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Triiodothyronine amplifies the adrenergic stimulation of uncoupling protein expression in rat brown adipocytes

ARTURO HERNÁNDEZ AND MARIA JESÚS OBREGÓN
Unidad de Endocrinología Molecular, Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas, 28029 Madrid, Spain

Hernández, Arturo, and Maria Jesús Obregón. Triiodothyronine amplifies the adrenergic stimulation of uncoupling protein expression in rat brown adipocytes. *Am J Physiol Endocrinol Metab* 278: E769–E777, 2000.—Uncoupling protein (UCP), the mitochondrial protein specific to brown adipose tissue, is activated transcriptionally in response to cold and adrenergic agents. We studied the role of triiodothyronine (T_3) on the adrenergic stimulation of UCP mRNA expression by use of primary cultures of rat brown adipocytes. Basal UCP mRNA levels are undetectable. Norepinephrine (NE) increases UCP mRNA during differentiation, not during proliferation. In hypothyroid conditions, UCP mRNA response to NE is almost absent. The presence of T_3 (0.2–20 nM) greatly increases the adrenergic response (30-fold). The sensitivity of UCP mRNA responses to NE is potentiated ~100-fold by the presence of T_3. The effect is proportional to the dose and time of preexposure to T_3. The increases obtained with NE and T_3 are prevented by actinomycin and cycloheximide. T_3 greatly stabilizes UCP mRNA transcripts. The effects of thyroxine and retinoic acid are weaker than those of T_3. In conclusion, in cultured rat brown adipocytes, T_3 is required and both synergizes with NE to increase UCP mRNA and stabilizes its mRNA transcripts.

thyroid hormones; brown adipose tissue

BROWN ADIPOSE TISSUE (BAT) is a thermogenic tissue that plays an important role in hibernating animals, newborns, and cold-exposed mammals. BAT also seems important in maintaining energy balance, as it is activated in response to diet (diet-induced thermogenesis). The main function of BAT is to produce heat under adrenergic stimulation (facultative thermogenesis). This function is accomplished by the uncoupling protein (UCP), nowadays called UCP-1 (UCP in this paper), a BAT-specific protein present only in the inner mitochondrial membrane of BAT (37). When activated adrenergically, UCP provides heat by uncoupling the oxidative phosphorylation, dissipating as heat the energy otherwise stored as ATP (25).

BAT thermogenesis is activated in response to the norepinephrine (NE) released by sympathetic nerve endings. The adrenergic stimulation induces UCP synthesis, a process that has been studied in vivo in several species and different situations, measuring increases in the activity, protein, or UCP mRNA expression (4, 6, 16, 24, 27, 38, 46, 47). The presence of UCP has also been characterized in isolated floating rat brown adipocytes (2) or in primary cultures of brown adipocytes obtained from mice or hamsters as donor animals (9, 17, 36). In the latter culture systems, UCP mRNA is stimulated in response to NE, adrenergic analogs, or cAMP, and the stimulation has been characterized as β-adrenergic, acting through the β_1- and β_3-adrenergic receptors (9, 17, 30, 36). Other studies have been done using murine cell lines from hibernomas in which different sensitivity to NE was observed (18, 21, 39, 40).

Thyroid hormones are also important in thermogenesis. Hypothyroid animals are intolerant to cold and even die when exposed to cold for several hours (4). This is due to a defective basal and facultative thermogenesis in the hypothyroid animals, because during cold exposure the increases in UCP expression are lower than in control animals. This deficiency can be restored by adequate replacement therapy with thyroxine (T_4) (4, 46). Basal and stimulated UCP mRNA is also low in hypothyroid fetuses and newborns, and either triiodothyronine (T_3) or T_4 restores UCP levels in fetuses (26, 28). Therefore, thyroid hormones seem necessary for the full expression of UCP in vivo (4, 46), and T_3 amplifies the adrenergic stimulation of UCP mRNA expression and transcription in cold-exposed rats (3). In addition, there is a high correlation between the occupancy of nuclear T_3 receptors and UCP increases (5). Recently, certain regions of the rat UCP promoter have been identified as thyroid hormone and retinoic acid response elements (TRE and RARE, respectively) (1, 8, 33, 34).

The production of T_3 in BAT is catalyzed by the type II 5′-iodothyronine deiodinase (DII), an enzyme stimulated mainly by cold and adrenergic agents (44); its role is to produce T_3 locally. DII seems necessary for an optimal thermogenic response of BAT to cold (4).

Although the role of thyroid hormones for the full expression of UCP has been well documented in rats in vivo (3), studies using cultured rat brown adipocytes are scarce (9), and the role and need of thyroid hormones for the adrenergic stimulation of UCP are not always recognized (9, 36) because the results depend on the culture model used. Several groups include T_3 as a so-called "differentiation medium," a beneficial factor for the differentiation of cultured brown preadipocytes (1, 18). Nevertheless, the precise role of T_3 has not been fully clarified in cultures of brown adipocytes.

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The aim of the present study is to study the effect of thyroid hormones on the adrenergic stimulation of UCP mRNA expression by use of primary cultures of rat brown adipocytes, a system in which the stimulation of UCP by NE is not easily achieved. Precursor cells were isolated from rat BAT pads and, after a period of active proliferation, were used during the differentiation period. We analyze the role of T_3 that is required and that potentiates the adrenergic activation of UCP mRNA in cultured rat brown preadipocytes. The present model remarks on the importance of T_3, which seems more important than T_3 as previously reported in other culture systems.

MATERIALS AND METHODS

Materials. DMEM was obtained from Life Technologies (Uxbridge, UK). Newborn calf serum (NCS) was purchased from Flow (Paisley, Scotland). Antibiotics were obtained from the local pharmacy. BSA (in solution at 22%, pH = 7.2) was obtained from Ortho Diagnostic Systems, Johnson & Johnson (Raritan, NJ). Collagenase, bovine insulin, ascorbic acid, guanidinium HCl, MOPS, T_3, T_4, NE, and retinoic acid (RA) were obtained from Sigma (St. Louis, MO). Cycloheximide (CHX), actinomycin D (Act D), and restriction enzymes were obtained from Boehringer Mannheim (Mannheim, Germany). Ion exchange resin AG1-X8 was from Bio-Rad (Richmond, CA). All other chemicals were reagent or molecular biology grade. Radiolabeled deoxy-[α-^{32}P]CTP (3,000 Ci/mmol) was purchased from Amersham International (Aylesbury, UK), and the oligolabeling system was obtained from Pharmacia (Uppsala, Sweden). Nytran membranes were purchased from Schleicher & Schuell (Dassel, Germany).

Cultures of brown adipocytes. Precursor cells were obtained from the interscapular brown adipose tissue of 20-day-old rats (Sprague-Dawley), isolated according to the method described by Néchad et al. (23), with modifications (13) by use of 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 in 25-cm^2 culture flasks to get 1,500–2,000 cells/cm^2 on *day 1* and were grown in DMEM supplemented with 10% NCS, 3 nM insulin, 10 mM HEPES, 50 IU penicillin, 50 μg streptomycin/ml, and 15 μM ascorbic acid. Culture medium was changed on *day 1* and every 2nd day thereafter. Precursor cells proliferated actively under these conditions, reached confluence on the 4th or 5th day after seeding (40,000–80,000 cells/cm^2), and by *day 8* were fully differentiated into mature brown adipocytes. Studies were performed during the period of differentiation (5th-8th culture day) using NCS or hypothyroid serum in the presence of thyroid hormones or other treatments, as specified.

Both NCS and hypothyroid serum (HS) were used for culture. The latter was obtained by depleting NCS of thyroid hormones with the anion exchange resin AG1X8, as previously described (43). HS contained ≤10% of the original amount of thyroid hormones, as assessed by RIA (22). In NCS, concentrations were 77 mM T_4 and 0.7 nM T_3, respectively. These levels were decreased to 2.2 nM T_4 and 0.13 nM T_3 in HS (13). 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). We also measured the free T_3 concentrations when using DMEM+ 10% HS+T_3. The free T_3 concentrations (at the start of the experiment) were 4% of the total T_3 added, and after 20 h, incubation decreased in variable amounts, depending on the T_3 concentration used: 50 and 170 pM T_3 when 1 and 10 nM T_3+10% HS, respectively, were used. In these conditions the cellular T_3 concentrations were 2.3 and 9 nM T_3 for 1 and 10 nM T_3, respectively (unpublished observations).

The incorporation of ^{35}S-labeled methionine into TCA-precipitable proteins was determined after cells were incubated for 16 h with ^{35}S-methionine, in the presence of several CHX doses.

RNA preparation and Northern blot analysis. Total cellular RNA was extracted in guanidinium-HCl as described (13), with use of ethanol precipitation. The recovery was 50–90 μg total RNA/25-cm^2 flasks, ~5 × 10^6 cells (~25 μg RNA in the 4th–5th days). For Northern analysis, total RNA (15–20 μg) was denatured and electrophoresed on a 2.2 M formaldehyde/1% agarose gel in 1× MOPS buffer and transferred to nylon membranes. A 1,200-bp fragment of the rat UCP cDNA clone [kindly provided by Dr. D. Ricquier (7)] was used as a probe by labeling with deoxy-[α-^{32}P]CTP by use of random primers (>10^8 cpm/μg DNA). Filters were hybridized for 20 h at 50°C [40% formamide, 5× sodium saline citrate (SSC), 2× Denhardt's solution, and 0.1% SDS] and washed 4 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 Dymanics, Sunnyvale, CA). The membranes were routinely dyed using methylene blue to visualize the ribosomal RNAs (rRNA), and differences between lanes were used to correct the results obtained from UCP mRNA. Usually rRNA is presented in the figures. Some filters were also hybridized with the cDNA for cyclophilin or vimentin as a loading control between lanes. All experiments were repeated 2–3 times using duplicates. The more complete and representative experiments are shown in the figures, and the graphs show the means of 2–3 experiments.

RESULTS

Adrenergic stimulation of UCP mRNA during differentiation. The expression of UCP mRNA during the differentiation of rat preadipocytes into adipocytes was examined. The basal expression of UCP is undetectable under standard culture conditions, namely 10% NCS (Fig. 1, odd lanes). The presence of NE (10 μM) significantly increased UCP mRNA (Fig. 1, even lanes, $P < 0.05$) on any day after the cells reached confluence, being maximal around confluence and decreasing thereafter ($P < 0.05$). NE does not increase UCP mRNA during proliferation (*day 3*). The two bands correspond to the two molecular sizes of the UCP mRNA, 1.6 and 1.9 kb. In Fig. 1 we also show, as control, the rRNA pattern and the expression of vimentin, a constitutive protein of the intermediate filaments in brown adipocytes. Therefore, rat confluent cells are true brown adipocytes, as they express UCP mRNA.

When 10% NCS is used, dose-response and time-course experiments show that maximal effects are obtained at 1 μM NE, a plateau is reached at higher doses, and 2-h exposure to NE is enough to induce significant increases (results not shown).

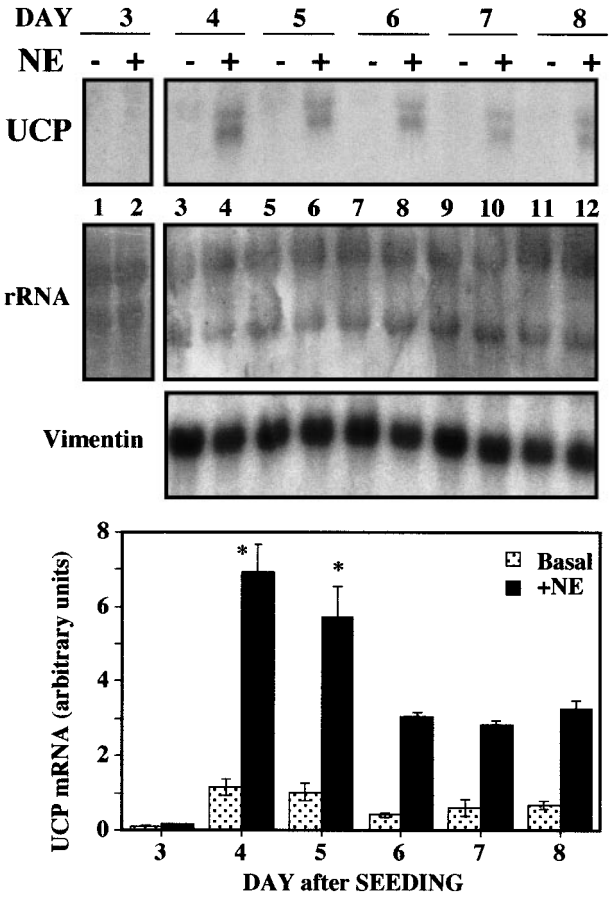

Fig. 1. Adrenergic stimulation of uncoupling protein (UCP) mRNA during differentiation. Rat brown precursor cells were grown in standard conditions [10% newborn calf serum (NCS)] and, at different days after seeding, incubated with 10 μM norepinephrine (NE) during the last 4 h before recollection. Basal and stimulated UCP mRNA, the ribosomal RNA (rRNA) stained with methylene blue, vimentin mRNA, and densitometric analysis are depicted. Autorradiograms presented correspond to cells obtained from the same culture. The experiment was repeated several times, checking 2 or 3 days/experiment. Graph represents means ± SE from several experiments. All increases due to NE were significant ($P < 0.05$) vs. basal levels. *$P < 0.05$ vs. *days 6*, *7*, and *8*.

Effect of T_3 on the adrenergic response of UCP. Because the responses to NE in the presence of NCS were frequently very low, we analyzed the effect of T_3 by using several T_3 concentrations, the effect of the differentiation state of the cells, and the kinetics of induction by NE and T_3. The effect of T_3 on the adrenergic response of UCP mRNA expression was studied at *days 6* and *8* (Fig. 2) by using 2 or 40 nM T_3 for 20 h and 10 μM NE during the last 4 h (*day 7* was also studied). In the absence of NE, T_3 per se does not increase UCP mRNA levels. The adrenergic response when NE is used in hypothyroid medium is barely detectable at *day 6* and absent at *day 8*. But when T_3 is added together with NE, there is a marked potentiation of the adrenergic response [5–30 times, depending on the T_3 concentration used ($P < 0.05$), as well as on the time of exposure to T_3 (lower at 4 h, data not shown)]. This effect of T_3 is observed on any day after confluence.

The relationship between T_3 and NE concentrations was also examined to analyze the minimal dose of NE or T_3 required for the response of UCP mRNA. A dose-response curve of NE in the presence of increasing doses of T_3 is represented in Fig. 3. Cells were exposed to three different doses of T_3 (0.1, 2, and 20 nM) for 20 h, and for each T_3 concentration, increasing doses of NE were added for 4 h. When hypothyroid medium was supplemented with a minimal dose of T_3 (0.1 nM T_3), UCP mRNA expression was very low, even at the highest NE dose (10 μM). When cells were preexposed to 2 or 20 nM T_3, the sensitivity of the response to NE was multiplied severalfold, so that a detectable signal was already observed when 0.1 μM NE and 2 nM T_3 were used (*lane 7*) and became a strong signal when 0.1 μM NE and 20 nM T_3 were used (*lane 12*) ($P < 0.05$). In summary, the sensitivity of the adrenergic response is increased ~100 times by preexposure to a tenfold higher T_3 dose, so that a similar UCP expression is observed whether 10 μM NE + 2 nM T_3 or 0.1 μM NE + 20 nM T_3 is used. This experiment also shows that, at very low T_3 concentrations, the adrenergic response is minimal and demands an increased NE concentration.

The smallest T_3 concentration that increases UCP mRNA expression is 0.2 nM T_3 (Fig. 4, $P < 0.05$). This

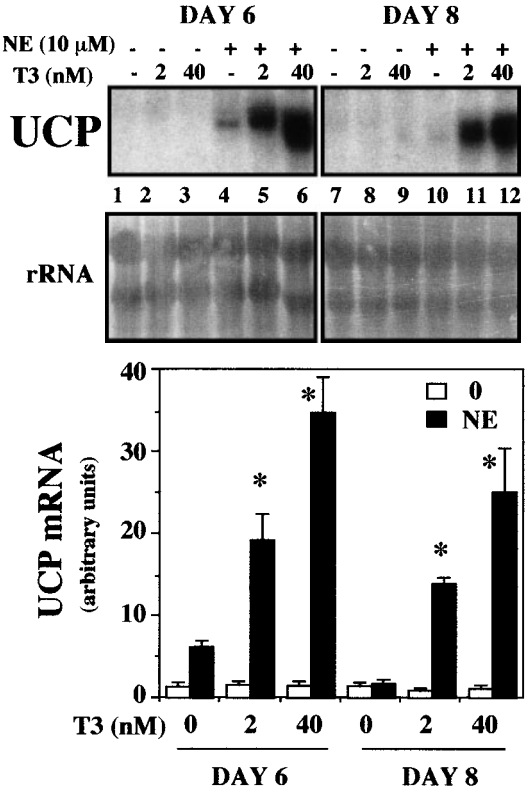

Fig. 2. Effect of triiodothyronine (T_3) on the adrenergic response of UCP mRNA. Cultures of rat brown preadipocytes grown in standard conditions were collected at *days 6* and *8* after corresponding treatments. Cells were maintained during the last 24 h in medium containing 10% hypothyroid serum, supplemented or not with 2 or 40 nM T_3, and 10 μM NE were added during the last 4 h before recollection. UCP mRNA, rRNA stained with methylene blue, and densitometric analysis are shown (means ± SE of 2 experiments). All increases due to NE were significant ($P < 0.05$) vs. basal levels, except hypothyroid at *day 8*. *$P < 0.05$ vs. preceding T_3 dose.

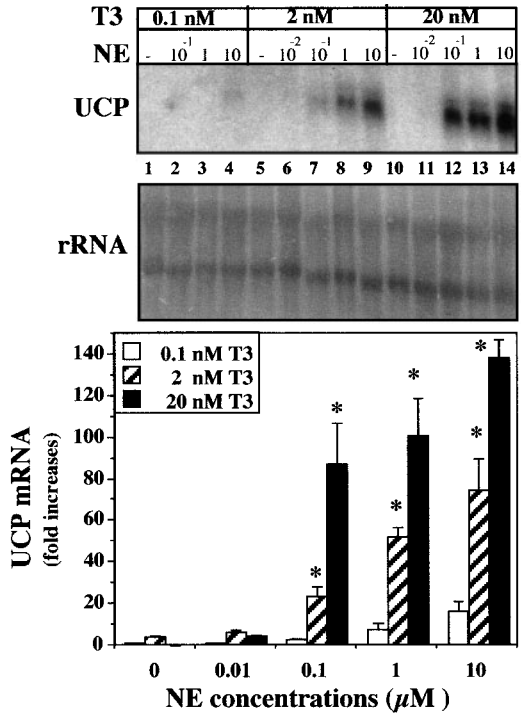

Fig. 3. T_3 potentiates effect of NE on UCP expression. Cultures of rat brown preadipocytes, grown in standard conditions, were maintained during the last 24 h in medium containing 10% hypothyroid serum, supplemented with 0.1, 2, or 20 nM T_3. Increasing doses of NE (0, 0.1, 1, or 10 μM) were added during the last 4 h before recollection at *day 7*. UCP mRNA, ribosomol RNAs (rRNA) stained with methylene blue, and densitometric analysis are shown (means ± SE of 2 experiments). *$P < 0.05$ vs. basal levels.

demonstrates that the potentiation of the adrenergic response by T_3 is also obtained at very low T_3 concentrations. Higher T_3 doses further increased UCP mRNA (see Figs. 2, 3, and 5), a plateau being reached at 20 nM T_3 (with DMEM+10% hypothyroid medium).

Kinetics of induction of UCP mRNA with NE and T_3. We also examined the minimal time required for both NE and T_3 to increase UCP mRNA levels. The kinetics of induction by NE are shown in Fig. 5*A* in cells preexposed for 24 h to 2 or 20 nM T_3. NE requires <2 h to increase UCP mRNA, and the effect is quicker when 20 nM T_3 is used, because at 2 h a strong signal is already observed. The effects are prolonged throughout 24 h and amplified at the larger T_3 dose.

The kinetics of induction for T_3 were also studied using two T_3 doses (2 and 20 nM) during 4, 8, 16, or 28 h, when NE (10 μM) was added during the last 4 h (Fig. 5*B*). The effect of T_3 on the adrenergic response is quick, as it is obtained in <4 h, and it is proportional to the dose of T_3 used ($P < 0.05$).

Effect of CHX and Act D on UCP gene expression. The effect of T_3 on the adrenergic stimulation is inhibited by CHX, so it requires de novo protein synthesis. Figure 6*A* shows that CHX inhibits the induction observed by use of NE, as well as NE+T_3, at two different doses, after either short (4 h) or long incubations (16–24 h). The experiment was repeated several times and in different conditions, and usually the inhibition was not complete (50–70%).

We tested the effect of several doses of CHX (25, 15, and 7.5 μM). These doses inhibited protein synthesis by 95.8, 94.1, and 91.8%, respectively, during 18 h, as measured by the incorporation of ^{35}S-methionine into TCA-precipitable proteins. The CHX doses were added 0.5 h before T_3 (18.5 h) or 0.5 h before NE (6.5 h). Figure 6*B* shows that all the CHX doses inhibited UCP expression, with a diminution of the effect at the lower CHX dose used (7.5 μM) and at shorter times (6.5 h, just before NE, *lanes 7*, *8*, and *9*). Act D (*lane 3*) completely blocked the induction observed with the combined use of NE+T_3 (Fig. 6*B*).

Stabilization of UCP mRNA by T_3. Figure 7 shows the effect of T_3 on the stabilization of the UCP mRNA transcripts. The adrenergic stimulation of UCP mRNA was tested by using two doses of T_3, 10 nM and 0.5 nM T_3, in HS in the presence of NE. Act D was added after 12-h exposure to NE+T_3, and cells were collected at different times from 2 to 9 h. Results show that T_3 stabilizes the UCP mRNA transcripts, increasing UCP mRNA half-life from 5 to >24 h.

Other experiments were done in which different T_3 doses were used (from 0.1 and 10 nM T_3 in hypothyroid medium), and the results obtained were similar, but frequently the effect of the lower T_3 doses (0.1–0.5 nM T_3) already stabilized UCP mRNA transcripts, preventing the discrimination between the low and high T_3 doses. The experiment presented above rendered the clearest results. Experiments in the complete absence of T_3, with HS, gave undetectable UCP mRNA levels. Experiments in the presence of 10% NCS rendered a UCP mRNA half-life of nearly 5 h.

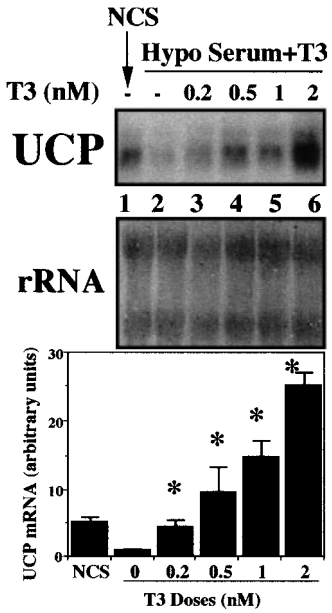

Fig. 4. Dose-dependent induction of UCP mRNA using NE and T_3. Cultures of rat brown preadipocytes, grown in standard conditions, were maintained during the last 24 h in medium containing 10% hypothyroid serum, supplemented with increasing doses of T_3 (0, 0.2, 0.5, 1, or 2 nM). NE (10 μM) was added during the last 4 h before recollection at *day 8*. UCP mRNA, rRNA stained with methylene blue, and densitometric analysis are shown (means ± SE of 2 experiments). All increases due to T_3 doses were significant ($P < 0.05$) vs. basal levels.

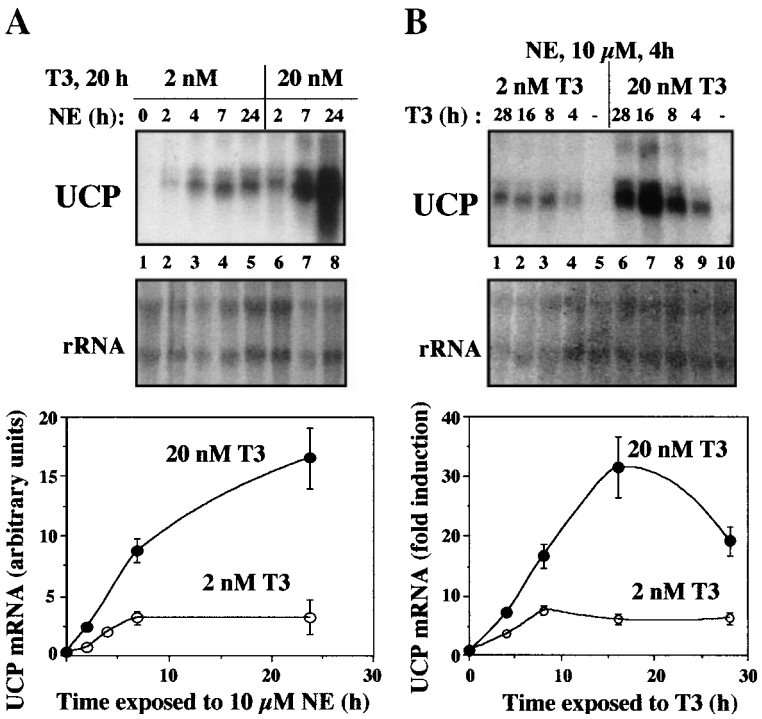

Fig. 5. Kinetics of induction of UCP mRNA by NE and T_3. *A*: time course for UCP induction by use of NE. Cultures of rat brown preadipocytes, grown in standard conditions, were maintained during the last 24 h in medium containing 10% hypothyroid serum, supplemented with 2 or 20 nM T_3. NE (10 μM) was added and cells were collected 2, 4, 7, or 24 h later. *B*: time course for UCP induction by use of T_3. Cultures of rat brown preadipocytes, grown in standard conditions, were maintained during the last 24 h in medium containing 10% hypothyroid serum, supplemented with 2 or 20 nM T_3 for different times (4, 8, 16, or 28 h). In each case, 10 μM NE were added during the last 4 h before RNA extraction. UCP mRNA, rRNA stained with methylene blue, and densitometric analysis are shown (means ± SE of 2 experiments). All increases were significant ($P < 0.05$) vs. basal levels and different between T_3 doses.

Effects of RA and T_4. The effects of RA and T_4 on the adrenergic response were also examined and, in general, were found to be less strong than when T_3 was used. Figure 8 shows a dose-response curve for T_4+NE. The adrenergic stimulation is higher at either low or high T_4 concentrations, and lower at the intermediate T_4 doses. This experiment was repeated 3 times, with doses ranging from 0.1 to 20 nM T_4. The effect of T_4+NE (at low doses) is much lower than that of T_3+NE (5 times lower, results not shown). In Fig. 9 we show the effect of RA alone or with NE or NE and T_3, compared with T_3. A clear effect of RA+NE is observed, although it is lower than that of T_3+NE. RA does not synergize with T_3 or T_3+NE.

DISCUSSION

BAT thermogenesis is stimulated in vivo by the NE released in the sympathetic nerve endings. The adrenergic stimuli induce the expression of UCP at the transcriptional level, as demonstrated in vivo in rats (3, 6, 16, 35, 38) as well as in vitro, by use of cultures of mouse brown adipocytes or cell lines (9, 21, 36). Most of these increases are mediated through β-adrenergic receptors, mainly β_3-adrenergic receptors (9, 17–19, 21, 30, 36, 39), although the participation of an α_1-adrenergic component has also been suggested (36). Most of the studies published on the regulation of UCP mRNA expression have been performed using cultures of brown adipocytes from mice or hamsters, species in which the adrenergic stimulation is more easily demonstrated (36), whereas studies using rat brown adipocytes are scarce (9, 11). The reasons for this lack of successful studies have not been clarified.

In the present paper, we show that UCP mRNA can be stimulated adrenergically in primary cultures of rat brown adipocytes obtained from precursor cells, demonstrating that the cells obtained after proliferation and differentiation in culture are true brown adipocytes, because UCP expression is the specific marker of BAT. The adrenergic stimulation of UCP, although weak, is obtained at any day after the cells reach confluence, indicating that, even when the accumulation of lipids and lipogenic markers begins on later days (10, 29), the cells are equipped for thermogenic functions and behave earlier as brown adipocytes. In the absence of NE, the basal expression of UCP is absent when standard culture conditions are used. Maximal adrenergic stimulation of UCP is obtained around confluence, and no increases are observed during active proliferation. In this aspect, the results obtained in rat primary cultures are similar to the ones described when primary cultures of mouse preadipocytes are used (36). The stimulation by NE is quick (2 h), suggesting, as described in vivo, an activation at the transcriptional level (3, 35). We observed that the stimulation is via β-adrenergic receptors, in agreement with data obtained by other authors (9, 19, 21, 36), and it is fully mimicked by β_3-adrenergic agonists (unpublished observations).

Nevertheless, the adrenergic stimulation of UCP mRNA in primary cultures of rat adipocytes is generally weak or absent (even when different sources of serum are used), and the optimal conditions for such studies have not been described, except for one brief study that compares UCP mRNA expression in mouse and rat primary cultures (9). Thus studies on the regulation of UCP mRNA in cultured rat brown adipocytes are scarce (11). The low levels of UCP mRNA observed in rat cultures after adrenergic stimulation can be attributed to *1*) growth factors or hormones present in the serum used in the cultures that might

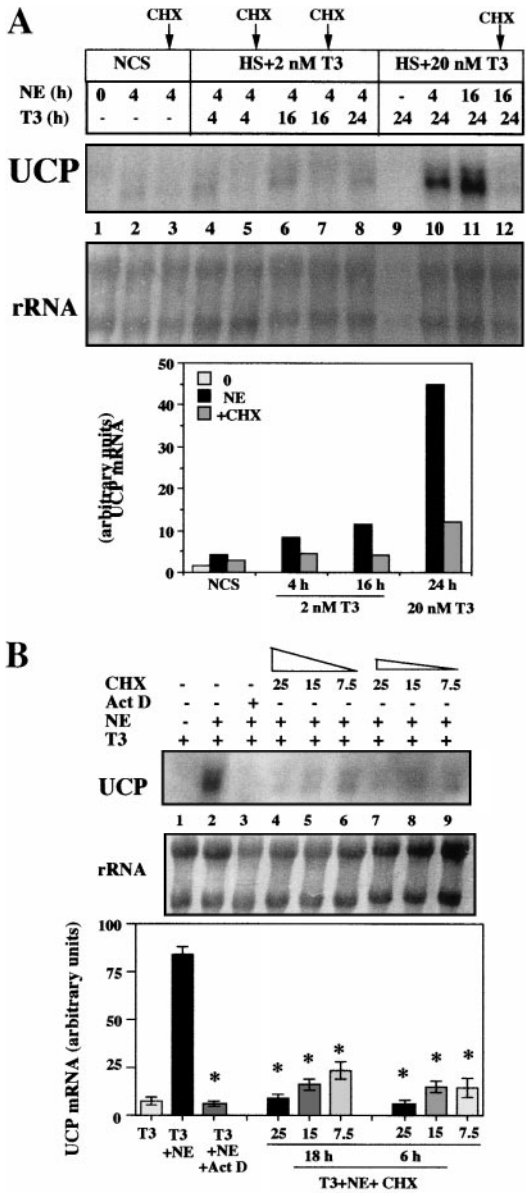

Fig. 6. Effect of cycloheximide (CHX) and actinomycin D (Act D) on UCP mRNA. *A*: cultures of rat brown preadipocytes, grown in standard conditions until *day 6*, were maintained during the last 24 h in medium containing 10% NCS (*lanes 1–3*) or 10% hypothyroid serum (HS). HS was supplemented with 2 or 20 nM T_3 for 4, 16, or 24 h. NE (10 μM) was added during the last 4 or 16 h. CHX (15 μM) was added to some tubes 30 min before NE (*lanes 3, 5, 7,* and *12*). *B*: cultures of rat brown preadipocytes, grown in standard conditions until *day 7*, were maintained during the last 18 h in DMEM containing 10% HS supplemented with 5 nM T_3. NE (3 μM) was added during the last 6 h before cell recollection. Act D (5 μg/ml) was added 30 min before T_3 addition (*lane 3*). Three doses of CHX (25, 15, and 7.5 μM) were added 30 min before T_3 addition (18.5 h, *lanes 4, 5,* and *6*) or 30 min before NE addition (6.5 h, *lanes 7, 8,* and *9*). Hybridization with a cyclophilin cDNA was used to correct for differences between lanes (means ± SE of 3 experiments). $*P < 0.05$ vs. NE+T_3.

inhibit the expression of UCP or *2*) the low amount of T_3 present in the culture medium, which we demonstrated in this study to be essential for the NE effect. In fact, although cellular T_4 concentrations are normal, T_4 concentrations measured in cultures using 10% NCS were ~0.07 nM T_3 in the medium and 0.7 nM T_3 in the cells, resulting in a cellular T_3 concentration 5–6 times lower than that found in BAT in vivo (3–4 nM, which increases to 8 nM T_3 after cold exposure; our unpublished results). Thus we came to the conclusion that our cells are really in hypothyroid conditions. This seems to be due to the presence of active pathways for T_3 degradation, namely, inner ring deiodinase activity (DIII), activated in response to the serum used for culture (13, 15). We have also demonstrated that, in the same cultures of brown adipocytes (14), a minimal amount of T_3 is required for the adrenergic activation of DII, the enzyme responsible for T_3 production in BAT.

The main finding of the present work is that T_3 is absolutely necessary for the adrenergic stimulation of UCP expression, reinforcing the results obtained in rats in vivo and in floating adipocytes (2, 3, 46). In the absence of thyroid hormones (hypothyroid medium), the adrenergic stimulation of UCP is almost undetectable, and T_3 potentiates the effect of a given NE dose. We demonstrate that the effect of T_3 takes place at any day after confluence and is proportional to the dose and time exposure to T_3. T_3 amplifies the adrenergic stimulation of UCP by ~30-fold, depending on the T_3 dose used, and a 10-fold increase in T_3 concentrations multiplies the NE effect by 100-fold. This gives an idea of the magnitude and importance of the T_3 effect. This T_3 effect is already observed at very low T_3 concentrations (0.2 nM) in the lower physiological range. These facts are easier to demonstrate in culture systems, where those small changes can be handled, whereas in vivo, potent mechanisms are present to maintain stable and high T_3 concentrations in BAT (increased T_3 uptake and high 5' deiodination), so that the diminution of T_3 concentrations and therefore of UCP expression would be more difficult to show (26).

The effect of T_3 is detected in <4 h, suggesting an effect on transcription, as already described (3, 35, 12), although a maximal effect requires longer times with the use of physiological T_3 concentrations. This fact suggests that the effect of T_3 requires the synthesis of an intermediate product and possibly de novo protein synthesis. The experiments using CHX demonstrate that this is the case. It is difficult to discern whether the inhibitory effect of CHX is exerted on the action of T_3 or of NE, as separately each does not have an effect. The inhibition seems to be exerted on the combined use of NE+T_3, possibly on a protein required for its combined action. Nevertheless, the effect of CHX is more potent when added before T_3 (18 h) than when added after T_3 and just before NE (6 h), suggesting that T_3 per se is able to induce the synthesis of a protein or transcription factor required for the adrenergic stimulation of UCP. These data are contrary to those reported in vivo, where the T_3 effect did not require de novo protein synthesis (3).

Our data using Act D and the prompt responses observed suggest that the effect is clearly at the transcriptional level, confirming previous results (12, 35, 36). However, the full T_3 effect is not only exerted at the transcriptional level, because the effect on the stabiliza-

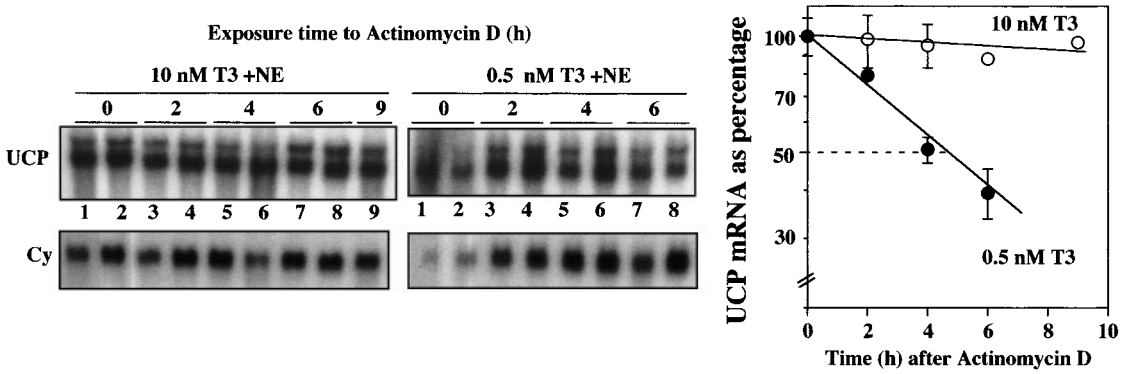

Fig. 7. Stabilization by T_3 of UCP mRNA transcripts. Cultures of brown preadipocytes, grown in standard conditions until *day 6*, were maintained during the last 20 h in medium containing 10% HS supplemented with 10 or 0.5 nM T_3, and NE (2 μM) was added during the last 12 h. Thereafter, Act D (5 μg/ml) was added and cells were harvested 2, 4, 6, and 9 h later. UCP mRNA, cyclophilin cDNA, and densitometric analysis are shown (means ± SE of 2 experiments).

tion of the mRNA transcripts seems even more important, contributing to a marked elongation of UCP mRNA half-life. Previous in vivo studies (35) described a brisk increase in transcription during the first hour of exposure of rats to cold, whereas the stabilization effect takes place at longer times (8 h). In our cultured cells, the stabilization effect is important even at low doses of T_3, making difficult a discrimination between low and high T_3 doses.

Important studies have been conducted to study the effect of T_3 on UCP at the promoter level, and an enhancer region has been defined in the −2490/−2280 bp of the rat UCP gene promoter (8, 34). Two TREs have been defined at −2391/−2376 (upTRE) and at −2348/−2334 (downTRE) (34), both of which seem needed for the stimulation of UCP and the downTRE for the potentiation of the cAMP effect by T_3. Another group of investigators (1) has identified three RAREs, in the same region as the downTRE, at −2357/−2330 (1), whereas the former authors (33) define the RAREs further upstream of the upTRE region (−2490/−2399). It is very much a possibility that this enhancer region might act promiscuously with both RA and T_3 to enhance UCP mRNA expression, and the results depend on the specific proteins present in the transfection cell system used or the origin of the cells used (32). In contrast to the mouse UCP gene promoter in which cAMP response elements (CREs) were described in an enhancer element located in an enhancer region similar to that of the rat promoter (20), CREs were not defined in the rat UCP promoter until recently (48), one near the proximal promoter region and a second one within the enhancer element described above (32). It seems that the synergism between NE and T_3 involves interaction with a protein or coactivator, but this area demands further investigation.

T_3 has also been described to induce per se the transcription of UCP mRNA without the concurrence of NE (12) and to stabilize its mRNA in primary cultures of fetal brown adipocytes. It is not clear whether this fact is linked to the fetal state of the donor animals or to the culture conditions used (serum-free medium), pointing to a possible inhibitory effect of serum on UCP mRNA expression.

In our cultures, T_4 also has an effect on the adrenergic stimulation of UCP mRNA expression. This effect is much lower than that of T_3 and follows a biphasic pattern, suggesting that at low T_4 doses (0.2 nM), the effect is mediated through the generation of T_3 from T_4

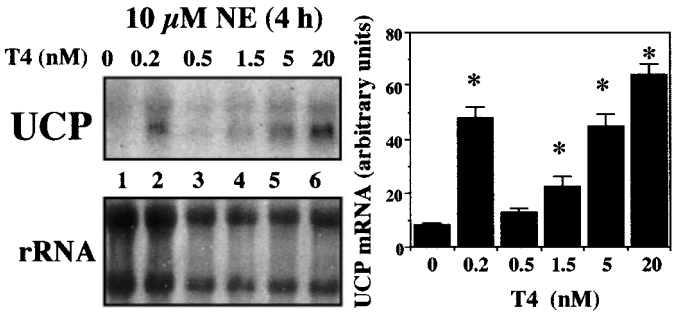

Fig. 8. Effect of thyroxine (T_4) on adrenergic increases of UCP mRNA expression. Cultures of rat brown preadipocytes, grown in standard conditions until *day 7*, were maintained during the last 16 h in serum-free medium supplemented with increasing doses of T_4 (0, 0.2, 0.5, 1.5, 5, or 20 nM T_4). NE (10 μM) was added during the last 4 h before recollection. UCP mRNA, rRNA stained with methylene blue, and densitometric analysis are shown (means ± SE of 3 experiments). *$P < 0.05$ vs. basal levels.

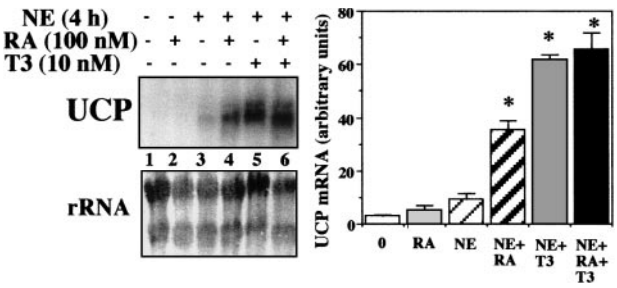

Fig. 9. Effect of retinoic acid (RA) on UCP mRNA expression. Cultures of rat brown preadipocytes, grown in standard conditions until *day 7*, were maintained during the last 24 h in medium containing 10% HS. T_3 (5 nM), RA (100 nM), or both were added during the last 18 h, and NE was added during the last 4 h. UCP mRNA, rRNA stained with methylene blue, and densitometric analysis are shown (means ± SE of 4 experiments). *$P < 0.05$ vs. basal levels.

through DII activity, when DII is not inhibited by T_4 (45), whereas at higher T_4 doses that inhibit DII, it might be mediated through the occupancy of nuclear T_3 receptors by T_4. We find striking similarities between the adrenergic stimulation of DII activity and UCP mRNA expression with respect to the T_3 requirements (14). Both genes require the combined presence of T_3 and NE to get adrenergic increases. In both, the potentiating effect of T_3 is large, increases with time, and requires protein synthesis, and in both UCP and DII, NE or T_3 alone does not exert per se a significant effect.

We have also found an effect of RA on the adrenergic stimulation of UCP, somewhat lower than that of T_3. This is contrary to other reports using cultured murine adipocytes (1, 31), where RA exerts a higher effect than T_3. Nevertheless, it has to be taken into account that, in the last report, T_3 was added to the medium to induce differentiation, and the effect of RA should only be considered as "pure" when depleted serum + RA were used. The effects we observed are not synergic or additive. This suggests that RA and T_3 use the same pathway to increase the adrenergic stimulation of UCP, competing for the same sites or for the retinoid X receptor present, because no synergism is found between T_3 and RA. These results are also different from the only report using floating rat brown adipocytes stimulated with NE (33).

In summary, we have studied the conditions required for the adrenergic stimulation of UCP in primary cultures of rat brown adipocytes, especially with respect to the need for T_3. We conclude that there is an absolute need of T_3 for the adrenergic stimulation of UCP mRNA, because in hypothyroid conditions, basal and adrenergically induced UCP expression is absent. The role of T_3 in the stabilization of the mRNA transcripts is also of major importance. However, the T_3 effects are exerted at multiple levels: at the UCP promoter, contributing to the stabilization of the transcripts, and at other levels, such as the modulation of the β-adrenergic receptor population (41, 42).

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Address for reprint requests and other correspondence: M. J. Obregón, Instituto Investigaciones Biomédicas (C.S.I.C.), Arturo Duperier 4, 28029 Madrid, Spain (E-mail: mjobregon@iib.uam.es).

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