Triiodothyronine is required for the stimulation of type II 5′-deiodinase mRNA in rat brown adipocytes

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Martinez-deMena, Raquel, Arturo Hernández, and Maria-Jesús Obregón. Triiodothyronine is required for the stimulation of type II 5′-deiodinase mRNA in rat brown adipocytes. *Am J Physiol Endocrinol Metab* 282: E1119–E1127, 2002; 10.1152/ajpendo.00433.2001.—Type II 5′-iodothyronine deiodinase (D2), produces triiodothyronine (T_3) and is stimulated by cold exposure via norepinephrine (NE) release in brown adipose tissue. Cultured rat brown adipocytes require T_3 for the adrenergic stimulation of D2 activity. D2 mRNA expression in cultured brown adipocytes is undetectable with the use of basal conditions or NE without T_3. Full D2 expression is achieved using NE + T_3, especially after prolonged T_3 exposure. β_3-Adrenergic agonists mimic the NE action, whereas cAMP analogs do not. Prolonged exposure to T_3 alone increases D2 mRNA. High T_3 doses (500 nM) inhibit the adrenergic stimulation of D2 activity while increasing D2 mRNA. The effects obtained with NE + T_3 or T_3 alone are suppressed by actinomycin, but not by cycloheximide, which leads to accumulation of short D2 mRNA transcripts. Prolonged or short exposure to T_3 did not change D2 mRNA half-life, but T_3 seemed to elongate it. In conclusion, T_3 is an absolute requirement for the adrenergic stimulation of D2 mRNA in brown adipocytes. T_3 upregulates D2 mRNA, an effect that might involve stimulation of factors required for transcription or for stabilization of D2 mRNA.

norepinephrine; uncoupling protein-1

BROWN ADIPOSE TISSUE (BAT) is a thermogenic tissue specialized in the production of heat in demanding situations such as cold exposure, during arousal from hibernation, or by the cold experienced after birth. This process is called facultative thermogenesis. The production of heat is accomplished by a mitochondrial protein, called uncoupling protein-1 (UCP1), the specific marker of BAT. Adrenergic stimulation is the main effector for the activation of UCP1, and thyroid hormones, specifically triiodothyronine (T_3), have been described as being necessary for the full expression of UCP1 in rats (3, 4). Moreover, T_3 in BAT is locally produced from thyroxine (T_4) via a deiodinase that plays an important role generating the T_3 required for UCP1 expression (5).

The deiodinases are enzymes that regulate thyroid hormone availability in peripheral tissues. Three different deiodinases have been described that catalyze outer- and inner-ring deiodination. Outer-ring deiodination is a key pathway of thyroid hormone metabolism that leads to the production of T_3, whereas inner-ring deiodination results in the formation of inactive compounds. Most of the T_3 present in tissues is produced from T_4 via outer ring 5′-deiodination. Two isoenzymes catalyze this activating pathway: type I and type II 5′-iodothyronine deiodinases (D1 and D2). D1 and D2 differ in their kinetic characteristics, sensitivity to inhibition by 6-*N*-propyl-2-thiouracil (PTU), tissular distribution, and response to thyroid status (23). Different from D1, D2 is insensitive to PTU, it prefers T_4 as substrate, and its Michaelis-Menten constant is in the nanomolar range. D2 activity is upregulated in hypothyroidism and inhibited by T_4 (26, 47, 48). D2 activity is localized in brain, adenohypophysis, BAT, pineal gland, and the maternal side of the placenta, among other tissues (24, 26, 39, 44, 53). D2 activity is regulated in rat tissues, glial cells, or brown adipocytes by a number of factors such as thyroid hormones (26, 27, 47), adrenergic agents (19, 22, 32, 35, 38, 39, 44), cAMP (10, 17, 22), growth factors (11), and insulin (29, 46).

The cDNAs coding for the three deiodinases have been isolated in rat, human, and other species (2, 12, 13). Sequence analysis has demonstrated that all of them contain an in-frame TGA codon that is translated as selenocysteine due to the presence of a specific structure, named selenocysteine insertion sequence (SECIS), in the 3′ untranslated region of their mRNAs (2, 6). Analysis of D2 expression in human tissues has shown that D2 is also present in human heart, skeletal muscle, and thyroid (12, 42).

In rat BAT, D2 activity is stimulated by hypothyroidism (27) and by cold exposure, norepinephrine (NE), and adrenergic stimuli (44). T_3-induced hyperthyroidism augmented the response to NE (47). These situations also increase D2 mRNA levels (12). As already mentioned, D2 activity produces most of the T_3 found in BAT (45), which saturates nuclear T_3 receptors, and the T_3 produced is necessary for a complete thermo-

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genic function, namely the full expression of the uncoupling protein UCP1, specific marker of BAT (5). Besides, BAT D2 activity is upregulated by insulin (46), as shown after insulin injection, or in diabetic rats, as well as in floating rat brown adipocytes (29). In these cells, D2 activity is stimulated by adrenergic agents, and a synergy between α_1- and β-adrenergic pathways has been described (32, 38), whereas in cultures of mouse brown adipocytes the main pathway is β-adrenergic (35).

Using primary cultures of rat brown adipocytes, we have previously shown that D2 activity is poorly stimulated by NE or cAMP analogs and that T_3 is required for and amplifies the adrenergic response 10- to 20-fold (19). The effect of T_3 is exerted when NE or β_3-adrenergic agents are used and is only poorly mimicked when cAMP-elevating agents are used. The stimulatory effect of T_3 on the adrenergic responses of D2 activity overcomes the inhibition caused by T_4, and such increases are proportional to the time of preexposure to T_3 (19). The T_3 + NE effect requires de novo protein synthesis and is fully inhibited by actinomycin.

In the present study, we have studied the regulation of D2 mRNA expression by adrenergic stimulation and by T_3 by use of primary cultures of rat brown adipocytes differentiated in culture from preadipocytes. Our results disclose a novel role of T_3 in the regulation of D2 mRNA levels, both in cooperation with NE and by itself, indicating the importance of T_3 for the brown adipocyte.

MATERIALS AND METHODS

Materials. The source of most of the reagents used has been previously described (21). Newborn calf serum (NCS) was purchased from Flow (Paisley, Scotland) or from GIBCO Life Technologies (Uxbridge, UK). Isoproterenol (ISO) and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP) were obtained from Sigma Chemical (St. Louis, MO). BRL-37344 was kindly provided by SmithKline Beecham Pharmaceuticals (Welwyn, UK). Ion exchange resins AG1-X8 and AG50W-X2 were obtained from Bio-Rad (Richmond, CA). Oligo(dT) cellulose was obtained from New England Biolabs (Beverly, MA), TRIzol from GIBCO-BRL Life Technologies, AmpliTaq Gold DNA polymerase from Roche Molecular Systems (Branchburg, NJ), and AMV reverse transcriptase (RT) from Promega (Madison, WI).

Cultures of brown adipocytes. Precursor cells were obtained from the interscapular BAT of 20-day-old rats (Sprague-Dawley), isolated according to the method described by Néchad et al. (30) by using collagenase digestion (0.2%) in DMEM + 1.5% BSA at 37°C and filtered 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-cm² culture flasks or 145-cm² plates at a density of 1,500–2,000 cells/cm² on *day 1* and grown in DMEM supplemented with 10% NCS, 3 nM insulin, 10 mM HEPES, 50 IU of penicillin, and 50 μg of streptomycin/ml and 15 μM ascorbic acid. Culture medium was changed on *day 1* and every 2–3 days until the experiment was performed. Precursor cells proliferate actively under these conditions, reach confluence on the 4th or 5th day after seeding (40,000–60,000 cells/cm²), and then differentiate into mature brown adipocytes. Most studies and treat-

ments were done in fully differentiated brown adipocytes (on the 9th day after seeding). For deiodinase activity, 25-cm² flasks were used, and for mRNA studies, 2–3 plates (145 cm²), seeded at the same cellular density, were pooled.

Both NCS and hypothyroid (Hypo) serum were used for culture. The latter was obtained by depleting NCS of thyroid hormones with the anion exchange resin AG1-X8, as described (43). Hypo serum contained ~10% or less of the original amount of thyroid hormones, as assessed by RIA (36). In NCS, concentrations of T_4 and T_3 were 77 and 1.3 nM, respectively, before its dilution with medium. These levels decreased to 2.2 nM T_4 and 0.13 nM T_3 in Hypo serum. In some experiments, T_3 and T_4 concentrations were measured in medium and cells (after purification of the extracts) by means of specific RIAs (36).

Free T_3 concentrations were measured by ultrafiltration as described (36). Free T_3 concentration was 2 or 4% of the total T_3 when 10 or 5% serum, respectively, (either NCS or Hypo serum) was used. The actual free T_3 concentrations were 5–10 pM when 10% NCS was used and 200–500 pM T_3 when 10 nM T_3 was added to the culture medium supplemented with 10 or 5% Hypo serum, respectively.

RNA preparation and Northern blot analysis. For the isolation of poly(A)$^+$ RNA, cells were collected and mRNA isolated using oligo(dT) cellulose as described (52). For Northern analysis, poly(A)$^+$ (5–7 μg) was denatured and electrophoresed on a 2.2 M formaldehyde/1% agarose gel in 1× MOPS buffer (pH 7.0) and transferred to nylon membranes (Nytran) as described (18, 21). A 1.988-kb fragment of the rat D2 cDNA clone (12), corresponding to the entire coding region of the rat D2, 580 nucleotides from the 5′ region and 30 nucleotides of the 3′ end, was used as a probe by labeling with [α-^{32}P]dCTP with the use of random primers (>10^8 cpm/μg DNA). Filters were hybridized for 20 h at 50°C [40% formamide, 20 μg/ml salmon sperm DNA, 50 μg poly(A), and 50 μg poly(C)/ml, 5× saline sodium citrate (SSC), 2× Denhardt's, 0.1% SDS] and 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, CA). The filters were hybridized with cyclophilin as a control to correct for differences between lanes in the amount of poly(A)$^+$ mRNA (14). All of the experiments were repeated 2–3 times, using Northern blot analysis in all. The more complete or representative experiments are shown in the figures.

RT-PCR assay. One microgram of total RNA (TRIzol, GIBCO) was used for RT-PCR amplification under the conditions previously described (12). We used the same RNA samples without RT reaction as controls. The PCR reaction conditions were 94°C for 5 min, and 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final 7-min period at 72°C, using AmpliTaq Gold DNA polymerase. The specific oligonucleotides used for amplification of a 590-bp product were: sense, 5′-ACT CGG TCA TTC TGC TCA AG-3′ and antisense, 5′-TTC AAA GGC TAC CCC ATA AG-3′, as previously described (12). After gel electrophoresis in 1% agarose, the PCR products were transferred to a filter and hybridized with a radiolabeled, nested oligonucleotide rat D2 probe (AAT GCC ACC TTC TTG ACT TT), using [γ-^{32}P]ATP, as previously described (12). The optimal conditions were chosen to avoid the presence of DNA in the samples, unspecific annealing in the PCR reaction, etc.

Determination of D2 activity. Cells were scraped, collected in *buffer A* [0.32 M sucrose, 10 mM HEPES, 10 mM dithiothreitol (DTT), pH 7.0] and homogenized. D2 activities were

determined in homogenates by measuring the release of iodide as described (27), with modifications (19, 33), using as final concentrations 2 nM T_4 (50.000 cpm $[^{125}I]T_4$), 1 μM T_3, 50 mM DTT, 1 mM PTU, 80–100 μg protein/100 μl of total volume, pH 7.0, during 1 h at 37°C. In these conditions, >95% of the activity was insensitive to inhibition with PTU. Each cell homogenate was tested in triplicate using 2–3 culture flasks per treatment. Protein content was determined by the method of Lowry (28) after precipitation of the homogenates with trichloroacetic acid to avoid interference of DTT in the colorimetric reaction (19). Results were expressed in femtomoles per hour per milligram of protein.

The high specific activity $[^{125}I]T_4$ used was obtained in our laboratory (>3,000 μCi/μg) with the use of chloramine T and T_3 as substrate (19, 33). 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 by use of increasing amounts of protein, and *3*) the within-assay coefficient of variation that was <5%.

Statistical analysis. Mean values ± SE are given. One-way analysis of variance (ANOVA) was done, after homogeneity of variance was ensured, using square roots or logarithmic transformations if the homogeneity was not found with the raw data. Significance of differences between groups was assessed using the protected least significant difference test. All calculations were done as described (49).

RESULTS

We have previously shown that, in primary cultures of rat brown adipocytes, the adrenergic stimulation of D2 activity requires the presence of T_3 (19). This T_3 requirement is similar to the one observed for the adrenergic stimulation of UCP1 mRNA in the same cell model (20).

D2 mRNA induction by NE and T_3. In rat BAT, D2 mRNA presents several bands at 7.5, 4.0, 2.5, and 2.0 kb, the first being the most abundant. D2 mRNA expression is clearly stimulated in the BAT of cold-exposed rats (12). In the present study, we used Northern blot analysis of poly(A)$^+$-enriched mRNA from differentiated brown adipocytes. D2 mRNA expression was undetectable using regular culture conditions no matter the amount or type of serum used (not shown) or when the cells were treated with NE alone (Fig. 1*A*, *lanes 1* and *4*). The combined addition of NE and T_3 led to increases in D2 mRNA expression (*lane 3*), in parallel with the findings for the adrenergic stimulation of D2 activity (19), where T_3 was a requirement for NE to become effective whereas NE or T_3 separately had no effect. Moreover, in the absence of adrenergic stimulation, prolonged exposure (40 h) to T_3 increased D2 mRNA levels (2-fold) (Fig. 1*A*, *lane 2*), whereas short exposures to T_3 did not increase D2 mRNA (not shown).

We also compared the effect of 24- and 72-h exposure to T_3 on D2 mRNA in cells treated with or without NE (Fig. 1*B*). In the absence of NE, no effect was observed after 24-h exposure to T_3 (*lane 1*), whereas prolonged exposure (72 h) to T_3 increased D2 mRNA above basal
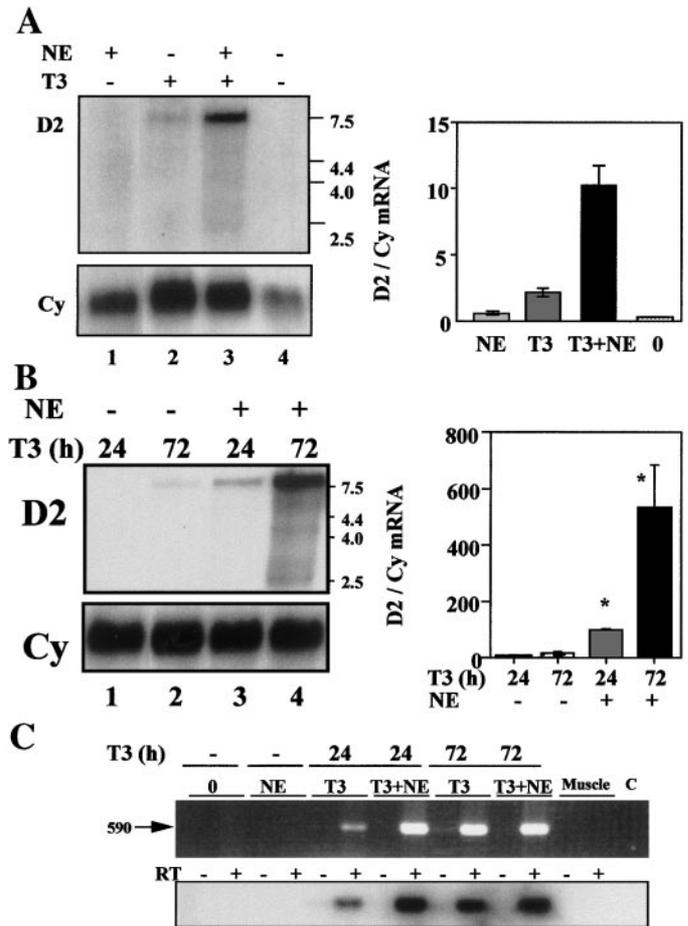

Fig. 1. *A*: adrenergic stimulation of 5′-iodothyronine deiodinase (D2) mRNA. Nine-day-old cultures of brown adipocytes were maintained during the last 40 h in medium supplemented with 5% hypothyroid serum (*lane 4*). Cells were cultured in the presence of 10 nM triiodothyronine (T_3) during the last 40 h (*lane 2*), 3 μM norepinephrine (NE) during the last 5 h (*lane 1*), or with 10 nM T_3 for 40 h and 3 μM NE for 5 h (*lane 3*). Seven micrograms of poly(A)$^+$ RNA were loaded per lane. D2 mRNA, cyclophilin (Cy) mRNA, and the D2-to-Cy ratio (D2/Cy) are shown (means ± SE from 3 experiments). *B*: effect of short and long exposure time to T_3 on the adrenergic induction of D2 mRNA. Nine-day-old cultures of brown adipocytes were maintained during the last 72 h in medium supplemented with 5% hypothyroid serum. Cells were cultured in the presence of 10 nM T_3 during the last 24 or 72 h and in the presence or absence of 5 μM NE during the last 6 h. Five micrograms of poly(A)$^+$ RNA were loaded per lane. Hybridization with a cyclophilin cDNA was used to correct for differences between lanes and D2/Cy is shown. Graph shows means ± SE of 5 different experiments. *$P < 0.05$, vs. treated with only T_3 for 72 h. *C*: RT-PCR assay. One microgram of total RNA from 9-day-old cultures of brown adipocytes was used for RT-PCR amplification. Cells were treated with 5% hypothyroid serum, with 10 nM T_3 for 24 or 72 h in the absence or presence of 5 μM NE during the last 6 h, or with NE alone. For each sample, reactions were performed with (+RT) and without (−RT) reverse transcriptase. As negative controls, mixtures with no RNA (C) or with muscle RNA were run. PCR products were separated on an agarose gel, transferred to a nylon membrane, and probed with a radiolabeled rat D2 oligonucleotide. PCR products are shown after ethidium bromide staining as well as the hybridization with the D2 oligonucletide probe.

levels (*lane 2*). In the presence of NE, D2 mRNA increased sixfold after a short exposure to T_3 and a further fivefold (a total of 30-fold) after a long (72 h) exposure to T_3 (*lanes 3* and *4*).

To see whether the NE response was too low to be measured using poly(A)$^+$ mRNA, we used RT-PCR amplification, with 24- or 72-h exposure to T_3, in the presence or absence of NE (Fig. 1C). After RT-PCR amplification, we observed essentially the same facts as with poly(A)$^+$ mRNA: NE alone was unable to increase D2 mRNA (undetectable levels comparable to basal levels or muscle), whereas NE + T_3 induced large increases (24 or 72 h); T_3 alone resulted in an intermediate response (lower, but detectable, at 24 h). Although this technique amplifies exponentially any small amount of DNA and does not allow a quantitative approach, it confirms that T_3 induces D2 mRNA expression by itself besides amplifying the adrenergic stimulation. With the use of NE alone, D2 induction is undetectable.

Induction of D2 activity by NE and T_3. In parallel, we examined the adrenergic stimulation of D2 activity using different exposure times to T_3 (Fig. 2). The presence of T_3 per se (or NE alone, Fig. 2, dashed line) did not increase D2 activity. Adrenergic increases of D2 activity were larger in cells exposed to T_3 from confluence (72 h) than in those exposed for a shorter time (24 h) to T_3. The effect of T_3 was also examined in the absence of insulin, which increases D2 activity in BAT, and the results were similar, although lower, indicating that the T_3 effect is independent of insulin. The increases observed in D2 activity after prolonged exposure to T_3 were modest compared with those observed at the mRNA level.

Effect of high doses of T_3. We then examined whether high doses of T_3 (100 nM) inhibit D2 mRNA in brown adipocytes, as described in pituitary cells (25). Figure 3A shows that D2 mRNA expression is not suppressed by a 100 nM dose of T_3 in either the presence or absence of NE. We further tested in the presence of NE a larger T_3 dose (500 nM), which did not inhibit D2 expression but induced further increases in D2 mRNA

Fig. 3. A: effect of high doses of T_3 on the adrenergic induction of D2 mRNA. Brown adipocytes were cultured as described. Cells were cultured in the presence of 10 or 100 (or 500) nM T_3 during the last 72 h and in the presence or absence of 5 μM NE during the last 6 h. Five micrograms of poly(A)$^+$ RNA were loaded per lane. Hybridization with a cyclophilin cDNA was used to correct for differences between lanes and D2/Cy is shown. Graph shows the mean ± SE of 2 different experiments, with 10 nM T_3 + NE taken as 100%. B: effect of high doses of T_3 on the adrenergic induction of D2 activity. Eight-day-old cultures of brown adipocytes were cultured in medium supplemented with 10% hypothyroid serum in the presence of increasing amounts of T_3 (10, 20, 50, 100, 200, 500, and 1,000 nM) during the last 24 h and NE (5 μM) during the last 6 h. The values from 10 nM T_3 + NE were taken as 100%. Line shows the values for NE or T_3 alone. Data are means ± SE (n = 4, done in triplicate) of 2 flasks from 2 different experiments. *P < 0.05, vs. cells treated with 10 nM T_3 for 24 h (100%).

(2.5-fold vs. 10 nM T_3 + NE). T_3 concentrations were very high, as measured by specific RIAs (50- and 250-fold increases in the medium and cells, when 100 and 500 nM T_3, respectively, were used vs. 10 nM T_3). In parallel, we tested whether these T_3 doses inhibited D2 activity (Fig. 3B). Increasing amounts of T_3 inhibited the adrenergic stimulation of D2 activity, effects that were detectable from 20 nM T_3, and maximal inhibition was observed when using 100 nM T_3.

Effect of adrenergic agonists. We have previously shown (19) that, in cultured brown adipocytes, the adrenergic responses of D2 activities were mediated mainly by NE, whereas cAMP analogs did not reproduce the NE effect. The thermogenic responses in BAT have been shown to be mediated via β_3-adrenergic receptors (55). We tested the effect of the β_3-adrenergic agonist BRL-37344, compared with NE and ISO, on D2 activity and D2 mRNA expression (Fig. 4, A and B). Using dose-response curves, we observed that BRL-37344 is more potent than NE and ISO at the dose of 0.1 μM (P < 0.05) in the stimulation of D2 activities (half-maximal concentrations were 0.05 μM for BRL-
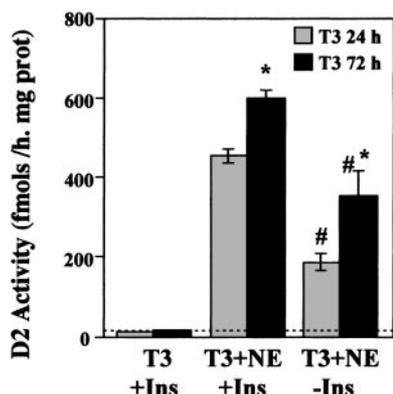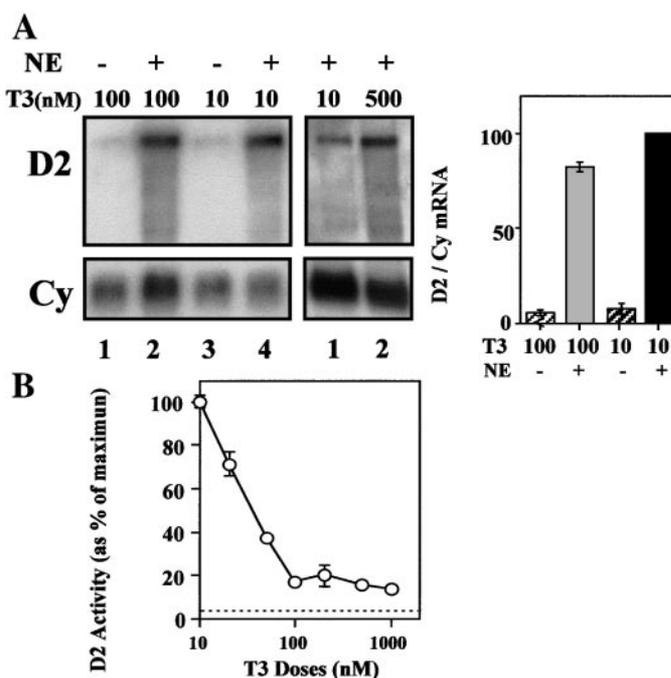

Fig. 2. Effect of short and long exposure time to T_3 on the adrenergic induction of D2 activity. Eight-day-old cultures of brown adipocytes were cultured in the presence of 10 nM T_3 during the last 24 or 72 h with or without NE (5 μM) during the last 6 h by use of medium supplemented with 10% hypothyroid serum. One-half of the cells was cultured in insulin (Ins)-depleted medium for the last 24 h. Data are means ± SE (n = 6) of 2–3 flasks from 2 different experiments. *P < 0.05, treated with T_3 for 24 vs. 72 h. #P < 0.05, vs. their respective control cells.
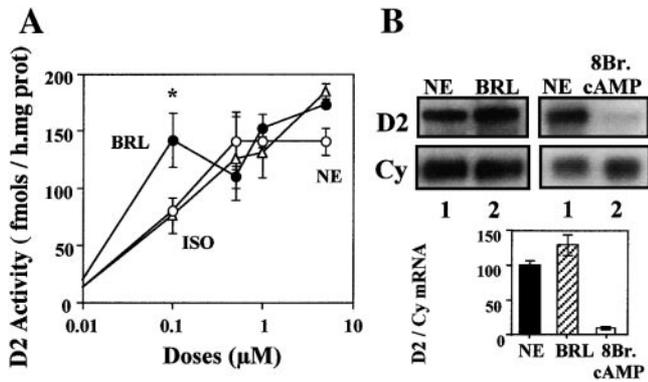
Fig. 4. *A*: dose-response curves for the adrenergic stimulation of D2 activity using NE, isoproterenol (ISO), and BRL-37344 (BRL). Eight-day-old cultures of brown adipocytes were maintained during the last 24 h in medium supplemented with 10% hypothyroid serum and 10 nM T_3. Cells were exposed to different concentrations of NE, ISO, and BRL during the last 6 h. Data are means $\pm$ SE ($n = 4$, triplicates) of 2 flasks from 2 different experiments. *$P < 0.05$, BRL vs. NE. *B*: adrenergic stimulation of D2 mRNA using NE, BRL, and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP). Nine-day-old cultures of brown adipocytes were maintained during the last 72 h in medium supplemented with 5% hypothyroid serum. T_3 (10 nM) was added during the last 24 h, and 0.2 μM NE or BRL or 0.5 mM 8-BrcAMP was added during the last 6 h. Five micrograms of poly(A)$^+$ RNA were loaded per lane. D2 mRNA, cyclophilin mRNA, and D2/Cy are shown (mean $\pm$ SE of two experiments).

37344 vs. 0.2 μM for NE and ISO). A plateau was reached at higher concentrations.

With respect to D2 mRNA expression, similar levels were detected with the use of equimolar concentrations of NE or the β_3-adrenergic agonist BRL-37344 in the presence of T_3 (Fig. 4*B*), although the latter seemed slightly more potent (not significant). Additionally, we tested the effect of 8-BrcAMP and found poor effects compared with NE ($P < 0.05$ vs. NE; Fig. 4*B*). This is in agreement with the low effect of cAMP analogs that we reported for D2 activity (19).

D2 mRNA inductions are blocked by actinomycin D and increased by cycloheximide. We studied whether D2 mRNA adrenergic inductions were dependent on increases in gene transcription and required de novo protein synthesis. Cells were exposed for 1 or 3 days to T_3 and to NE for 7 h, in the presence or absence of either actinomycin D (Act) or cycloheximide (CHX) (both added 15 min before NE). We had previously tested the complete inhibition of protein synthesis using these doses of CHX in culture (20). Addition of Act or CHX for periods longer than 20 h was toxic for the cells. Figure 5*A* shows that Act inhibits the induction of D2 mRNA, suggesting that the adrenergic response requires increases in gene transcription, whereas CHX increases D2 mRNA levels, indicating that inhibition of de novo protein synthesis for 7 h results in accumulation of the D2 mRNA, especially of a short mRNA species ($\sim$1.9 kb). Similar results were obtained when T_3 was added for a short or a long time (+NE) (Fig. 5*A*). When both inhibitors were added to T_3 alone (in the absence of NE), the results were similar to those using NE + T_3 (see Fig. 5*B*). Addition of CHX to cells in basal conditions or using NE alone did not result in accumu-

lation of mRNA (data not shown), indicating that such an accumulation is found only in the presence of T_3.

D2 mRNA stability. We explored whether the effect of T_3 could be due to stabilization of D2 mRNA transcripts leading to increases in its half-life, as this effect of T_3 is observed in other T_3-dependent genes in brown adipocytes, such as UCP1, malic enzyme, or S14 (3, 18, 20, 37).

To measure the stability of the D2 mRNA, brown adipocytes were cultured in the presence of T_3 for 1 or 3 days and exposed to NE (5 μM) during the last 10 h to stimulate D2 mRNA expression. Thereafter, Act was added to some of the flasks, and cells were harvested at different times for mRNA preparation. Because D2 mRNA expression was decreasing during this time, the appropriate controls were run in parallel. Figure 6 shows first that, in the absence of Act, a decrease in D2 mRNA expression occurred during the period studied, as the effect of NE decreased between 10 and 18 h, so D2 mRNA expression decreased by 50% after 7 h, both when T_3 was added for long or short times (1 or 3 days). Act induced a further reduction in D2 mRNA, but no significant changes were observed between exposure to T_3 for 1 or 3 days. D2 mRNA half-life was $\sim$6 h, so the
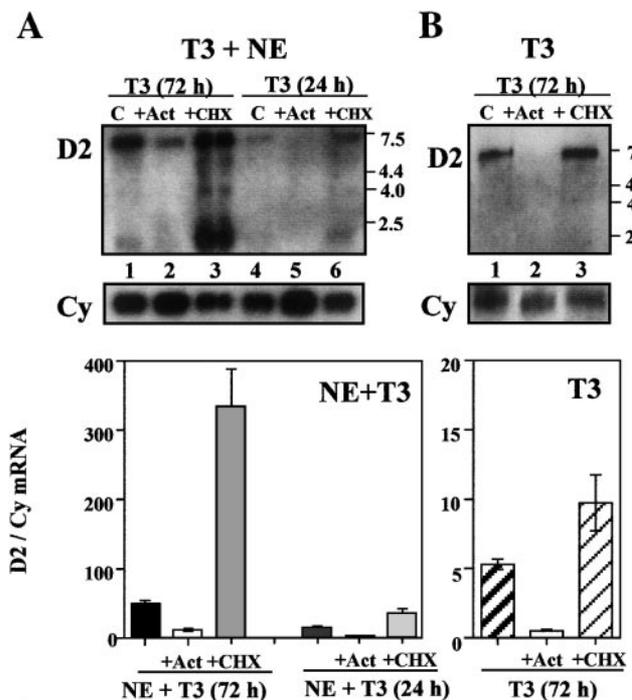

Fig. 5. Effect of actinomycin D (Act) and cycloheximide (CHX) on D2 mRNA. *A*: by use of NE and T_3 for 1 or 3 days, 9-day-old cultures of brown adipocytes were maintained during the last 72 h in medium supplemented with 5% hypothyroid serum. Cells were cultured in the presence of 10 nM T_3 during the last 24 or 72 h, and 5 μM NE was added during the last 7 h. Act (5 μg/ml) or CHX (25 μM) was added 15 min before NE. Five micrograms of poly(A)$^+$ RNA were loaded per lane. Hybridization with a cyclophilin cDNA was used to correct for differences between lanes and D2/Cy is shown (mean $\pm$ SE from 3 experiments). *B*: with T_3 alone, the same protocol was used as in *A*, except that T_3 was added for 72 h (without NE), and Act (5 μg/ml) or CHX (15 μM) was added during the last 14 h (mean $\pm$ SE from 2 experiments).
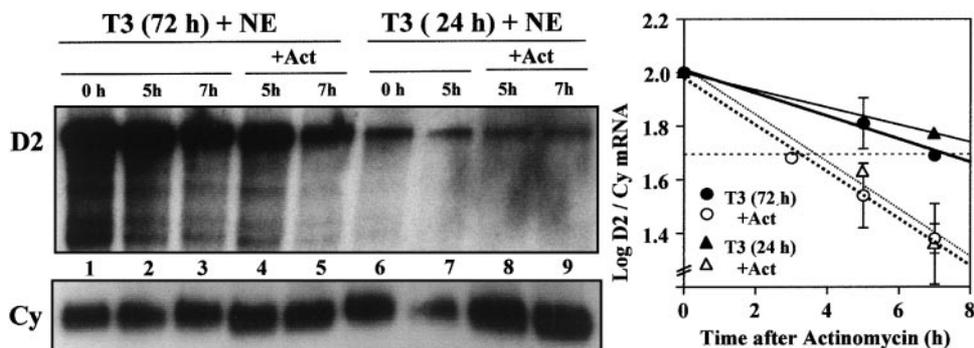
Fig. 6. Determination of half-life of D2 mRNA. Nine-day-old cultures of brown adipocytes were maintained during the last 72 h in medium supplemented with 5% hypothyroid serum. Cells were cultured in the presence of 10 nM T_3 during the last 72 or 24 h (circles and triangles, respectively), and 5 μM NE was added during the last 10 h. Thereafter, Act (5 μg/ml) was added and cells harvested at 3, 5, and 7 h. The natural decrease in D2 mRNA expression at 5 and 7 h is shown (filled symbols and solid lines) as well as Act-treated cells (open symbols and dashed lines). Five micrograms of poly(A)$^+$ RNA were loaded per lane. Hybridization with a cyclophilin cDNA was used to correct for differences between lanes. Figure shows the mean $\pm$ SE from 3 different experiments.

presence of T_3 for short or long times does not seem to play a role in increasing D2 mRNA stability. Nevertheless, as we cannot measure D2 mRNA half-life in the absence of T_3, it is likely that T_3 is playing a role in the stabilization of D2 mRNA transcripts.

DISCUSSION

The main regulator of D2 activity in BAT is cold exposure, via the adrenergic stimulation (44), a situation in which D2 provides the T_3 required for the full expression of UCP1, the specific marker of BAT (5). T_3 plays other important roles in brown adipocytes, increasing mitocondriogenesis, the expression of lipogenic markers (18, 37), and favoring differentiation programs. The action of T_3 on D2 expression does not seem to be an indirect effect linked to the differentiation program, but rather an action required for the NE action.

NE. D2 is stimulated adrenergically in floating adipocytes (32), and a synergy between the β- and α_1-adrenergic pathways was found (38), the adrenergic stimulation of D2 being mostly β-adrenergic in mouse brown adipocytes (35). We have shown that T_3 is an absolute requirement for the adrenergic stimulation of D2 and amplifies this response (19).

In the present study, we analyzed the mechanisms involved in the stimulation of D2 mRNA expression by use of rat brown adipocytes in culture. A predominant band of 7.5 kb and several other weaker bands were observed for D2 mRNA, as described (12). Using poly(A)$^+$, we did not detect D2 mRNA in basal conditions or when adding NE alone. Using an RT-PCR technique, we were also unable to detect increases in D2 mRNA in response to NE alone, and the presence of T_3 was required. This lack of response could be due to the hypothyroidism-induced impairment of the β-adrenergic response that results in a lower density of β_1-adrenergic receptors (AR), and lower cAMP levels (both decreased by 60%) (40). This effect of hypothyroidism has been reported for many tissues (51) and recently has been addressed extensively in BAT for β_1-,

β_2-, and β_3-AR (40, 41). Treatment with T_3 slowly restores first the density of β_1-AR and later the capacity to produce cAMP, suggesting that the lower cAMP production reflects a fault at the postreceptor level (40). Nevertheless genes such as UCP1, which are stimulated adrenergically and whose expression is low in hypothyroid rats, restore their levels a few hours after T_3 injection (4), much before cAMP production is normalized. The lack of response to NE and the T_3 requirement for NE action goes much further than the simple restoration of the β_1-AR density. Moreover, the changes in β_3-AR density in response to hypothyroidism and T_3 are opposite to the ones observed for β_1-AR density (41).

Indeed, a cAMP response element (CRE) has been identified in the 5' UT region of the human D2 promoter ($-92/-85$) (1), and D2 mRNA responds quickly to forskolin and 8-BrcAMP in astrocytes (34), indicating that CRE seems to be an important regulatory element in the D2 promoter. To our knowledge, no functional CRE has been identified yet in the rat D2 promoter.

T_3 and NE. The adrenergic stimulation of D2 mRNA in cultured brown adipocytes requires the presence of T_3. This finding is similar to what is found for the adrenergic stimulation of UCP1 mRNA, where a synergy between NE and T_3 is required, postulated to be exerted via CREs. In the UCP1 gene, an enhancer region has been found that has several thyroid response elements, retinoic acid response elements, and possibly peroxisome proliferator-activated response elements that synergized with the response of NE via cAMP. The cooperative interaction between the T_3 and NE actions is firmly stablished (3, 4), but the specific proteins involved (coactivators) have not yet been identified. The recruitment of coactivators would increase the affinity or phosphorylation of CCAAT/enhancer-binding protein/p300 to increase transcription. Another possible candidate is PGC-1, the coactivator 1 for peroxisome proliferator-activated receptor-γ (PPARγ), induced in BAT upon cold exposure. The participation

of these proteins in the synergistic action of NE and T_3 is not elucidated. Recent reports using mice with targeted disruption of the D2 gene suggest that the T_3 derived from D2 is essential for the thyroid-sympathetic synergy required for adaptive thermogenesis (15).

The adrenergic responses behave as β_3-adrenergic at the mRNA level, in agreement with previous reports using CGP-12177A (19, 35), and the responses are similar for D2 activity and mRNA. The effect of NE on D2 activity and mRNA is only poorly reproduced when using 8-BrcAMP (19). This suggests either that cAMP by itself is not the only pathway of activation or that T_3 interferes in its transduction pathway. Other signaling pathways, such as p38 mitogen-activated protein kinase, have been recently reported to be activated in response to β_3-agonists in brown adipocytes, indicating the need for this signaling pathway, in addition to elevation of cAMP levels (8). We have measured cAMP levels in our cells (unpublished results), which increase with the use of NE, BRL-37344, or other β_3-agonists to levels comparable to those obtained using 8-BrcAMP (150–200 up to 500 pmol cAMP/mg protein), levels that increase twofold in the presence of T_3. The possibility that the CRE is inhibited by the action of serum, via c-jun, as has been described in the UCP1 promoter (54), is unlikely, because we find no response to NE either in the absence of serum.

T_3. In addition, we have demonstrated that T_3 by itself increases D2 mRNA expression. Although the extent of this increase may seem modest, it is an unexpected finding. The increased D2 mRNA expression observed with the use of T_3 could be due to direct stimulation of transcription (as Act blunts it), stimulation of factors required for transcription, or stabilization of the preexisting mRNA, as the T_3 effect requires long times to be observed. T_3 could also stimulate basal D2 expression by increasing the basal adenylyl cyclase activity, as T_3 reduces G_i levels (9). Nevertheless, basal cAMP levels are not modified by addition of T_3 in our cells. Of note, RT-PCR is able to amplify D2 transcripts after 24 h of T_3 treatment alone, a fact not observed when NE is used alone.

This effect of T_3 is in contrast to what is found in vivo in other tissues such as brain (7, 12, 26) or pituitary cells (25), where T_3 inhibits D2 mRNA by 50%, suggesting specific modulation of D2 in each cell. The inhibition of D2 activity by T_3 is much lower than that of T_4 and reverse T_3 (47, 48). In BAT, T_3 has little ability, if any, in inhibiting D2 activity and even 2-fold increases were reported (47). We found that constant infusion of T_3 into rats stimulated D2 activity in BAT and brain from fetal and adult rats (16, 31). In the present study, we observe clear inhibition of D2 activity when using 20 nM T_3 or higher, but at the mRNA level, we do not observe decreases in D2 expression, even when very high T_3 doses are used, and further increases in D2 mRNA are observed with the use of 500 nM T_3 (a T_3 dose that increase T_3 concentrations $\geq$250-fold in cells and medium).

Mechanisms. The experiments done using CHX and Act indicate that D2 mRNA induction using NE + T_3 or T_3 is activated at the transcriptional level and that the inhibition of de novo protein synthesis results in accumulation of mRNA, especially of a shorter mRNA species. CHX treatment of T_3-exposed cells produces a marked increase in a 1.9-kb mRNA species that was barely detectable in control cells (Fig. 5). This transcript might be a short-lived product generated by alternative mRNA processing. The nature of this mRNA is unknown, but it might correspond to a 1.9-kb D2 cDNA isolated from brown fat (12) that does not code for an active protein because it is lacking a putative SECIS element. If this is the case, expression of this shorter mRNA might be an important regulatory mechanism of expression of active D2 enzyme. The accumulation of D2 mRNA with the use of CHX has been reported in pituitary cells (25) (mRNAs sizes were not described) and in pineal glands, where a short band is also observed (22). Of note, CHX per se, or in the presence of NE without T_3, is unable to accumulate shorter or larger mRNA species (results not shown).

The studies performed to determine whether a prolonged exposure to T_3 contributes to increases in D2 mRNA half-life suggest that this is not the case. Nevertheless, we do not exclude the possibility that T_3 is inducing a stabilization of the D2 mRNA transcripts, as we were unable to measure D2 mRNA half-life in the absence of T_3. A much shorter D2 mRNA half-life (2 h instead of the 6 h we find) has been reported in pituitary cells (25), suggesting that the presence of T_3 might increase D2 mRNA half-life.

In summary, we have demonstrated that T_3 is an absolute requirement for the adrenergic stimulation of D2 mRNA (and activity) in brown adipocytes. This stimulation occurs mainly via β_3-adrenergic receptors. T_3 by itself is able to increase D2 mRNA after prolonged exposure, and its presence is required for the accumulation of D2 mRNA species after treatment with CHX. Additional work is necessary to elucidate the mechanisms by which this novel role of T_3 takes place. The findings reported here are important, as D2 mRNA expression has not been studied in brown adipocytes, and give a new dimension to the important role of T_3 in the regulation and maintenance of its own production in BAT besides its importance in the stimulation of the thermogenic function, namely the expression of UCP1, the specific marker of brown adipocytes.

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