Triiodothyronine Induces the Transcription of the Uncoupling Protein Gene and Stabilizes Its mRNA in Fetal Rat Brown Adipocyte Primary Cultures*

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The main feature of brown adipose tissue (BAT) is heat production by a mechanism generally called “non-shivering thermogenesis.” This function is carried out by a protein known as uncoupling protein (UCP), localized in the mitochondrial inner membrane, which uncouples the electron transport chain from ATP synthesis, dissipating the proton-electrochemical gradient as heat (1). BAT is activated under certain conditions: in newborn mammals, during cold adaptation, in arousal from hibernation, and in overfeeding (for review, see Ref. 2).

BAT starts to differentiate in rat fetuses during late gestation and reaches its maximal development and thermogenic capacity during the first postnatal week (3, 4). The postnatal development could be due to the noradrenaline (NA) released by the sympathetic fibers as a consequence of the environmental temperature drop after birth, when the newborn faces extrauterine life (5). Noradrenaline induces UCP expression through a β-adrenergic mechanism at the transcriptional level (6, 7). Triiodothyronine (T3) has been reported to be required for the optimal UCP gene expression in response to the noradrenergic stimulus in vivo (8), in brown adipocyte primary cultures (9) and in dispersed brown adipocytes (10). During the perinatal period, the earliest UCP expression is detected in the last days of fetal life, under euthermic conditions (4, 11). Therefore, UCP gene expression before birth must be controlled by mechanisms different from the noradrenergic-dependent induction occurring after cold stimulation. At this stage, other hormones or growth factors can influence BAT development and UCP gene expression. One candidate is the thyroid hormone, which can be maternally provided at early gestation (12, 13) or later produced by the fetus itself (14).

Studies in vivo to determine the signal(s) involved in the development of BAT during fetal stages are rather limited. However, primary cultures provide a useful tool for such studies. We have recently described that T3 per se induces UCP expression after long term treatment of fetal brown adipocytes in culture (15). The mechanism by which T3 induces UCP remains to be established. Accordingly, the aim of this work was to study the T3 mechanism of action on the UCP gene expression using rat fetal brown adipocyte primary cultures. Our results not only confirm our previous data showing the role of T3 per se in the UCP expression in the absence of catecholamines (15), but also show that T3 increases the transcription rate of UCP expression and stabilizes the UCP mRNA. The UCP mRNA and functional UCP content are thereby increased.

EXPERIMENTAL PROCEDURES

Materials—Fetal and neonatal calf serum and culture media were from Imperial Laboratories (Hampshire, United Kingdom). AG-1-XB anion exchange resin was from Bio-Rad. Proteinase K and collagenase were from Boehringer Mannheim. Triiodothyronine, noradrenaline, cycloheximide, and actinomycin D were from Sigma. [3H]GDP; [α-32P]dCTP and [α-32P]UTP (650 Ci/mmol) were from Amersham (Buckinghamshire, United Kingdom). Nick-translation DNA-labeling system kit was from Life Technologies, Inc. (Middlesex, United Kingdom). Nucleotides for transcription assays were from Pharmacia Biotech Inc. Rabbit anti-UCP serum and rat cDNA probe for the UCP were kindly provided by Dr. E. Rial and Dr. D. Ricquier, respectively. All other chemicals were of reagent grade or of the highest purity available.

Isolation and Culture of Brown Adipocytes—Immature fetal brown adipocytes were isolated from 20-day-old Wistar rat fetuses by collagenase disgregation method according to Lorenzo et al. (16) and cultured until confluence under conditions similar to those previously described by Guerra et al. (17). The neonatal calf serum used was depleted of thyroid hormones by anion exchange chromatography, using resin AG-1-XB from Bio-Rad (18); the extent of removal and final concentrations of thyroid hormones were checked by solid-phase RIA as described previously (15). After confluence, cells were maintained for 24 h in a serum-free culture medium before starting hormonal treatments. Cells were subsequently maintained for additional periods of time up to 72 h (as stated in the figure legends) in a serum-free medium supplemented with 100 μM ascorbate (to prevent oxidation of adrenergic agents), in the absence or presence of 10 nM T3, 1 μM NA, or T3 plus NA. Culture medium was always replaced every 24 h.

Cells were harvested after confluence plus 24 h in the absence of serum (CC), or after different periods (as stated in the figure legends) of subsequent culture in the absence (control cells, C) or presence of hormones.

UCP mRNA stability (half-life, τ1/2) was measured in confluent 24-h serum-starved cells after exposure to T3 or NA for 16 h in the culture conditions above. UCP mRNA was quantified by nick-translated rat cDNA probe by a method similar to that described by Lorenzo et al. (16). 

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medium in the absence of serum, in order to induce UCP gene expression. After T3 or NA treatment, actinomycin D or cycloheximide (CHX) were added to the cultures at 10 and 7 μg/ml, respectively, in the absence of hormones, and cells were harvested at different periods of time, as indicated in the figure legends. Control experiments established that these concentrations of inhibitors reduced radiolabeled uridine and leucine incorporation, respectively, by more than 96% during the course of the experimental time periods.

RNA Extraction and Northern Blot Analysis—Total RNA was isolated by the guanidinium thiocyanate-phenol-chloroform method (19), separated by electrophoresis on agarose/formaldehyde gels, then transferred to membranes and hybridized as described previously (17). cDNA probes for UCP (20) and 18 S ribosomal RNA were labeled with [α-32P]deoxy-CTP by nick translation.

Nuclear Run-on Transcription Assays—Nuclei were isolated from the pooled cells of 10–12 plates (21, 22) and stored at −70°C in 50 mM Hepes, pH 7.9, 150 mM NaCl, 0.1 mM EDTA, 5 mM dithiothreitol, 0.125 mM phenylmethylsulfonyl fluoride, and 50% glycerol. The in vitro elongation reactions were carried out as described (23, 24). 32P-Labeled RNA transcripts were purified by the method of Linial et al. (25) using Nick columns (Pharmacia) and following the manufacturer’s instructions. Denaturation of DNA probes, application of DNA to GeneScreen membranes, hybridization of the 32P-labeled RNA transcripts to the membrane-bound DNA, posthybridization washes, and autoradiography were carried out as described by Roncero and Goodridge (26).

Quantitation of Results from Northern Blots and Nuclear Run-on Transcription Assays—Northern blots from the mRNA experiments and slot blots from the transcription run-on assays were subjected to autoradiography. Different exposure times were used for computer-assisted densitometry (laser densitometer from Molecular Dynamics, Sunnyvale, CA) being linear at all densities studied. The results were expressed as mean ± S.E. of the number of independent experiments, as indicated in the figure legends.

Mitochondria Isolation and Determination of GDP-binding Capacity—Mitochondria were isolated and the GDP-binding capacity determined as described previously (27). Protein content was determined by the Lowry method as modified by Petterson (28).

Immunological Detection of the Uncoupling Protein—Western blot analysis and immunodetection of the UCP by specific rabbit anti-UCP serum were performed as described previously (27).

Statistical Analysis—Differences between means were statistically compared by analysis of variance, using Statview 1.03 for the Macintosh. Data were compared among them by the Scheffe F-test and considered significantly different when p < 0.05.

RESULTS

Effect of T3 on UCP mRNA Content—Fetal rat brown adipocyte primary cultures were used throughout this work. After 7 days under our culture conditions, cells reached confluence, which declined when the hormone was present up to 24 h (25- and 30-fold increase, respectively, compared to time 0), which declined when the hormone was present up to 24 h (8-fold). Treatment with T3 progressively increased the UCP mRNA content up to 12-fold after 24 h. The increase of UCP mRNA levels induced by T3 was significantly diminished by the presence of cycloheximide, which suggests that the effect of T3 or NA depends on the ongoing protein synthesis.

Effect of T3 on UCP Gene Transcription Rate—Nuclear run-on assays were performed to examine the effect of T3 on UCP mRNA synthesis (Fig. 3). Transcriptionally active nuclei were isolated from 24-h serum-starved confluent cells maintained for 1 h in the presence of 10 nM T3 or 1 μM NA to induce gene transcription. Parallel experiments were carried out in the presence of CHX. The UCP transcription rate in the T3- or NA-treated cells was 7- and 5.5-fold higher than that obtained in the control cells (C), respectively. These data demonstrate that T3 increases UCP gene transcription in fetal rat brown adipocytes, as does the positive control (NA). T3 and NA effects were significantly reduced when protein synthesis was abolished by the presence

\[ T_3 \text{ induces UCP gene transcription and UCP mRNA stabilization} \]
of cycloheximide (CHX +) (1.5- and 2-fold compared to control), confirming their dependence on protein synthesis, as suggested by results in Fig. 2.

Effect of T3 on UCP mRNA Stability—24-h serum-starved confluent cells were treated with 10 nM T3 or 1 μM NA for 16 h to induce UCP gene expression and obtain sufficient UCP mRNA levels for the study of its decay as a function of time (up to 24 h), after removing T3 or NA from the medium. This was performed in the presence or absence of actinomycin D or CHX.

At the indicated periods of time, total RNA was isolated and UCP mRNA levels calculated by densitometric scanning of the Northern blots (Fig. 4). UCP mRNA half-life values were calculated from data shown in Fig. 4 and confirmed by semilogarithmic representations.

The NA-induced UCP mRNA showed a very short half-life (4 ± 1 h), which was increased in the presence of actinomycin D (10 ± 2 h) or cycloheximide (22 ± 2 h; p < 0.05). However, the UCP mRNA induced by T3 was very stable, showing a calculated half-life of more than 24 h (p < 0.001 compared to the NA value). This mRNA stability decreased in the presence of actinomycin D or CHX.

Effect of T3 on the Mitochondrial UCP Levels—The UCP levels were studied by Western blot analysis of the mitochondrial proteins isolated from confluent cells and from cells maintained in culture for additional 72 h period in the absence (C) or presence of 10 nM T3, and/or 1 μM NA. Results from Western blot analysis (Fig. 5) revealed that confluent cells contained a very low amount of UCP (observed as a band of 32 kDa of molecular mass), which diminished in those samples from C cells. However, the presence of T3 or/and NA in the culture
medium produced an ~2-2.5-fold increase in the UCP level.

In order to assess the functional activity of the UCP and its correlation with UCP content, the mitochondrial GDP-binding capacity of the cells in culture was measured (Table I). B$_{\text{max}}$ values under the different hormonal treatments were calculated and showed a good correlation with the UCP content derived from the Western blot analysis. No statistically significant changes in the dissociation constants (K$_d$) values were observed.

These results show that fetal rat brown adipocytes in culture respond to the T$_3$ treatment, increasing both the amount of UCP present in the mitochondria and its functional content.

**DISCUSSION**

The immature brown adipocytes from rat fetuses proliferated and reached confluence under our culture conditions. Confluent cells show low levels of UCP mRNA, providing useful starting material to study the induction of UCP gene expression. These cells were used to study whether T$_3$ plays a direct role on the UCP gene expression, in the absence of noradrenergic stimulation.

Maintenance of confluent cells (Cc) in culture in the absence of serum for various periods of time, up to 72 h (C) produced a decrease in the levels of UCP mRNA (Fig. 1). However, treatment of cells with T$_3$ during these periods of time induced an increase in the UCP mRNA content, reaching maximum levels after 48 h of treatment. These UCP mRNA levels were significantly higher than those obtained after a 48-h treatment with 1 $\mu$m NA used as a positive control. When shorter periods of treatment were analyzed (Fig. 2), results showed that T$_3$ induced a slow but progressive increase in the levels of the UCP mRNA. Klaus et al. (29) reported that treatment of brown adipocytes differentiated in culture with T$_3$ plus insulin caused a slow increase of UCP mRNA content. However, in their cell model T$_3$ alone did not change UCP mRNA levels but it did prevent the loss of UCP mRNA content after serum removal. Fig. 2 also shows that NA induced a quick and potent increase in the UCP mRNA content, reaching a maximum between 5 and 12 h of treatment. A similar pattern of rapid response to NA or $\beta$-adrenergic agonists was reported to occur in primary cultures of brown adipocytes (29) and in brown adipocyte cell lines derived from brown fat tumors of transgenic mice (30, 31).

The UCP mRNA levels observed in cells treated for 48 or 72 h with NA were significantly lower than those in cells treated with T$_3$. This fact could be explained by a shorter half-life of the UCP mRNA in the NA-treated cells, as deduced from results shown in Fig. 4 and Table I. Moreover, a $\beta$-adrenergic receptor desensitization during long treatment with NA, which has been described for many systems (32), cannot be excluded.

Results from nuclear run-on assays (Fig. 3) revealed that addition of T$_3$ to the culture medium increased the transcription rate of UCP gene up to values similar to those induced by

**Table I**

| Tissue | B$_{\text{max}}$ (cpm/10$^5$ cells) | K$_d$ (nmol/mg protein) | NA < 0.001 |
|---|---|---|---|
| NA | 2.96 | 0.25 | 0.0001 |
| NA + T$_3$ | 3.16 | 0.23 | 0.0001 |

a Significantly different from control cells: p < 0.05.
b Significantly different from control cells: p < 0.01.
NA treatment. Yeh et al. (33) obtained indirect evidence of the \( T_3 \) effect on UCP expression in vivo, where \( T_3 \) treatment of derenervated BAT produced an increment in UCP expression. Cassard-Doulcier et al. (34) identified a domain in the UCP gene promoter containing a directly repeated sequence that was able to bind retinoic X and triiodothyronine receptors. Furthermore, after submission of this manuscript, Rabelo et al. (35) reported that \( T_3 \) was able to directly stimulate the rat UCP gene, via its receptors acting on two far upstream thyroid hormone response elements (positioned between \(-2391\) and \(-2334\)). In that report, transient transfection experiments were performed in \( JEG-3 \) cells (a human placental cell line) as well as in \( H1B-1B \) cells (a brown fat cell line established from hibernoma induced in transgenic mice). These results obtained by means of a transfected reporter gene support the \( T_3 \) effect on the endogenous UCP gene activation here described.

The presence of cycloheximide in the culture media significantly reduced the induction of UCP transcription rates (Fig. 3) in response to \( T_3 \) and NA. This suggests that the \( T_3 \) and NA action depends on ongoing protein synthesis. We have shown previously (15) that \( T_3 \) induces the expression of its own nuclear receptors in fetal brown adipocytes in culture after a long term treatment, leading to an increased nuclear \( T_3 \) binding capacity and to an increased ability to respond to \( T_3 \). Therefore, the requirements for the ongoing protein synthesis in the \( T_3 \) increased UCP gene transcription rate are probably related to the \( T_3 \) induction of its own nuclear receptors expression, although other explanations cannot be excluded. From in vivo experiments carried out in adult rats, Bianco et al. (36) reported that the \( T_3 \) amplification of NA stimulation of UCP gene transcription was a mechanism not requiring protein synthesis. The discrepancy between our results obtained in fetal cells and those from in vivo can be explained by the following: (i) in the in vivo model the \( T_3 \) effect was observed using rats treated also by NA, and (ii) adult brown adipose tissue has a higher nuclear \( T_3 \) binding capacity (37) than fetal brown adipocytes in culture where a regulation of the thyroid hormone receptors by \( T_3 \) has also been described (15).

\( T_3 \) treatment of cells increased the UCP gene transcription rate and stabilized the UCP mRNA. The stabilization is deduced from the maintenance of UCP mRNA levels after removal of the hormone (Fig. 4). These experimental conditions are different from those reported by Bianco et al. (10) using isolated rat brown adipocytes, in which UCP mRNA levels decrease in cells in the absence of adrenergic stimulation but the presence of \( T_3 \) prevented this decline. However, they also reported that \( T_3 \) per se failed to increase UCP mRNA levels.

The short half-life of the UCP mRNA in the NA-treated cells (Fig. 4) agrees with similar data obtained from in vivo experiments, both in mice (38) and rats (39) after cold stimulation, where the main action is due to the NA released from the sympathetic innervation of the tissue. Picó et al. (40) also reported that the UCP mRNA levels induced in vitro by NA treatment of mouse brown adipocytes maintained in primary cultures decayed very rapidly after NA removal from the medium (half-life was 3.7 h). From our results and those already cited (38 - 40), it is deduced that NA not only increases the UCP gene transcription rate but also destabilizes the messenger, assuring a rapid decrease of UCP mRNA levels once the signal is abolished.

The NA and/or \( T_3 \) treatment increased the UCP mRNA content in the cells (by 8–18-fold depending on hormonal treatment after 48 – 72 h) and also the UCP present in the mitochondrial fraction (by 2–2.5 fold, Fig. 5, Table I). In terms of quantity, the increase in the mitochondrial UCP does not parallel the increase in UCP mRNA levels. It should be pointed out that the amount of functional UCP reached in the mitochondria after the in vitro treatments reported here is similar to that described as the maximum value reached in vivo during the perinatal development of the rat (4, 41). The difference between the increase in the UCP mRNA and the mitochondrial UCP levels in the NA- and/or \( T_3 \)-treated cells suggests the existence of posttranscriptional regulatory mechanisms. Puigserver et al. (42) have previously reported that the pool of newly synthesized UCP, after NA treatment of brown adipocytes in culture, is more susceptible to degradation than that which has become fully incorporated into the mitochondria.

In conclusion, our results show a direct effect of \( T_3 \) on UCP gene transcription and UCP mRNA stabilization, which produces increased levels of UCP mRNA in fetal brown adipocytes as well as a higher content of functional UCP in the mitochondrial fraction (Fig. 5, Table I). This effect was obtained in the absence of adrenergic stimulation, stressing the direct role of \( T_3 \) in UCP gene expression in fetal cells. Thus, \( T_3 \) might play a significant role in the in utero development of the fetal brown adipose tissue, where the noradrenergic mechanisms involved in cold stimulation are not operating. Furthermore, due to the \( \beta \)-adrenergic desensitization process, thyroid hormones might have a physiological relevance in the maintenance of high levels of UCP acutely induced by noradrenergic stimulation of brown adipose tissue.

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