CMLS, Cell. Mol. Life Sci. 60 (2003) 1957–1967
1420-682X/03/091957-11
DOI 10.1007/s00018-003-3158-9

CMLS Cellular and Molecular Life Sciences

Research Article

Potent thermogenic action of triiodothyroacetic acid in brown adipocytes

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Received 29 April 2003; received after revision 10 June 2003; accepted 16 June 2003

Abstract. Triiodothyroacetic acid (TRIAC) is a triiodothyronine (T3) metabolite with high affinity for T3 nuclear receptors. We compared the thermogenic action of TRIAC versus T3 in brown adipocytes, by studying target genes known to mediate thermogenic action: uncoupling protein 1 (UCP-1), a marker of brown adipocytes, and type II-5′deiodinase (D2), which provides the T3 required for thermogenesis. TRIAC is 10–50 times more potent than T3 at increasing the adrenergic induction of UCP-1 mRNA and D2 activities. TRIAC action on UCP-1 is exerted at the transcriptional level. In the presence of an adrenergic stimulus, TRIAC is also more potent than T3, inducing lipoprotein lipase mRNA and 5 deiodinase (D3) activity and mRNA. Maximal effects occur at very low concentrations (0.2 nM). The greater potency of TRIAC is not due to preferential cellular or nuclear uptake. Therefore, TRIAC is a potent thermogenic agent that might increase energy expenditure and regulate T3 production in brown adipocytes.

Key words. Thermogenesis; T3; UCP-1; LPL; deiodinase.

Triiodothyroacetic acid (TRIAC, also known as Tiratricol), is a naturally occurring metabolite of triiodothyronine (T3), derived by deamination and oxidative decarboxylation of the alanine chain [1]. In humans, the amount of TRIAC produced by the liver and other tissues accounts for about 14% of T3 metabolism [2]. This production is increased in some situations, such as fasting [3, 4].

Serum TRIAC concentrations in humans are very low (42–140 pM) [2, 5] or undetectable [6], and the serum free fraction is also lower than that of T3, because TRIAC is more firmly bound to plasma proteins than T3, especially to transthyretin [7]. Plasma clearance of TRIAC is also very rapid [6]: TRIAC has a faster metabolic clearance rate (MCR) and shorter half-life than T3 [6]. This is due to its rapid hepatic metabolism, via formation of sulfates and glucuronides, especially in humans [8, 9]. This short half-life has led to the use of very high doses of TRIAC (0.4 to 1–2 mg TRIAC/day), 200 times higher than those considered physiological for T3.

TRIAC has similar or higher affinity than T3 for the thyroid hormone nuclear receptors in different cells [10] and in rat brown adipocytes [11]. TRIAC has greater affinity than T3 for the β1 T3 receptor isoform (TR-β1) (345%) and similar affinity for the α1 isoform (TR-α1) [10]. TRIAC has been used to suppress thyroid-stimulating hormone (TSH) secretion in patients with thyroid hormone resistance, inappropriate TSH secretion or thyroid carcinoma [12, 13] due to its low cardiac effects compared to T3. TRIAC thyromimetic actions include decreases in cholesterol or lipids and increases in several hepatic T3-dependent genes [14–18].

Multiple attempts aimed to demonstrate a role for TRIAC in increasing oxygen consumption or energy expenditure have been contradictory or inconclusive. Early experi-

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ments reported that TRIAC increased oxygen consumption [19] and suggested its potential use to increase energy expenditure [20], but other studies reported negative results [15, 21]. The exogenous administration of TRIAC [22] induced persistent hypothyroidisms due to TSH suppression.

Brown adipose tissue (BAT) is specialized in the production of heat in facultative thermogenesis. This function is accomplished at the cellular level by the uncoupling protein (UCP-1), a mitochondrial protein that uncouples the oxidative phosphorylation, dissipating as heat the energy that otherwise is converted to ATP. Cold exposure and diet increases BAT activity and UCP-1 expression, via the norepinephrine (NE) released from the sympathetic nervous system. The maintenance of energy balance is also related to the level of BAT activity, which is low in animal models of obesity [23].

Euthyroid status is essential for the complete thermogenic activity of BAT, and for the full expression of UCP-1 [24, 25]. Indeed, UCP-1 expression and its response to cold are low in hypothyroid rats and newborns and its responses to cold are lower than in control rats [26, 27]. Thyroid hormone administration restores UCP-1 mRNA and its response to cold [26, 28]. T3 increases the transcription rate, stabilizes UCP-1 mRNA transcripts and potentiates the effect of NE [24, 29, 30].

BAT T3 concentrations are very high, especially under cold exposure and during fetal life [31, 32]. The production of T3 in BAT is accomplished by the type II 5′ deiodinase activity (D2) that is activated in response to cold [33]. Most of the T3 in BAT is produced from local deiodination of T4, via D2 [32], and this local T3 production is required for the high saturation of the nuclear T3 receptors in response to cold exposure [34]. Recent experiments using mice with targeted disruption of the D2 gene have shown that D2 is essential for thermal homeostasis and the synergism between sympathetic and thyroid hormone responses [35]. Using brown adipocytes in primary culture, we found that T3 is required for and amplifies the adrenergic stimulation of UCP-1 mRNA and D2 activity and mRNA in brown adipocytes differentiated in culture [36–38]. Therefore, T3 acts as a positive signal for D2 regulating its own production in brown adipocytes.

The aim of this work was to compare the effects of T3 and TRIAC on the adrenergically mediated stimulation of UCP-1 mRNA and D2 activity in brown adipocytes. We also compared their effects on lipoproteinlipase (LPL) mRNA expression, a gene activated in BAT by adrenergic stimulus [39] and that provides free fatty acids required for thermogenesis. A further comparison was made on the induction of 5 deiodinase (D3) activity and mRNA, an imprinted gene involved in growth [40] and in the homeostasis of T3; D3 is regulated by growth factors and thyroid hormones in brown adipocytes [41, 42]. Our results disclose a superior effect of TRIAC in the regulation of these genes involved in thermoregulation, an effect particularly important at very low concentrations.

Materials and methods

Materials

All chemicals were reagent grade or molecular biology grade. Collagenase, dithiothreitol (DTT), 3-N-morpholino-propanesulfonic acid (MOPS), 6-N-propyl-2-thiouracil (PTU), NE and DEAE-Dextran were from Sigma (St. Louis, Mo.). T3, thyroxine (T4), TRIAC and 3, 5-diiodothyroacetic acid (Diac) were from Henning GMBH (Berlin, Germany). [3, 5-^{125}I]-T3 (inner ring labeled T3) was first provided by Dr. Rokos (Henning, Berlin, Germany) and later obtained from Dr. Thoma, (Formula, Berlin, Germany). BRL 37344 was from SKB Pharmaceuticals (Worthing, UK) and ZD7114 from Zeneca Pharmaceuticals (Macclesfield, UK).

Cultures of brown adipocytes

Precursor cells were obtained from the interscapular BAT of 20-day-old rats (Sprague-Dawley). They were isolated according to the method described by Néchad et al. [43] with modifications [36], using collagenase digestion (0.2%) in DMEM + 1.5% bovine serum albumin (BSA) at 37°C, and filtration through 250-µm silk filters. Mature cells were allowed to float and the infranatant was filtered through 25-µm silk filters and centrifuged. Precursor cells were seeded in 25-cm^2 culture flasks to get 1500–2000 cells/cm^2 on day 1, and grown in culture medium [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 medium was changed on day 1 and every second day thereafter, until the experiment was performed. Precursor cells actively proliferate under these conditions and reached confluence at the 4th–5th day after seeding (40–60,000 cells/cm^2) prior to differentiating into mature brown adipocytes. Studies were performed in differentiated brown adipocytes (day 8). T3 and TRIAC were diluted in serum depleted of thyroid hormones.

Hypothyroid serum was obtained as described elsewhere [44] and depleted serum was obtained by treatment of hypothyroid serum with charcoal overnight (20 mg charcoal/ml serum). This treatment further depleted serum of the remaining thyroid hormones. Hypothyroid or depleted serum contained about 10% or less of the original amount of thyroid hormones, as assessed by RIA [45]. Thyroid hormone concentrations were 77 nM T4 and 1.3 nM T3 in NCS and decreased to 2.2 nM T4 and 0.13 nM T3 in hypothyroid serum and to 0.8 nM T4 and 0.06 nM T3 in depleted serum. These were the concentrations before the final dilution in culture medium.

Free T3 and TRIAC concentrations were measured by ultrafiltration as described previously [46]. Free T3 was 5% and 19% of total T3 when using 10% and 2% serum, respectively. Free TRIAC concentrations were lower: 1.4% and 8.1% of the total TRIAC when using 10% and 2% serum, respectively. As an example, the free concentrations were 190 pM T3 and 81 pM TRIAC when using 1 nM T3 or 1 nM TRIAC in 2% serum, respectively.

Synthesis of labeled iodothyronines

The high-specific-activity [^{125}I]-T4 and [^{125}I]-TRIAC used were obtained in our laboratory (specific activity > 3000 µCi/µg) using chloramine T and T3 or Diac as substrates, respectively [31].

Determination of D2 and D3 activities

D2 and D3 activities were determined as previously described [37, 47]. Briefly, cells were scraped, collected in buffer (0.32 sucrose, 10 mM Hepes, 10 mM DTT, pH 7.0) and homogenized. D2 activities were determined in cell homogenates, as described elsewhere [31] with modifications [37] using as final concentrations: 50,000–100,000 cpm [^{125}I]-T4, 2 nM T4, 1 µM T3, 50 mM DTT, 1 mM PTU, 80–100 µg protein/100 µl, for 1 h at 37 °C. Before each assay, [^{125}I]-T4 was purified by paper electrophoresis to separate iodide. Detection limits were 2–5 fmol/h per milligram of protein. D3 activity was determined measuring the iodide released after incubation of cell homogenates with 40,000 cpm of inner-ring-labeled T3, [3, 5-^{125}I]-T3 (80 µCi/µg) at 37 °C for 1 h. Final assay conditions were: 25 nM [3, 5-^{125}I]-T3, 20 mM DTT, 80–100 µg protein/100 µl, 1 mM PTU, pH 7.5 [47]. Results for D3 were expressed in pmol/h per milligram protein; detection limits were 0.075–0.100 pmol/h per milligram protein. The amount of iodide in the blanks was less than 0.5–1% of the total radioactivity. Enzymatic activities were always determined in duplicates or triplicates, using two to three culture flasks per treatment.

RNA preparation and Northern blot analysis

Total cellular RNA was extracted with guanidinium-HCl and ethanol precipitation as described elsewhere [36]. The recovery was 60–90 µg total RNA/25 cm^2 flask (approx. 4–5 × 10^6 cells). Total RNA (15 µg) was denatured and electrophoresed on a 2.2 M formaldehyde/1% agarose gel in 1 × MOPS buffer and transferred to nylon membranes. Total rRNA was stained using methylene blue. The corresponding cDNAs were used as template for [α-^{32}P]-dCTP-labeled probes using random primers (>10^8 cpm/µg DNA). Filters were hybridized for 20 h at 50 °C [40% formamide, 5 × SSC (0.75 M saline, 0.075 M sodium citrate), 2 × Denhardt's, 0.1% SDS], washed four times in 2 × SSC/0.2% SDS at room temperature for 15 min and then twice in 0.1 × SSC/0.2% SDS at 65 °C for 20 min. Autoradiograms were obtained from the filters and quantified by laser computer-assisted densitometry (Molecular Dynamics, Sunnyvale, Calif.). The membranes were hybridized with probes for cyclophilin to correct for loading differences between lanes. Total rRNA staining was also used as control when the Cy/rRNA ratio was different from 1.

The following probes were used: a 1200-bp fragment of a UCP-1 cDNA clone (provided by Dr. Ricquier [48]); a 1480-bp fragment of the LPL cDNA clone (provided by Dr. Kirchgessner); a 1562-bp fragment of a D3 cDNA clone (provided by Dr. St. Germain).

Transfection reporter assays

Transient transfection studies were done in brown adipocytes in primary culture using DEAE-dextran, according to standard methods previously described [49]. Brown adipocytes were transfected when they reached 80% confluence (approx. 4–5 day of culture). The medium was withdrawn and the cells washed twice with culture medium at 37 °C.

A rat genomic library (Clontech, Palo Alto, Calif.) was screened using the UCP-1 cDNA as probe and several UCP-1 promoter restriction fragments (from –4.5 kb to –1 kb) were subcloned in the luciferase reporter vector pXp2 [50]. One of the constructs used (TRE-Bstx-pXp2) contained the TRE enhancer region (–2493/–2253), which was amplified by PCR using the appropriate primers, linked to the basal UCP-1 promoter (Bstx I, –157/+114), which contains a CRE element. From all the constructs tested, the best results were found using either the TRE-Bstx-pXp2 construct or a longer 2.5-pXp2 (–2491/+114) construct of the UCP-1 promoter.

Cells were cotransfected with 3 µg of these plasmids (TRE-Bstx-pXp2 or 2.5-pXp2) and 0.5 µg of a CMV-β-galactosidase control vector, using 1 mg/ml of DEAE-dextran in Hepes-DMEM. After 2 h of incubation at 37 °C, the cells were washed with medium DMEM+ Hepes+antibiotic at 37 °C, and once with medium + 10% NCS. The cells were then incubated with T3 or TRIAC for 24 h and 3 µM NE for 6 h at 37 °C and harvested to measure luciferase and β-galactosidase activities. Luciferase activities were normalized to those of β-galactosidase.

Uptake studies

T3 and TRIAC uptake studies were performed as described by Everts et al. [51], with minor modifications. Cells were cultured as described above. At day 7, the cells were incubated for 24 h with a lower percentage of serum (2%) in the culture medium. At day 8, the cells were preincubated with medium containing 0.1% BSA, for 30 min at 37 °C. T3 or TRIAC uptake was then measured using 250–350,000 cpm [^{125}I]-T3 or [^{125}I]-TRIAC (approx. 0.4 nM) in 0.1% BSA and the culture medium and cells were collected at 20 and 40 min, 2.5 and 6 h and nuclei isolated [11]. For each time point, flasks with an ex-

cess of TRIAC or T3 (10 µM) were run. Cells were centrifuged at 3000 rpm for 10 min using STM (0.25 M sucrose, 20 mM Tris-HCl, 1.1 mM $MgCl_2$)+10% Triton X-100. Nuclei were obtained from the cells homogenized and centrifuged at 1000 rpm in STM+Triton X-100 at 0.5%. The integrity of the radioactive products was verified by paper chromatography.

Statistics

Mean values (± SE) are given. All the experiments were done at least twice, but many figures are the mean of six to eight different experiments (see details in each figure). When not visible in the figures, SEs were smaller than the symbols. One-way analysis of variance was applied after ensuring homogeneity of variance by Bartlett's test. Statistically significant differences between mean values were then identified by the least significant difference (LSD) method. All calculations were performed as described by Snedecor and Cochran [47].

Results

TRIAC at low concentrations enhances the adrenergic stimulation of UCP-1 mRNA

UCP-1 is considered the best marker for the thermogenic status of BAT. T3 has previously been shown to be required for the adrenergic stimulation of UCP-1 mRNA in rats and in brown adipocytes [29, 36]. As already described, fig. 1 confirms that, in the absence of T3, NE does not increase UCP-1 mRNA in cultured rat brown adipocytes [36]. In the presence of both T3 and TRIAC, NE induced UCP-1 mRNA levels (up to 40-fold). TRIAC, already effective at 0.025 nM, is more potent than T3 at increasing the adrenergic stimulation of UCP-1 mRNA. At higher concentrations (2–20 nM, right panel), the actions of T3 and TRIAC on UCP-1 mRNA expression levels are similar. TRIAC stabilizes UCP-1 mRNA (results not shown) in a similar way as T3 [36].

To investigate whether this observed effect is produced via the β3 adrenergic receptors, we compared the effect of TRIAC and T3 on UCP-1 mRNA when stimulated with NE or other β3 adrenergic agents, such as BRL-37344 or ZD-7114. In fig. 2, we show that TRIAC is again more potent than T3 when using other β3 adrenoagonists, BRL-37344 being the most potent β3 agonist tested.

To verify whether this potency of TRIAC was apparent at the transcriptional level, transient transfections were done in primary cultures of rat brown adipocytes using a reporter construct (TRE-Bstx-pXp2). Fig. 3 shows the effect of several treatments on the reporter activity. NE has a low effect that increases in the presence of T3 and TRIAC. The lowest TRIAC dose (0.02 nM) was more effective than the lowest T3 dose (0.2 nM). This was confirmed using a larger reporter construct (2.5-pXp2) (fig. 3). These data confirm that the TRIAC effect is also transcriptional, already reaching a maximal induction using the lowest dose of TRIAC and NE.

TRIAC and the adrenergic stimulation of D2 activity

In BAT, D2 accounts for the local production of T3. This is required for the induction of UCP-1. We have previously shown that T3 is also necessary for the adrenergic induction of D2 activity and mRNA in brown adipocytes [37, 38]. To compare the effects of TRIAC and T3 on the adrenergic stimulation of D2 activity, cells were exposed for 24 h to increasing concentrations of T3 or TRIAC (from 0.025 to 50 nM) and to NE (3 µM) during the last 12–14 h, the time at which a maximum is obtained [37]. All the experiments were done during the differentiation period (day 8 of culture). Fig. 4A shows that TRIAC is more potent than T3 in promoting the adrenergic stimulation of D2 activity. TRIAC is already active at a dose of 0.025 nM, reaching a maximum at 1 nM, while T3 requires higher concentrations to enhance D2 activity. A

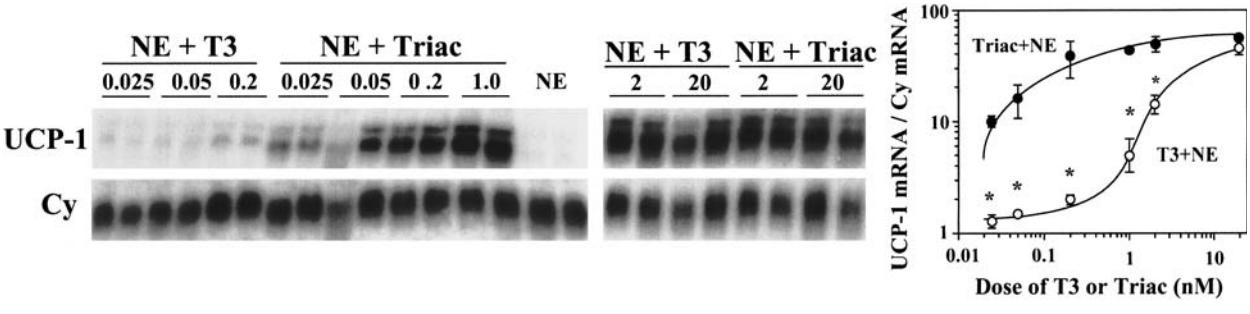

Figure 1. Effect of TRIAC and T3 on the adrenergic stimulation of UCP-1 mRNA in brown adipocytes. Primary cultures of brown adipocytes (day 8) were treated with T3 (0.025, 0.05 and 0.2 nM) or TRIAC (0.025, 0.05, 0.2 and 1 nM) for 24 h, using 2% depleted serum. NE (3 µM) was added to all the cells during the last 12 h (left). A representative experiment is also shown using 2 and 20 nM TRIAC or T3 under the same conditions (right). Hybridization with UCP-1, cyclophilin and the densitometric analysis (means ± SE) with the UCP-1/Cy ratio are shown from two to four different flasks from four different experiments. Both bands of UCP-1 were used for the densitometric analysis. * $p < 0.05$, vs TRIAC+NE.

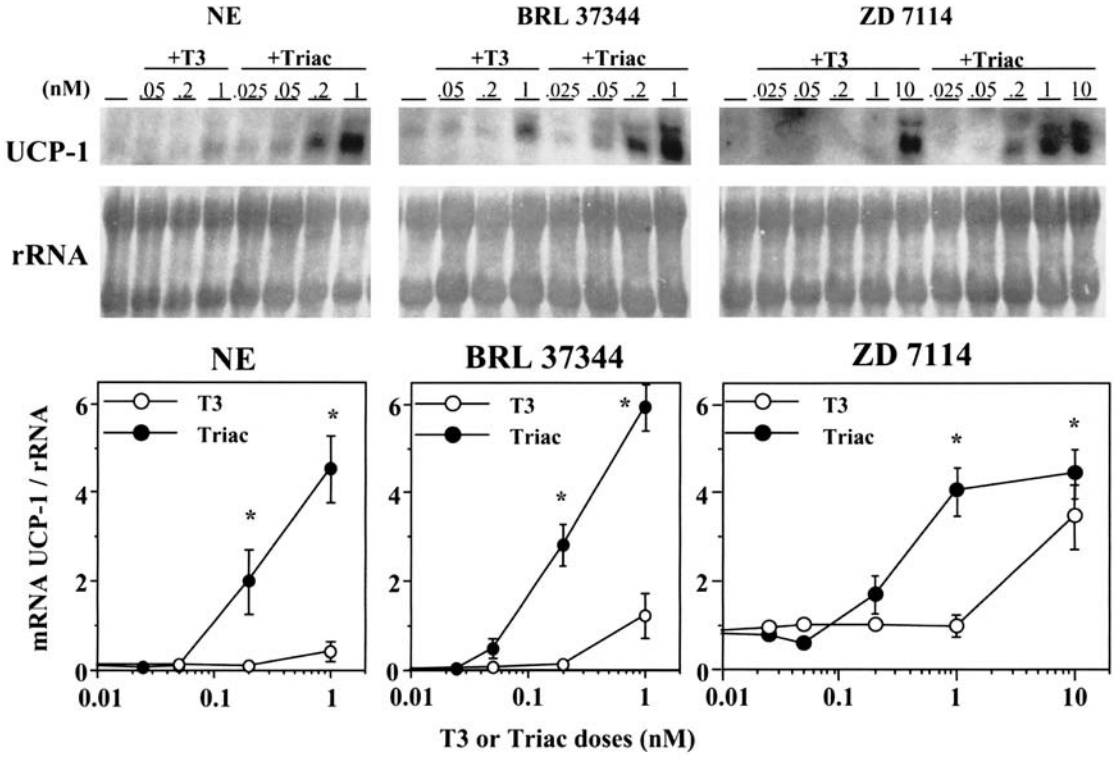

Figure 2. Effect of TRIAC and T3 on NE and the β3 adrenoagonists (BRL 37344 and ZD 7114) on the stimulation of UCP-1 mRNA in brown adipocytes. Primary cultures of brown adipocytes were treated with T3 (0.05, 0.2 and 1 nM) or TRIAC (0.025, 0.05, 0.2 and 1 nM) for 24 h, using 2% depleted serum. NE, BRL 37344 or ZD 7114 (all at 3 µM) were added during the last 6 h. Hybridization with UCP-1, rRNA staining and the densitometric analysis with the UCP-1/ rRNA ratio are shown. Means ± SE from two to three different flasks from two different experiments. * $p < 0.05$ vs T3.

dose of 0.2 nM TRIAC produced a similar effect to 5 nM T3. Higher concentrations of TRIAC (5–50 nM) had a paradoxic effect, decreasing D2 activity, an inhibition similar to that observed using high T3 concentrations (>50 nM) [38]. Hence, TRIAC is at least 25 times more potent than T3 in inducing the adrenergic stimulation of D2 activity.

The effect at low TRIAC concentrations (0.2 nM) was also observed when using the β3 adrenergic agent BRL37344 (fig. 4B), although NE was more potent than BRL when using 0.2 nM TRIAC, both reaching a maximum at 1 nM TRIAC.

TRIAC increases D3 activity and mRNA gene expression

Another gene regulated by T3 and NE in our cultures is the 5 deiodinase, D3 [47]. This enzyme is the main degradative pathway for T3, and is stimulated by growth factors and thyroid hormones. We explored if T3 and TRIAC increase D3 activity and mRNA both in the absence and presence of NE. Preliminary experiments showed that both T3 and TRIAC increased D3 activity. We confirmed that a lower TRIAC dose (0.2 nM) had a greater effect on D3 activity than did 2 nM T3 (not shown).

A dose-response curve for T3 and TRIAC revealed that a small dose (0.05 nM) of TRIAC reached a maximum effect (fig. 5A) while the effect of T3 on D3 activity was only observed at a dose 20 times higher than TRIAC (1 nM). Higher D3 activities were found when T3 or TRIAC were used in the presence of NE (fig. 5B). Cells exposed to 0.05 nM TRIAC+NE already had a similar effect as 1 nM T3+NE on D3 activity. Maximal activities were observed at 5–10 nM of T3+NE and TRIAC+NE. These changes in D3 activity were associated with parallel changes in D3 mRNA levels (fig. 5C). In fact, 0.5 nM TRIAC was twice as potent as 5 nM TRIAC or 10 nM T3.

Effects of TRIAC on LPL mRNA expression

We further explored whether T3 and TRIAC exerted any effect on LPL mRNA expression. LPL increases during the adrenergic stimulation of BAT [39], to recruit the lipids required for mitochondrial combustion. While T3 decreases LPL mRNA levels in brown adipocytes, LPL increases when T3 is added together with NE [52], as NE has a stimulatory effect [39]. Fig. 6A shows that TRIAC exerts the same inhibitory effect at a dose of 1 nM as 10 nM T3 and also shows that addition of NE has a stimulatory effect that prevents the inhibition of high concen-

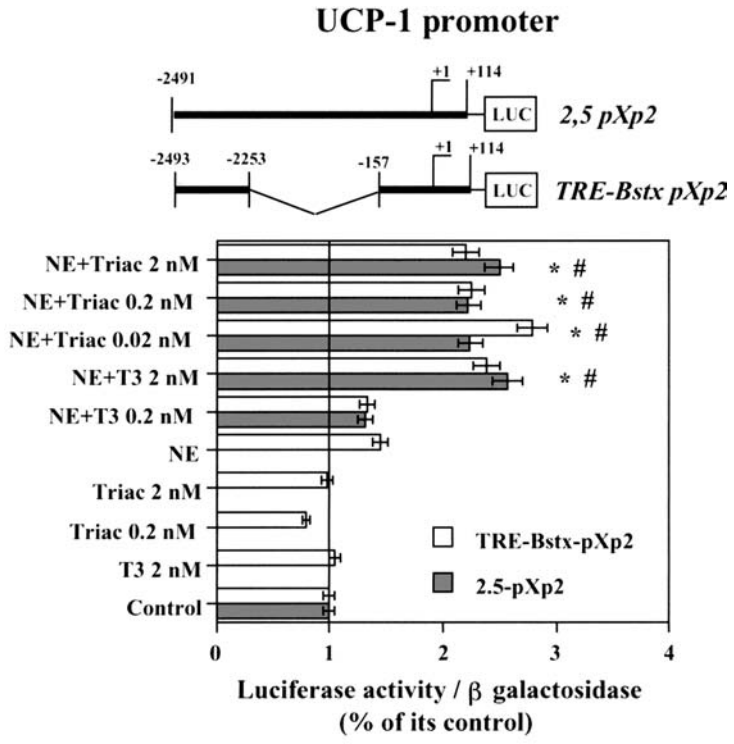

Figure 3. Effect of TRIAC, T3 and NE on the UCP-1 gene promoter using the TRE-Bstx-pXp2 and 2.5-pXp2 constructs. Primary cultures of brown adipocytes were transfected on day 5 after seeding with 3 µg of the constructs TRE-Bstx-pXp2 or 2.5-pXp2, and CMV-β-galactosidase to check for efficiency. Cells were then treated with 0.02, 0.2 or 2 nM TRIAC or T3 for 24 h, alone or together with 3 µM NE during the last 6 h. Luciferase activity was measured and corrected by β-galactosidase activity, and is expressed as percentage of their respective basal values. Data are the means ± SE (n = 6–8/point) from three to eight different experiments, using duplicates. *$p < 0.05$ vs control, $^{\#}p < 0.05$ vs NE+T3 0.2 nM.

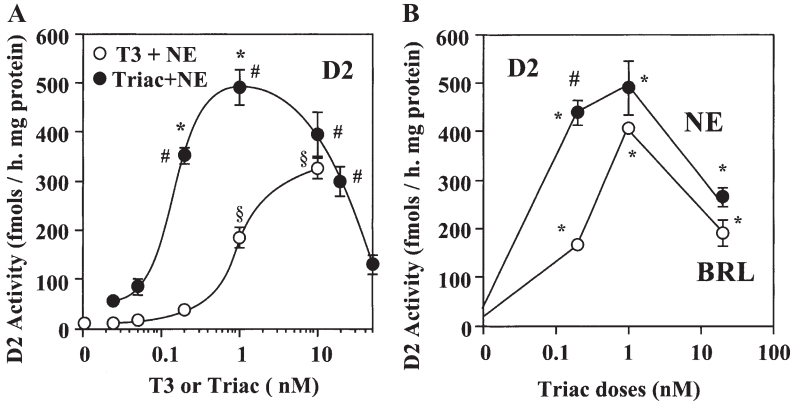

Figure 4. (*A*) Effect of increasing concentrations of TRIAC or T3 on the adrenergic stimulation of D2 activity in brown adipocytes. Primary cultures of brown adipocytes were treated with NE in the presence of T3 or TRIAC. Cells were treated during the last 24 h with 2% depleted serum and increasing amounts of T3 or TRIAC (0.025, 0.05, 0.2, 1, 10, 20 and 50 nM). NE (3 µM) was added during the last 12–14 h. Cells were collected on day 8 of culture. Data are the mean ± SE of nine different experiments. *$p < 0.05$ TRIAC vs the same dose of T3, $^{\#}p < 0.05$ vs the lowest TRIAC dose (0.025 nM), $^{\S}p < 0.05$ vs the lowest T3 dose (0.025 nM). (*B*) Effect of NE and the β3 adrenergic agonist BRL 37344, and increasing concentrations of TRIAC on the adrenergic stimulation of D2 activity in brown adipocytes. Primary cultures of brown adipocytes were treated with NE+TRIAC or BRL 37344 + TRIAC. Cells were treated during the last 22 h with 2% depleted serum and increasing amounts of TRIAC (0.2, 1 and 20 nM). NE or BRL 37344 (5 µM) were added during the last 14 h. Data are the mean ± SE of triplicate plates from two different experiments. *$p < 0.05$ vs its basal NE or BRL, $^{\#}p < 0.05$ NE vs BRL at the same TRIAC dose.

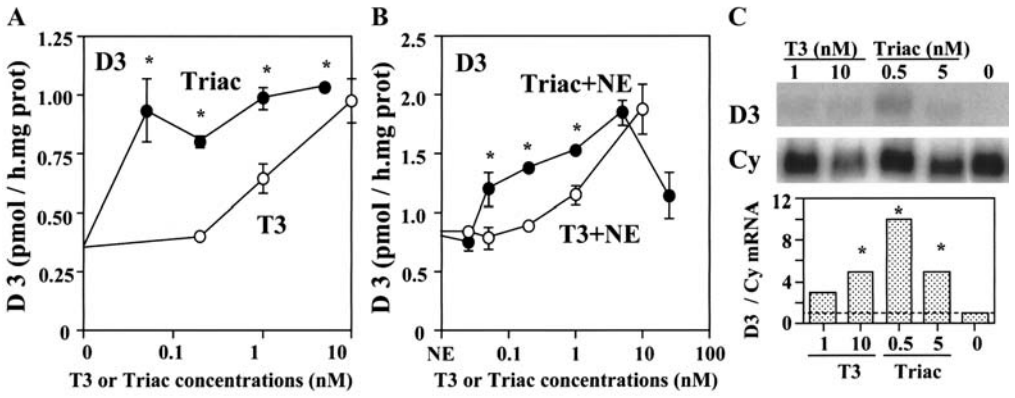

Figure 5. (*A*) Effect of increasing concentrations of TRIAC or T3 on D3 activity in brown adipocytes. Primary cultures of brown adipocytes were treated during the last 24 h with 2% depleted serum and increasing amounts of T3 (0.2, 1 and 10 nM) or TRIAC (0.05, 0.2, 1 and 5 nM). Data are the mean ± SE of two experiments. * $p < 0.05$ TRIAC vs T3. (*B*) Effect of increasing concentrations of TRIAC+NE or T3+NE on D3 activity in brown adipocytes. Primary cultures of brown adipocytes were treated during the last 24 h with 2% depleted serum and increasing amounts of T3 (0.025, 0.05, 0.2, 1 and 10 nM) or TRIAC (0.025, 0.05, 0.2, 1, 5 and 25 nM). NE (3 µM) was added during the last 9 h. Data are the mean ± SE of two experiments. * $p < 0.05$ TRIAC+NE vs T3+NE. (*C*) Effect of T3 and TRIAC on D3 mRNA in brown adipocytes. Primary cultures of brown adipocytes were treated during the last 24 h with 2% hypothyroid serum and 1 or 10 nM T3 or 0.5 or 5 nM TRIAC. Data are the mean ± SE of two experiments. * $p < 0.05$ vs Basal.

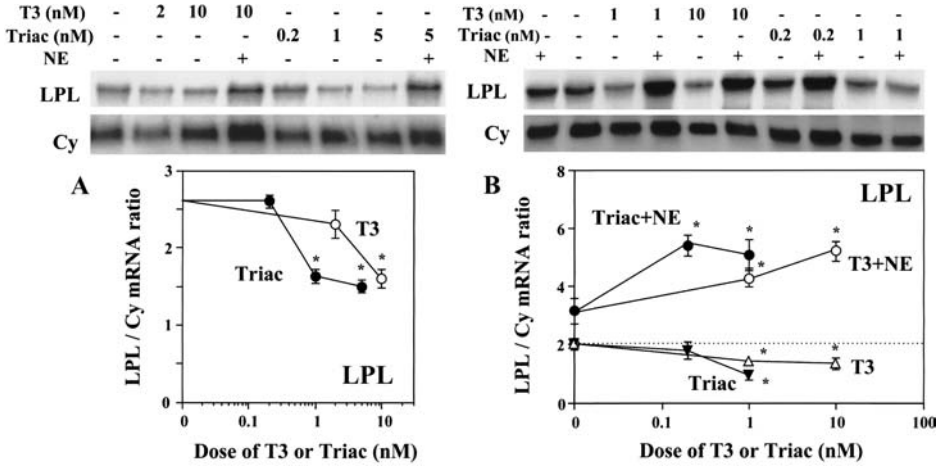

Figure 6. (*A*) Effect of TRIAC and T3 on LPL mRNA in brown adipocytes. Primary cultures of brown adipocytes were treated with 0.4, 2 or 10 nM T3 or 0.2, 1 or 5 nM TRIAC during 24 h, using 2% hypothyroid serum. NE (1 µM) was added during the last 7 h to the highest T3 or TRIAC concentrations. The hybridization with LPL, cyclophilin and the densitometric analysis with the LPL/Cy ratio are shown. Means ± SE from two different experiments. * $p < 0.05$ vs basal. (*B*) Effect of TRIAC and T3 ± NE on LPL mRNA in brown adipocytes. Primary cultures of brown adipocytes were treated with T3 (1 or 10 nM) or TRIAC (0.2 or 1 nM) for 24 h, using 5% hypothyroid serum. NE (2 µM) was added during the last 4 h. The hybridization with LPL, cyclophilin and the densitometric analysis with the LPL/Cy ratio are shown. Means ± SE from two to four different flasks from 2 different experiments. * $p < 0.05$ vs basal.

trations of T3 and TRIAC. When added together with NE (fig. 6B), TRIAC already increases LPL mRNA at concentrations of 0.2 nM, to levels higher than those observed using 10 nM T3+NE. Thus, TRIAC is 20 times more potent than T3 when added alone and 50 times more potent than T3 in the presence of NE on LPL mRNA.

TRIAC uptake studies

We next explored whether the increased potency of TRIAC may be due to a higher TRIAC uptake. Fig. 7A shows the time course of [^{125}I]-T3 and [^{125}I]-TRIAC cellular uptake, expressed as percentage of the radioactive dose used, and in the presence of an excess (10 µM) of their respective unlabeled hormones. The results show that [^{125}I]-T3 uptake was two- to fourfold higher than that of [^{125}I]-TRIAC at any time point ($p < 0.05$). Furthermore, [^{125}I]-TRIAC uptake was only 25% that of [^{125}I]-T3 at any time point, increasing up to 2.5 h and reaching a plateau thereafter. The presence of 10 µM of unlabeled TRIAC or T3 resulted in significantly lower [^{125}I]-T3 uptake, with a maximal inhibitory effect at the shortest times. These data might indicate the presence of a spe-

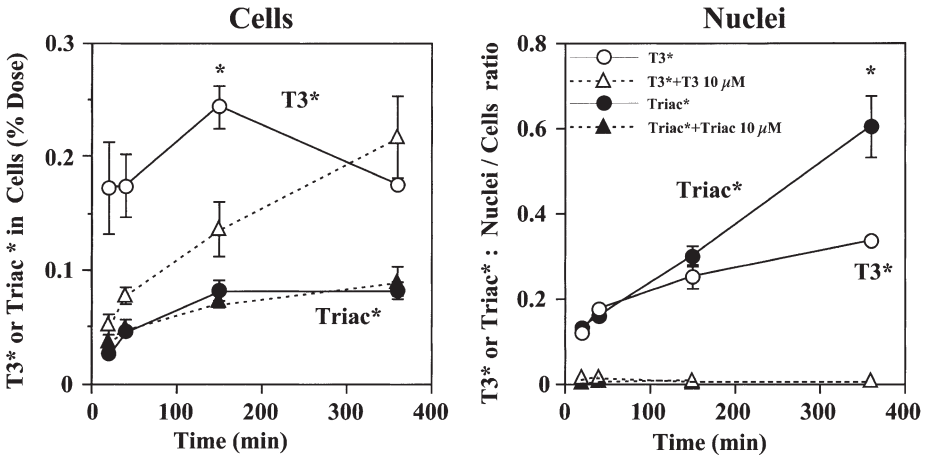

Figure 7. Cellular and nuclear uptake of ^{125}I-TRIAC and ^{125}I-T3 in cultured brown adipocytes. Primary cultures of brown adipocytes on day 7 of culture were treated with 2% depleted serum for 24 h. Cells were then treated with 5 ml of culture medium containing 0.1% BSA for 30 min, thereafter the medium was withdrawn and 2 ml of culture medium + 0.1% BSA containing 0.25 ×10^6 cpm ^{125}I-T3 or 0.35 × 10^6 cpm ^{125}I-TRIAC were added. Half of the flasks were supplemented with 10 µM T3 or TRIAC, respectively. Cells and medium were collected at 20, 40, 150 and 360 min and counted and the nuclear fraction was isolated. The cellular uptake (as % of the total dose) and nuclear to cell ratio are shown for ^{125}I-T3 and ^{125}I-TRIAC, together with the unspecific binding in cells and nuclear fraction. Means ± SE from four flasks from two different experiments. *$p < 0.05$ vs 20 min uptake in cells, and vs T3 nuclear uptake.

cific transport mechanism for T3 at short times, while [^{125}I]-T3 uptake is not displaced by 10 µM T3 at 6 h. In contrast, a high dose of TRIAC (10 µM) did not affect [^{125}I]-TRIAC uptake at any of the times studied, showing always a lower uptake than that observed for [^{125}I]-T3.

The nuclear binding of T3 and TRIAC in brown adipocytes is shown in fig. 7B. The nuclear [^{125}I]-TRIAC uptake is higher than that of [^{125}I]-T3, especially at longer incubation times (0.33 for [^{125}I]-T3 and 0.6 for [^{125}I]-TRIAC at 6 h). These data were expressed as nuclei/cell ratio because of the large difference between the cellular uptake of both hormones. The incubation with 10 µM unlabeled T3 or TRIAC reduced both nuclear uptakes at any time point, indicating that the nuclear binding of [^{125}I]-T3 and [^{125}I]-TRIAC is highly specific.

Comparison of T3 and TRIAC uptake in brown adipocytes in situ showed that the cellular TRIAC uptake was lower than that of T3, but the nuclear to cell ratio was twofold higher for TRIAC at 6 h. Thus our results show that TRIAC is more efficiently transported into the nucleus.

Discussion

Thyroid hormones play an important role in the regulation of growth, differentiation, and energy metabolism in different tissues, actions largely mediated by the nuclear T3 receptors, which modulate the expression of many genes. Although the classical thyroid hormones, T3 and T4, have been the subject of many studies, little consideration has been paid to their metabolites.

In the present study, we analyzed the biological effect of TRIAC, a physiological metabolite of T3, on several target genes involved in the thermogenic action of brown adipocytes. We clearly showed that TRIAC is more potent than T3 in facilitating the adrenergic stimulation of UCP-1, D2, D3 and LPL.

TRIAC is considered an active metabolite of T3 that binds selectively to nuclear T3 receptors, but its specific functions, different from those of T3, have not been described. These functions could be relevant when T3 concentrations are low, e.g., in the 'low T3 syndrome,' as has been suggested by increased TRIAC production during fasting [3]. TRIAC could play roles, now attributed to T3, or have specific roles in some tissues, or, alternatively, be the main effector in the activation of certain genes regulated by the TR-β1 isoform. We propose that TRIAC may have a specific role in the regulation of BAT thermogenesis that includes the facilitation of NE effects. We observed that TRIAC is active at low concentrations (10–50 times more potent than T3) in actions linked to the adrenergic stimulation of UCP-1 and in the maintenance of optimal T3 concentrations. We hypothesized that TRIAC might be the main effector of thermogenesis, because it is more potent than T3 or, at least, be an alternative to T3 in terms of stimulation of UCP-1 mRNA. Not only this, but TRIAC stimulates the mechanism responsible for the local production of T3 in BAT (D2), which is required absolutely for the adrenergic stimulation of UCP-1 mRNA [26, 36, 38]. Thus, it could play a fundamental role in the maintenance of T3 production in brown adipocytes, facilitating the entire thermogenic process.

The low range of TRIAC action is another important aspect. TRIAC effects are exerted at very low concentrations, suggesting that despite its lower cellular and nuclear concentration ranges, it may play an important

physiological function. In the present study, we observed that high concentrations of TRIAC result in inhibition of the adrenergic response of D2 (fig. 4). Indeed, when equimolar high concentrations of T3 and TRIAC were used (2–20 nM), no difference was observed in their effects on UCP-1 mRNA expression (fig 1). This may explain why the effects of TRIAC described in the present study had not been described before; previous studies used high doses of TRIAC in rats or cells that hide its specific effects at low doses.

To unravel the molecular mechanisms of the selective actions of TRIAC at low doses, we first analyzed the concentration of TRIAC and T3 in several rat tissues, including BAT. TRIAC concentrations were low, 10–20% of T3 for each tissue analyzed. The highest levels were found in BAT (0.45 ng TRIAC/g BAT vs 5 ng T3/g BAT) [unpublished results]. Therefore, our studies in rats show that TRIAC concentrations in BAT are about 10% those of T3. In our culture medium, the free TRIAC concentrations are lower than the free T3 concentrations, arguing against a higher influx of TRIAC into the cells. We find very low concentrations of TRIAC in cultured brown adipocytes. The uptake studies showed that TRIAC cellular uptake is two to four times lower than that of T3. However, the nuclear uptake of TRIAC is twofold higher than that of T3 at 6 h, possibly resulting in accumulation of TRIAC in nuclei. The possibility exists that TRIAC is locally generated in BAT under specific (and still unknown) conditions.

The studies at the promoter level provide evidence that TRIAC exerts its action at the transcriptional level, at least for the UCP-1 gene, and confirm that low concentrations (0.02 nM) of TRIAC+ NE are more active than T3+NE. As TRIAC binds to the TR-*β1* with an affinity twice that of T3, we might hypothesize that the specific TRIAC actions are exerted either as a very active ligand for the TR-*β1* isoform [53, 54], or by facilitating the binding of the coactivators required for the activation of the transcriptional machinery [55]. Most of the effects of TRIAC on NE parallel those of T3, but are exerted at very low concentrations. In fact, differences have been described in the transactivation of the TR ligand-binding domain (LBD) depending on the ligand: T3, TRIAC, or GC-1[1], the specific ligand of the TR-*β1* isoform [55–57]. This action might be more effective than that of T3 in the BAT thermogenic process, as has been demonstrated for GC-1 in relation with plasma lipids or thermogenesis [55, 57, 58]. However, a good explanation for the specific actions of TRIAC is still speculative and requires further research. Both T3 and TRIAC synergize with NE, and have a similar action on the stabilization of UCP-1 mRNA transcripts.

The effects of TRIAC versus T3 also affect the expression of LPL and D3, two genes regulated by T3 and NE. TRIAC can regulate the expression of both genes either by acting alone or together with NE. D3 plays an important role in regulating the exact T3 concentrations required for specific functions in different cells from the tadpole to mammalian tissues. TRIAC was also more active in regulating both D3 activity and D3 mRNA. And, finally, LPL, stimulated in BAT, but not in white adipose tissues by adrenergic stimulation [39]. In so doing, the free fatty acids are provided for mitochondrial combustion, an important fuel during the thermogenic process. TRIAC at low concentrations is also more effective than T3 in regulating LPL mRNA. TRIAC actions are exerted both when acting alone and together with NE.

A key question is whether this effect of TRIAC is specific for BAT. Recent experiments in rats [our unpublished results] indicate that the higher potency of TRIAC at low doses is exerted preferentially in BAT, not in liver or heart. If confirmed, this would imply that TRIAC action might be exerted on coactivators linked to the thermogenic process or specific to BAT, such as PGC-1.

TRIAC has a short half-life and very low plasma concentrations, due to its high clearance rate. High TRIAC doses have been used to obtain effects in vivo, a fact taken as indication of poor biological activity, which contrasts with its high affinity for the nuclear T3 receptors (TR-*β1*). In humans, TRIAC increases sex hormone binding-globulin and decreases cholesterol, LDL cholesterol and lipids [14–17]. But the main use of TRIAC has been the suppression of TSH in patients with peripheral or pituitary resistance to thyroid hormones [12, 13]. TRIAC has profound TSH-suppressing effects in vitro and in vivo [51]. In rats, T3 is more active than TRIAC at increasing hepatic or heart D1 and malic enzyme mRNA levels [17]. For brain D3 activity, TRIAC was more potent than T3, but had no effect on brain D2 activity [17].

In summary, we have shown that TRIAC is at least 10–20 times more potent than T3 at inducing the adrenergic response of UCP-1 mRNA, D2 activity and D3 and LPL gene expression. Our results suggest that TRIAC may play a physiological role in BAT thermogenesis that might be more important than that of T3. The higher thermogenic potency of TRIAC could provide a tool in the future to stimulate the thermogenic processes, especially if the conditions that increase its cellular production are found. Exogenous administration of TRIAC should be avoided due to its TSH-suppressing effects.

Acknowledgments. We want to thank Dr. Thoma and Dr. H. Rokos (Henning, Berlin, Division of Marion Merrell Dow Research Institute) for kindly providing [3, 5-^{125}I]-T3 at the beginning of this study. BRL37344 was a gift from SmithKline Beecham Pharmaceuticals (Worthing, UK) and ZD7114, a gift from Dr. B. Holloway (Zeneca Pharmaceuticals, Macclesfield, UK). We thank Dr. D. Ric-

[1] 3,5 dimethyl-4-(4′hydroy-3′-isopropylbenzyl)-phenoxy acetic acid.

quier (Paris), Dr. Kirchgessner, Dr. D. St.Germain and J. G. Sutcliffe (La Jolla) for the UCP-1, LPL, D3, and cyclophilin cDNAs, respectively. We thank Dr. A. J. Vidal-Puig for his suggestions and help in correcting the manuscript. This work was supported by research grants PB 95-0097 from Promoción General del Conocimiento (PGC) (Spain), FISS 94/0274 and 99/0813 from Fondo de Investigaciones Sanitarias (FIS), CAM 08.6/0030/1998 from Comunidad de Madrid (CAM) (Spain) and SAF2001-2243 from PN (Spain).

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