform, to optimize the therapeutic benefits, and minimize the toxic side effects of thyromimetic therapeutic agents.

The development of T_3 analogs that bind preferentially to TRß could be a strategy to obtain beneficial T_3 effects on lipids while avoiding undesirable side effects. The T_3 analog GC-1 (also called sobetirome or QRX-431) has an affinity for TRβ equal to that of T_3 and a 10-fold reduced affinity for TRα1, and shows TRβ1 selective actions (27). GC-1 lowers serum cholesterol and triglycerides in mice with equal or greater potency than T_3, without stimulation of heart rate (28–30). The effect of T_3 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 hypothyroid mice and failed to maintain BAT and core body temperature (30). The differential effects of GC-1 *vs.* T_3 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 T_3-induced UCP1 gene expression.

The aim of the present study is to compare the effect of T_3 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 infranatant was filtrated through 25-μm pore size silk filters and centrifuged. Precursor cells were seeded to get 1500–2000 cells/cm^2 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/cm^2), 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 hypothyroid serum in the presence of thyroid hormones or other treatments, as specified.

Both NCS and hypothyroid serum were used for cell culture. The hypothyroid 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. Hypothyroid serum contained less than 10% of the original amount of thyroid hormones, as assessed by RIA (34). In NCS, concentrations of T_4 and T_3 were 77 and 0.7 nM T_3, respectively. These levels were decreased to 2.2 nM T_4 and 0.13 nM T_3 in Hypo serum (21). These concentrations are before 10% dilution in the culture medium.

The free T_4 and T_3 concentrations were measured by ultrafiltration and RIA in the culture medium (DMEM + 10% NCS): 35 pM T_4 and 2.5 pM T_3 (0.45 and 4% of the total T_4 and T_3 concentrations, respectively) (21). We also measured the free T_3 concentrations, when using DMEM + 10% hypothyroid serum + T_3. The free T_3 concentration was 50 and 170 pM T_3 when using 1 and 10 nM T_3 in 10% hypothyroid serum, respectively. In these conditions, the cellular T_3 concentrations were 2.3 and 9 nM T_3 for 1 and 10 nM T_3, respectively. Cellular T_3 concentrations were 45 nM T_3 when using 50 nM T_3 added to the culture medium (DMEM + 10% hypothyroid serum).

D2 activity

Cells were scraped, collected in buffer A [0.32 M sucrose, 10 mM HEPES, and 10 mM dithiothreitol (DTT) (pH 7.0)], and

homogenized. D2 activities were determined in homogenates measuring the release of iodide, as described (35, 36), using as final concentrations: 2 nM T_4 (50.000 cpm [^{125}I]-T_4), 1 μM T_3, 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 express in fmol/h·mg protein.

The high specific activity [^{125}I]-T_4 used was obtained in our laboratory (>3000 μCi/μg) using chloramine T and T_3 as substrate (35, 36). Before each assay, [^{125}I]-T_4 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 T_3, 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-^{32}P] deoxy-CTP using random primers (>10^8 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 $2^{-\Delta\Delta Ct}$ method. All experiments have been 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 T_3, 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 T_3 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 T_3, separately, stimulate poorly *Ucp1* mRNA. In rat brown adipocytes, the adrenergic stimulation of *Ucp1* requires the presence of NE and T_3 (11).

Figure 1A shows the expression of *Ucp1*, as measured by Northern blot analysis, stimulated by NE and increasing doses of T_3 or GC-1 (0.2–50 nM). *Ucp1* mRNA reached maximal mRNA expression around 2–10 nM T_3. The effect of GC-1 on *Ucp1* mRNA was similar to that of

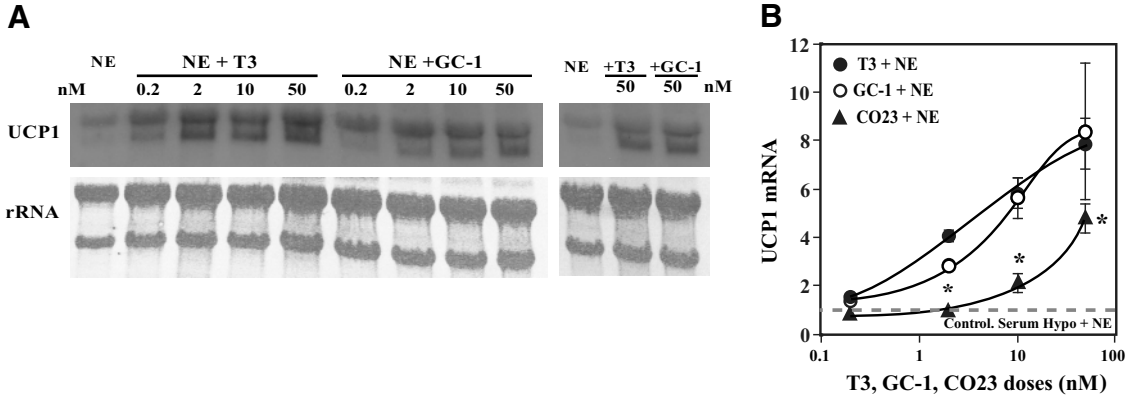

FIG. 1. Effect of T_3, GC-1, and CO23 on the adrenergic stimulation of *Ucp1* mRNA. Rat brown adipocytes were grown in standard conditions (10% NCS). Cells were treated during the last 24 h with 1% hypothyroid serum and different doses of T_3 and GC-1 (A) or different doses of T_3, GC-1, and CO23 (B). NE (5 μM) 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 $2^{-\Delta\Delta Ct}$ method, and expressed as fold increases *vs.* cells treated only with NE (no T_3, 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 T_3 or GC-1.

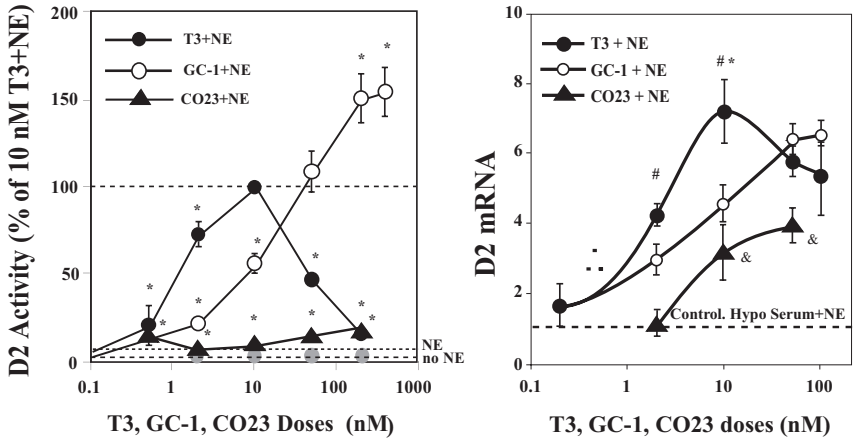

FIG. 2. Effect of T_3, GC-1, and CO23 on the adrenergic stimulation of D2 activity and mRNA. Rat brown adipocytes were grown in standard conditions (10% NCS). Cells were treated during the last 24 h with 10% hypothyroid serum for D2 activity and 1% hypothyroid serum for *D2* mRNA and different doses of T_3, GC-1, or CO23. NE (5 μM) was added during the last 15 h before recollection. *Left*, Results for D2 activity are expressed taking the 10 nM T_3 dose as 100%. *, $P < 0.05$ *vs.* 10 nM T_3 + NE. D2 activities at 10 nM T_3 (100%) were 200–400 fmol/h·mg protein. Data are means ± SEM (n = 4–10 per dose) from 10 experiments expressed as percentage of control. The *lines* indicate the effect of NE alone or "no NE" in the presence of T_3, GC-1, or CO23. *Right*, *D2* mRNA was measured using qRT-PCR, calculated by the $2^{-\Delta\Delta Ct}$ method, and expressed as fold increases *vs.* cells treated only with NE (no T_3 or GC-1 or CO23). Ubiquitin was used as reference gene. Data are means ± SEM (n = 6) from three experiments. *, $P < 0.05$ T_3 *vs.* GC-1; #, $P < 0.05$ T_3 *vs.* CO23; &, $P < 0.05$ CO23 *vs.* basal.

T_3 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). T_3 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 T_3 *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 T_3 or GC-1. We conclude that the effect of T_3 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. T_3, GC-1, or CO23 *per se* had no effect (data not shown).

Effects of T_3 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 T_3 (21, 35). Figure 2 shows the effect of T_3, GC-1, and CO23 on D2 activity (Fig. 2, *left*) and *D2* mRNA (Fig. 2, *right*). At low doses (2–10 nM), T_3 had a higher effect than GC-1, increasing the adrenergic stimulation of both D2 activity and mRNA, which reached a maximum at 10 nM T_3. At higher doses (>10 nM), T_3 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 T_3 and 10 and 100 nM GC-1, to exclude changes in D2 responses at different times. Before 6 h, 50 nM T_3 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 T_3 up to 10 nM, but at higher doses, T_3 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 T_3) but had some effect on *D2* mRNA at high doses (10 and 50 nM), about 50% the effect of GC-1. NE, T_3, GC-1, or CO23 *per se* did not increase *D2* activity. Therefore, the effect of T_3 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 T_3 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 T_3 but not using GC-1 suggests differences between the action of T_3 through both TR isoforms on the stability of D2 activity.

Effect of high doses of T_3 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 T_3 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 T_3 and GC-1. The inhibitory effect of high doses of T_3 (50 nM) was observed using both NE and BRL 37344, though the β3-adrenergic pathway seemed lower at the high T_3 doses (from 75% at 10 nM T_3 to 60% at 50 nM T_3), and again, no inhibition was observed using high GC-1 doses either using NE or BRL 37344.

We then analyzed whether the differences between T_3 and GC-1 could be due to changes in the stimulation of different

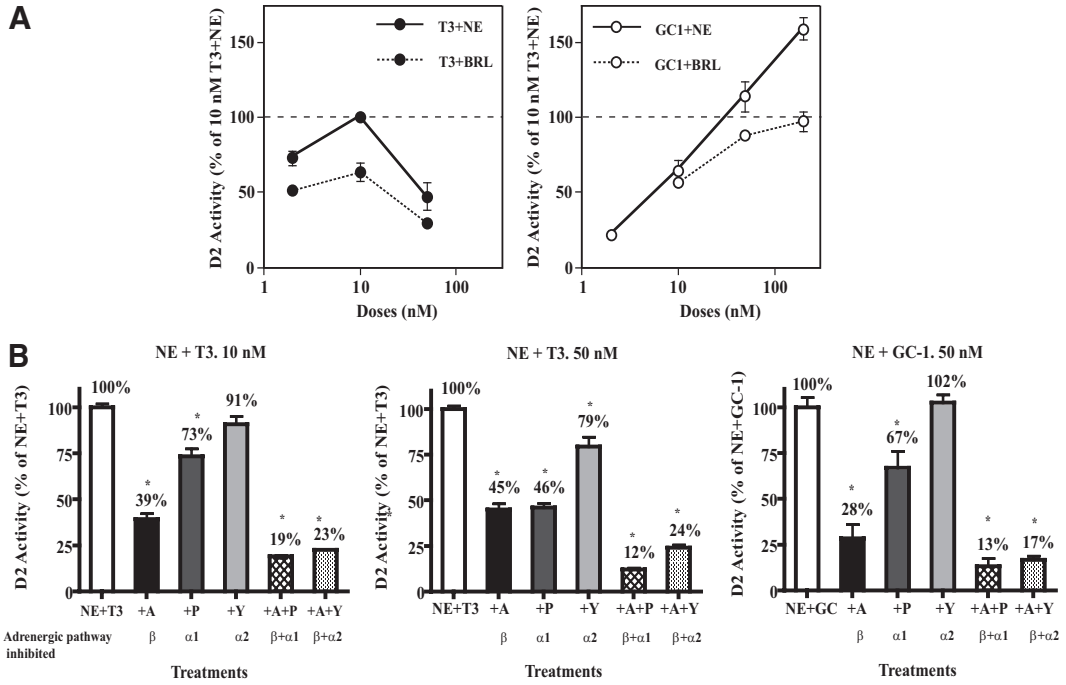

FIG. 3. A, Effects of high doses of T_3 and GC-1 on the adrenergic induction of D2 activity. Cells were treated for 24 h with increasing doses of T_3 (A) or GC-1 (B) in 10% hypothyroid serum, and NE or BRL 37344 (5 μM) were added during the last 15 h. Results for D2 activity are expressed taking 10 nM T_3 + NE as 100%. D2 activities at 10 nM T_3 + NE (100%) were 200–400 fmol/h·mg protein. Data are means ± SEM (n = 4–20 per point) from 10 experiments. B, D2 activity. Changes in the adrenergic pathways using 10 or 50 nM T_3 and 50 nM GC-1 in brown adipocytes. Cells were treated for 24 h with 10 or 50 nM T_3 or 50 nM GC-1 in 10% hypothyroid serum. NE (5 μM) was added during the last 15 h in the presence of different adrenegic antagonists [for α1, prazosin (P); α2, yohimbine (Y); β, alprenolol (A)] at 10 nM, added 1 h before NE. Results for D2 activity are expressed taking 10 nM T_3 + NE, 50 nM T_3 + NE, and 50 nM GC-1 + NE, respectively, as 100%. Data are means ± SEM (n = 8) from four experiments. *, $P < 0.05$ *vs.* 100% in *each panel*.

adrenergic pathways (ß- and α-adrenergic pathways). For this, we used NE and 10 or 50 nM T_3 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 T_3, 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 T_3), 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 T_3. 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 T_3 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 T_3, the lack of stimulation of D2 activity by CO23, and the inhibition of D2 activity at high doses of T_3 could be due to a change in cAMP production.

Brown adipocytes were incubated with increasing doses of T_3, 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). T_3 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.

T_3 and CO23 induced similar increases in cAMP, which were lower than using GC-1, indicating that CO23 is as effective as T_3 stimulating cAMP production. We did not observe inhibition in cAMP production in the presence of high doses of T_3 or using CO23. The levels of cAMP are higher using BRL 37344, increasing with high T_3 and GC-1 doses.

Mechanisms of degradation of D2 activity with T_3 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 T_3 and 200 nM GC-1.

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

Doses	NE (5 μM)	+T_3	+GC-1	+CO23
	4.69 ± 0.32			
0.2 nM		7.53 ± 1.75	6.48 ± 0.85[d]	6.60 ± 0.48
2 nM		5.57 ± 1.11	6.08 ± 0.31[d]	6.85 ± 0.67
10 nM		9.16 ± 1.81[a]	11.55 ± 2.18[a,b]	7.16 ± 0.94[a]
50 nM			16.15 ± 0.61[a,b,c,d]	7.64 ± 0.67[a]
100 nM		6.59 ± 0.11		
200 nM			16.35 ± 2.36[a,b,c,d]	
	BRL 37344 (5 μM) 5.65 ± 0.70			
0.2 nM		6.10 ± 0.59[d]	10.04 ± 1.70	
2 nM		7.97 ± 1.86[d]	12.49 ± 1.58[a]	
10 nM		13.63 ± 2.68[a,b]	14.04 ± 2.73[a]	
50 nM			15.34 ± 1.96[a,c]	
100 nM		9.65 ± 0.59		
200 nM			19.87 ± 1.90[a,b,c,d]	

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.

[a] $P < 0.05$ *vs.* NE or BRL 37344.

[b] $P < 0.05$ *vs.* its corresponding lower dose (0.2 nM).

[c] $P < 0.05$ *vs.* the equimolar T_3 dose.

[d] $P < 0.05$ *vs.* its corresponding 10 nM dose of T_3 or GC-1.

D2 half-life

Brown adipocytes were treated with 10 nM, 100 nM T_3, 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 T_3 or 200 nM GC-1, and both induced a stabilization of D2 activity, but the half-life in the presence of 100 nM T_3 was shorter than using 10 nM T_3 or 200 nM GC-1 (<1 h), indicating a higher turnover rate of D2 activity.

D2 degradation

We also tested whether high doses of T_3 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).

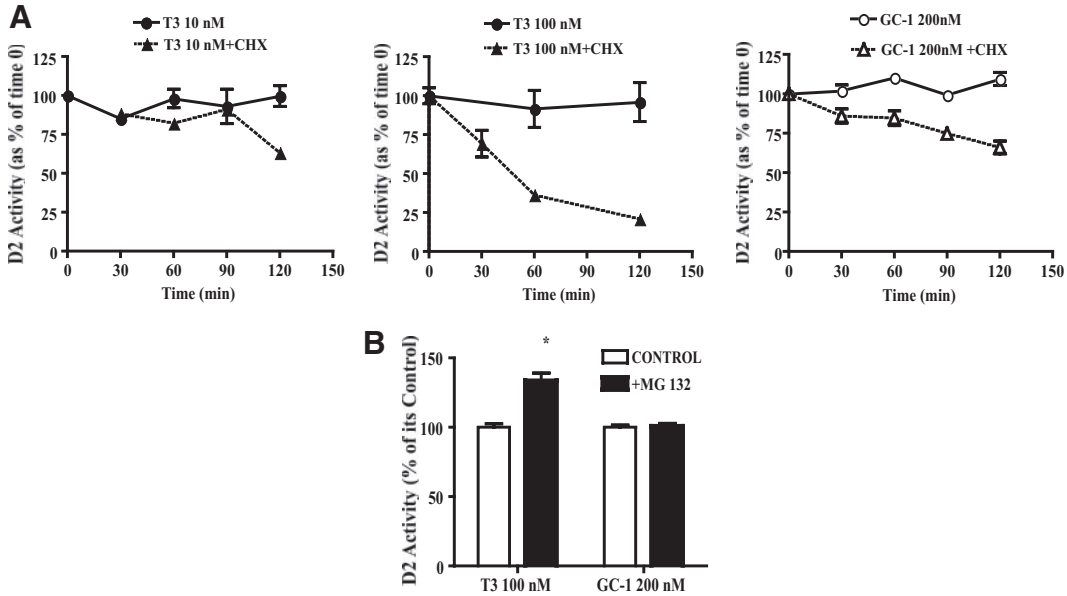

FIG. 4. A, Half-life of D2 using 10 or 100 nM T_3 and 200 nM GC-1. Cells were treated for 24 h with 10 or 100 nM T_3 or 200 nM GC-1 in 10% hypothyroid serum. NE (5 μM) was added during the last 15 h. Then, cycloheximide (CHX) (25 μM) was added, and cells were harvested at 0, 30, 60, 90, and 120 min, using controls without CHX at the same times. CHX by itself had no effect. Data are means ± SEM (n = 10–12 per point) from six experiments. B, Effect of proteasome inhibitor MG132 on D2 activity. Cells were treated for 24 h with 100 nM T_3 or 200 nM GC-1 in 10% hypothyroid serum. NE (5 μM) was added during the last 15 h. MG132 (10 μM) was added during the last hour of adrenergic stimulation. MG132 by itself had no effect. Results for D2 activity are expressed taking 100 nM T_3 + NE or 200 nM GC-1 + NE as 100%. *, $P < 0.05$. Data are means ± SEM (n = 14–18 per point) from nine experiments.

Now, we compare the effect of the proteasome inhibitor MG132 added during the last hour of adrenergic stimulation in cells treated with 100 nM T_3 or 200 nM GC-1. The use of MG132 (Fig. 4B) led to increases in D2 activity in 100 nM T_3-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 T_3 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 T_3 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 showed 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

T_3 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). T_3 is also involved in the regulation of energy balance by regulating body weight and basal and adaptative thermogenesis. Research is currently underway to identify the TR isoform responsible for each specific action of T_3, to enable and facilitate the development of selective thyromimetic therapeutic agents. The use of genetically modified mice for TRα, TRß, 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 TRß may help to identify

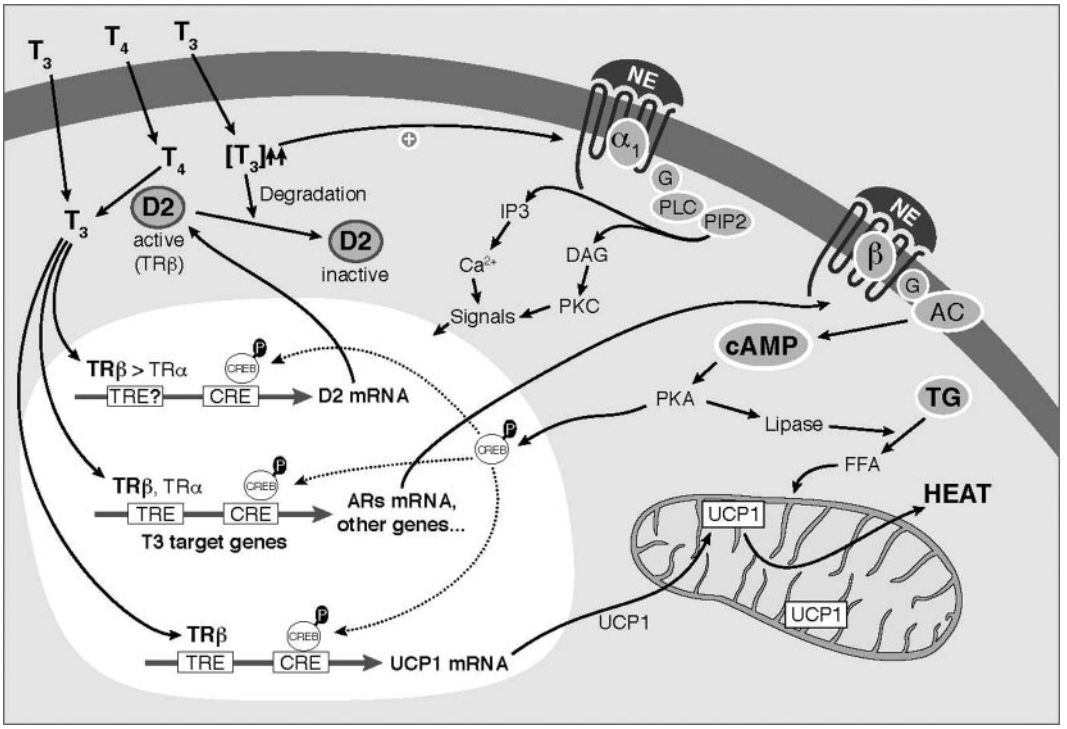

FIG. 5. Role of TRβ and TRα isoforms in brown adipocyte thermogenesis. Binding of NE to the β-adrenergic receptors activates adenylate cyclase (AC), increasing cAMP levels. This event activates lipolysis and the expression of cAMP-dependent genes, namely, UCP1, D2, and proteins involved in adrenergic responses. Intracellular T_3 increases through D2 activation, and there is a synergism between T_3 and cAMP actions. T_3 increases cAMP, through both TR isoforms. T_3, via TRβ1, increases UCP1 expression and D2 activity and expression, although a TRα effect on D2 expression is also present. High doses of T_3 change the pattern of D2 adrenergic stimulation increasing α1-adrenergic pathways. High T_3 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-bisphophate.

selective effects of T_3 mediated predominantly by one of the TR isoforms (43).

In the present study, we examine the effect of T_3 action on TRα1 and TRß1 in the adrenergic stimulation of UCP1 mRNA and D2 activity and mRNA, using specific agonists of the TRα1 and TRß1 isoforms. The T_3 effect on both genes is mediated preferentially via the TRß isoform, although the TRα isoform might participate in the regulation of D2 mRNA.

The adrenergic stimulation of UCP1 is clearly mediated by the TRß isoform as shown by using GC-1, and GC-1 is as effective as T_3. This agrees with previous work using injections of GC-1 in hypothyroid mice, describing that the T_3 effect on UCP1 occurs via TRß (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 T_3 + 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 TRß PV mutant mice, indicating the need of the TRß 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).

T_3 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 T_3 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 T_3 in BAT, especially under adrenergic stimulation, providing the T_3 required for thermogenesis. D2 is frequently used as a marker of thermogenesis and for the presence of BAT, because D2 is highly increased under adrenergic stimulation together with the up-regulation of UCP1. In rat brown adipocytes, the adrenergic stimulation of D2 expression requires T_3 (21, 35). The specific TR isoforms involved in the regulation of D2 activity and mRNA have not been previously studied. Our data indicate that TRß is the main pathway for the activation of the D2 adrenergic stimulation by T_3. However, we find several important differences between the effect of T_3 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 TRß, because the functional selectivity of this compound is about 10-fold (31). Second, the dose-response curves for GC-1 and T_3 are shaped differently: GC-1 displays a standard sigmoidal curve, whereas the T_3 curve is bell shaped, making it difficult to assess the dose dependence of T_3.

Another main difference is the inhibition of D2 activity by high T_3 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 T_3 with a maximum at 1 nM, and at higher doses, Triac inhibited D2 activity. The inhibition found using high T_3 doses might be a regulatory pathway down-regulating T_3 production by D2, against a T_3 excess. In hyperthyroidism, D2 is inhibited (47), and during chronic cold exposure, D2 activity decreases after some days, indicating that high T_3 concentrations in BAT switch off the mechanisms of T_3 production, via D2. T_3 concentrations in BAT are rather high (5–8 nM) in control rats and after 48-h cold exposure increase up to 25 nM T_3, as measured by RIA in BAT (48). The T_3 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 T_3 (49). Using 10 and 50 nM T_3, the T_3 concentrations in our cells are 9 and 45 nM T_3, as measured by RIA (see *Materials and Methods*). Therefore, using high T_3 doses, the T_3 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 T_3 on D2 stability. D2 is inactivated by the proteasome; rT_3 reduces D2 activity by accelerating its degradation (50). The effect of MG132 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 catalytical activity and dimerization (53). It has not been described that T_3 could regulate the proteasome activity or increase D2 degradation, as this process was always associated to degradation by the sustrate, rT_3, but it is evident that high T_3 doses increase D2 degradation as shown using the proteasome inhibitor MG132 and measuring D2 half-life. The TRß1 isoform could be participating in the stabilization of D2.

Another possibility is that T_3 induces changes in the adrenergic pathways used to stimulate D2 activity. In our culture, the adrenergic stimulation of D2 is mostly ß3-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 T_3 production. The changes we observed with high T_3 doses point in the same direction: an increase in the α1-adrenergic pathway. TRß 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 T_3 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 TRß agonists, used to control cholesterol in humans, might be useful tools to increase energy expenditure and facultative thermogenesis in humans (27). Other TRß 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 TRß analogs. GC-1 by increasing UCP1 and D2 activity and its stability could help to maintain an adequate T_3 production in BAT and to prolong the thermogenic effects of T_3, diminished in obesity, as well as in the low T_3 syndrome induced during caloric restriction and fasting, situations in which thermogenesis is diminished.

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References

1. **Cannon B, Nedergaard J** 2004 Brown adipose tissue: function and physiological significance. Physiol Rev 84:277–359
2. **Feldmann HM, Golozoubova V, Cannon B, Nedergaard J** 2009 UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. Cell Metab 9:203–209
3. **van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, Schrauwen P, Teule GJ** 2009 Cold-activated brown adipose tissue in healthy men. N Engl J Med 360:1500–1508
4. **Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto NJ, Enerbäck S, Nuutila P** 2009 Functional brown adipose tissue in healthy adults. N Engl J Med 360:1518–1525
5. **Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng YH, Doria A, Kolodny GM, Kahn CR** 2009 Identification and importance of brown adipose tissue in adult humans. N Engl J Med 360:1509–1517
6. **Jacobsson A, Nedergaard J, Cannon B** 1986 α- And β-adrenergic control of thermogenin mRNA expression in brown adipose tissue. Biosci Rep 6:621–631
7. **Rehnmark S, Néchad M, Herron D, Cannon B, Nedergaard J** 1990 α- And β-adrenergic induction of the expression of the uncoupling protein thermogenin in brown adipocytes differentiated in culture. J Biol Chem 265:16464–16471
8. **Bianco AC, Silva JE** 1987 Intracellular conversion of thyroxine to triiodothyronine is required for the optimal thermogenic function of brown adipose tissue. J Clin Invest 79:295–300
9. **Silva JE, Matthews PS** 1988 Full expression of uncoupling protein gene requires the concurrence of norepinephrine and triiodothyronine. Mol Endocrinol 2:706–713
10. **Bianco AC, Sheng XY, Silva JE** 1988 Triiodothyronine amplifies norepinephrine stimulation of uncoupling protein gene transcription by a mechanism not requiring protein synthesis. J Biol Chem 263:18168–18175
11. **Hernández A, Obregón MJ** 2000 Triiodothyronine amplifies the adrenergic stimulation of uncoupling protein expression in rat brown adipocytes. Am J Physiol Endocrinol Metab 278:E769–E777
12. **Guerra C, Roncero C, Porras A, Fernández M, Benito M** 1996 Triiodothyronine induces the transcription of the uncoupling protein gene and stabilizes its mRNA in fetal rat brown adipocyte primary cultures. J Biol Chem 271:2076–2081
13. **Rehnmark S, Bianco AC, Kieffer JD, Silva JE** 1992 Transcriptional and posttranscriptional mechanisms in uncoupling protein mRNA response to cold. Am J Physiol 262:E58–E67
14. **Rabelo R, Schifman A, Rubio A, Sheng X, Silva JE** 1995 Delineation of thyroid hormone-responsive sequences within a critical enhancer in the rat uncoupling protein gene. Endocrinology 136:1003–1013
15. **Bianco AC, Silva JE** 1987 Nuclear 3,5,3′-triiodothyronine (T3) in brown adipose tissue: receptor occupancy and sources of T3 as determined by *in vitro* techniques. Endocrinology 120:55–62
16. **Silva JE, Rabelo R** 1997 Regulation of the uncoupling protein gene expression. Eur J Endocrinol 136:251–264
17. **de Jesus LA, Carvalho SD, Ribeiro MO, Schneider M, Kim SW, Harney JW, Larsen PR, Bianco AC** 2001 The type 2 iodothyronine deiodinase is essential for adaptive thermogenesis in brown adipose tissue. J Clin Invest 108:1379–1385
18. **Christoffolete MA, Linardi CC, de Jesus L, Ebina KN, Carvalho SD,**

Ribeiro MO, Rabelo R, Curcio C, Martins L, Kimura ET, Bianco AC 2004 Mice with targeted disruption of the Dio2 gene have cold-induced overexpression of the uncoupling protein 1 gene but fail to increase brown adipose tissue lipogenesis and adaptive thermogenesis. Diabetes 53:577–584

19. **Obregon MJ** 2008 Thyroid hormones and adipocyte differentiation. Thyroid 18:18595
20. **Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR** 2002 Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. Endocr Rev 23:38–89
21. **Martinez-deMena R, Hernández A, Obregón MJ** 2002 Triiodothyronine is required for the stimulation of type II 5′-deiodinase mRNA in rat brown adipocytes. Am J Physiol Endocrinol Metab 282: E1119–E1127
22. **Yen PM** 2001 Physiological and molecular basis of thyroid hormone action. Physiol Rev 81:1097–1142
23. **Brent GA** 2000 Tissue-specific actions of thyroid hormone: insights from animal models. Rev Endocr Metab Disord 1:27–33
24. **Wu Y, Koenig RJ** 2000 Gene regulation by thyroid hormone. Trends Endocrinol Metab 11:207–211
25. **Baxter JD, Webb P, Grover G, Scanlan TS** 2004 Selective activation of thyroid hormone signaling pathways by GC-1: a new approach to controlling cholesterol and body weight. Trends Endocrinol Metab 15:154–157
26. **Hernández A, Obregón MJ** 1996 Presence and mRNA expression of T3 receptors in differentiating rat brown adipocytes. Mol Cell Endocrinol 121:37–46
27. **Scanlan TS** 2010 Sobetirome: a case history of bench-to-clinic drug discovery and development. Heart Fail Rev 15:177–182
28. **Trost SU, Swanson E, Gloss B, Wang-Iverson DB, Zhang H, Volodarsky T, Grover GJ, Baxter JD, Chiellini G, Scanlan TS, Dillmann WH** 2000 The thyroid hormone receptor-β-selective agonist GC-1 differentially affects plasma lipids and cardiac activity. Endocrinology 141:3057–3064
29. **Grover GJ, Mellström K, Ye L, Malm J, Li YL, Bladh LG, Sleph PG, Smith MA, George R, Vennström B, Mookhtiar K, Horvath R, Speelman J, Egan D, Baxter JD** 2003 Selective thyroid hormone receptor-β activation: a strategy for reduction of weight, cholesterol, and lipoprotein (a) with reduced cardiovascular liability. Proc Natl Acad Sci USA 100:10067–10072
30. **Ribeiro MO, Carvalho SD, Schultz JJ, Chiellini G, Scanlan TS, Bianco AC, Brent GA** 2001 Thyroid hormone-sympathetic interaction and adaptive thermogenesis are thyroid hormone receptor isoform-specific. J Clin Invest 108:97–105
31. **Ocasio CA, Scanlan TS** 2006 Design and characterization of a thyroid hormone receptor α (TRα)-specific agonist. ACS Chem Biol 1:585–593
32. **Néchad M, Kuusela P, Carneheim C, Björntorp P, Nedergaard J, Cannon B** 1983 Development of brown fat cells in monolayer culture. I. Morphological and biochemical distinction from white fat cells in culture. Exp Cell Res 149:105–118
33. **Samuels HH, Stanley F, Casanova J** 1979 Depletion of L-3,5,3′-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of action of thyroid hormone. Endocrinology 105:80–85
34. **Morreale de Escobar G, Pastor R, Obregon MJ, Escobar del Rey F** 1985 Effects of maternal hypothyroidism on the weight and thyroid hormone content of rat embryonic tissues. Endocrinology 117:1890–1900
35. **Hernández A, Obregón MJ** 1996 T3 potentiates the adrenergic stimulation of type II 5′-deiodinase in cultured rat brown adipocytes. Am J Physiol 271:E15–E23
36. **Obregón MJ, Ruiz de Oña C, Hernandez A, Calvo R, Escobar del Rey F, Morreale de Escobar G** 1989 Thyroid hormones and 5′-deiodinase in rat brown adipose tissue during fetal life. Am J Physiol 257:E625–E631
37. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
38. **Bouillaud F, Weissenbach J, Ricquier D** 1986 Complete cDNA-derived amino acid sequence of rat brown fat uncoupling protein. J Biol Chem 261:1487–1490
39. **Hernández A, Obregón MJ** 1995 Presence of growth factors-induced type III iodothyronine 5-deiodinase in cultured rat brown adipocytes. Endocrinology 136:4543–4550
40. **Snedecor GW, Cochran WG** 1980 Statistical methods. 7th ed. Ames, IA: Iowa State University Press
41. **Martinez-deMena R, Obregón MJ** 2005 Insulin increases the adrenergic stimulation of 5′ deiodinase activity and mRNA expression in rat brown adipocytes; role of MAPK and PI3K. J Mol Endocrinol 34:139–151
42. **Forrest D, Vennström B** 2000 Functions of thyroid hormone receptors in mice. Thyroid 10:41–52
43. **Baxter JD, Webb P** 2009 Thyroid hormone mimetics: potential applications in atherosclerosis, obesity and type 2 diabetes. Nat Rev Drug Discov 8:308–320
44. **Ribeiro MO, Bianco SD, Kaneshige M, Schultz JJ, Cheng SY, Bianco AC, Brent GA** 2010 Expression of uncoupling protein 1 in mouse brown adipose tissue is thyroid hormone receptor-β isoform specific and required for adaptive thermogenesis. Endocrinology 151:432–440
45. **Golozoubova V, Gullberg H, Matthias A, Cannon B, Vennström B, Nedergaard J** 2004 Depressed thermogenesis but competent brown adipose tissue recruitment in mice devoid of all hormone-binding thyroid hormone receptors. Mol Endocrinol 18:384–401
46. **Medina-Gomez G, Hernàndez A, Calvo RM, Martin E, Obregón MJ** 2003 Potent thermogenic action of triiodothyroacetic acid in brown adipocytes. Cell Mol Life Sci 60:1957–1967
47. **Silva JE** 2006 Thermogenic mechanisms and their hormonal regulation. Physiol Rev 86:435–464
48. **Medina-Gomez G, Calvo RM, Obregon MJ** 2008 Thermogenic effect of triiodothyroacetic acid at low doses in rat adipose tissue without adverse side effects in the thyroid axis. Am J Physiol Endocrinol Metab 294:E688–E697
49. **Hernandez A, Garcia B, Obregon MJ** 2007 Gene expression from the imprinted Dio3 locus is associated with cell proliferation of cultured brown adipocytes. Endocrinology 148:3968–3976
50. **Steinsapir J, Harney J, Larsen PR** 1998 Type 2 iodothyronine deiodinase in rat pituitary tumor cells is inactivated in proteasomes. J Clin Invest 102:1895–1899
51. **Gereben B, Goncalves C, Harney JW, Larsen PR, Bianco AC** 2000 Selective proteolysis of human type 2 deiodinase: a novel ubiquitin-proteasomal mediated mechanism for regulation of hormone activation. Mol Endocrinol 14:1697–1708
52. **Curcio-Morelli C, Zavacki AM, Christofollete M, Gereben B, de Freitas BC, Harney JW, Li Z, Wu G, Bianco AC** 2003 Deubiquitination of type 2 iodothyronine deiodinase by von Hippel-Lindau protein-interacting deubiquitinating enzymes regulates thyroid hormone activation. J Clin Invest 112:189–196
53. **Sagar GD, Gereben B, Callebaut I, Mornon JP, Zeöld A, da Silva WS, Luongo C, Dentice M, Tente SM, Freitas BC, Harney JW, Zavacki AM, Bianco AC** 2007 Ubiquitination-induced conformational change within the deiodinase dimer is a switch regulating enzyme activity. Mol Cell Biol 27:4774–4783
54. **Silva JE, Larsen PR** 1985 Potential of brown adipose tissue type II thyroxine 5′-deiodinase as a local and systemic source of triiodothyronine in rats. J Clin Invest 76:2296–2305
55. **Silva JE, Bianco SD** 2008 Thyroid-adrenergic interactions: physiological and clinical implications. Thyroid 18:157–165
56. **Amorim BS, Ueta CB, Freitas BC, Nassif RJ, Gouveia CH, Christoffolete MA, Moriscot AS, Lancelloti CL, Llimona F, Barbeiro HV, de Souza HP, Catanozi S, Passarelli M, Aoki MS, Bianco AC, Ribeiro MO** 2009 A TRβ-selective agonist confers resistance to diet-induced obesity. J Endocrinol 203:291–299
57. **Castillo M, Freitas BC, Rosene ML, Drigo RA, Grozovsky R, Maciel RM, Patti ME, Ribeiro MO, Bianco AC** 2010 Impaired metabolic effects of a thyroid hormone receptor β-selective agonist in a mouse model of diet-induced obesity. Thyroid 20:545–553