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Adenosine 3′,5′-Monophosphate and Thyroid Hormone Control of Uncoupling Protein Messenger Ribonucleic Acid in Freshly Dispersed Brown Adipocytes*

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ABSTRACT. We intend to develop *in vitro* model systems to study the hormonal regulation of uncoupling protein (UCP) and its role in brown adipose tissue (BAT) thermogenesis. We report here that UCP mRNA responses to adrenergic and thyroid hormone manipulations in freshly dispersed, mature brown adipocytes mimic *in vivo* observations. Studies were performed in brown adipocytes obtained from interscapular brown fat of euthyroid or hypothyroid rats. The tissue was dispersed with collagenase, and cells were isolated by floatation over 4% BSA. UCP mRNA in these cells is 2–3 times more abundant than that in the whole tissue, indicating a selection of cells expressing the gene. In cells from euthyroid rats, UCP mRNA is maximally elevated within 2 h of exposure to 1 μM forskolin and 50 ng T_3/ml (77 nM total, 0.43 nM free). T_3 significantly enhances the effect of forskolin. In the absence of stimulation, UCP mRNA rapidly disappears from euthyroid brown adipocytes, and this can be prevented with the addition of T_3 by a mechanism not requiring on-going transcription. In cells from hypothyroid rats, forskolin or isoproterenol plus phenylephrine fail to stimulate UCP mRNA, but within 3 h of exposure to T_3, cells recover full responsiveness. As *in vivo*, a high extracellular concentration of T_3 is required for maximal responsiveness of UCP mRNA to cAMP, while T_4 can restore responsiveness in physiological concentrations (40 pM). This effect of T_4 is prevented by iopanoic acid, a compound that blocks the type II T_4 5′-deiodinase. In conclusion, 1) freshly dispersed brown adipocytes retain all of the properties concerning UCP regulation by thyroid hormone and sympathetic nervous system described for brown fat *in vivo*; 2) the observations made *in vivo*, thus, represent direct action of norepinephrine and thyroid hormone on these cells; 3) as *in vivo*, T_4 is a better source of intracellular T_3 than extracellular T_3 for brown adipocytes; hence, the *in vivo* findings are the result of the cell biology of 5′-deiodinase type II rather than dynamic factors inherent to the *in vivo* condition; 4) stabilization of mature UCP mRNA by T_3 is an important mechanism to maintain the levels of this mRNA elevated under sustained stimulation; and 5) dispersed brown adipocytes and UCP gene products constitute a powerful model to study interactions between the sympathetic nervous system and thyroid hormone at a cellular or molecular level. (*Endocrinology* **130:** 2625–2633, 1992)

BROWN adipose tissue (BAT) appears to be the main site of facultative thermogenesis in small rodents and probably most mammalian species during the newborn period (1–3). The key element for the energy dissipation capacity of this tissue is uncoupling protein (UCP) or thermogenin, a 32,000-dalton mol wt protein uniquely expressed in the inner membrane of BAT mitochondria. UCP dissipates the proton gradient created by the respiratory chain, thereby accelerating respiration in an uncoupled fashion (2, 4). BAT thermogenesis is activated by the sympathetic nervous system. Among other effects, the adrenergic stimulation releases fatty acids and induces the synthesis of this protein. Fatty acids activate preexisting UCP and serve as fuel for the enhanced oxidations (2). UCP determines the thermogenic potential of BAT. This protein is encoded in the nuclear DNA, and its mRNA increases promptly after sympathetic stimulation (5–7).

We have previously demonstrated that T_3 plays a critical role in the expression of UCP. In hypothyroid rats, UCP does not increase after cold exposure or exogenous norepinephrine (NE), and the injection of T_3 rapidly restores the responsiveness of UCP to adrenergic stimulation in a dose-dependent fashion (7–10). While it is possible to demonstrate a weak stimulation of UCP gene transcription by NE in the absence of T_3, in the absence of NE, T_3 does not significantly increase the levels of this mRNA (7). T_3 amplifies the transcriptional response to NE by several-fold, probably interacting at

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the level of the gene with a NE-dependent signal (7). To emulate in thyroidectomized rats the maximal UCP response seen in euthyroid rats acutely exposed to cold, it is necessary to maintain plasma T_3 levels over 100 times the euthyroid concentrations if only T_3 is given, while restoration of the euthyroid concentration of T_4 suffices to normalize the response (8, 9). This higher efficiency of T_4 is due to the adrenergic activation of BAT type II T_4 5′-deiodinase (5′D-II) (8, 9). Accordingly, in the euthyroid rat (11) or thyroidectomized rats receiving T_4 replacement (12), the adrenergic activation of 5′D-II by acute cold exposure generates enough T_3 to virtually saturate its nuclear receptors.

So far, it has not been possible to duplicate these results *in vitro*. Studies in brown adipocytes differentiated in primary cultures do not show the same dramatic effect of T_3 in UCP mRNA expression seen *in vivo*, nor has it been possible to demonstrate in these cells a role for 5′D-II as important as that observed *in vivo* (13). The possibility remains that part or all of these effects are the consequence of thyroid hormone actions elsewhere. For example, the preponderance of plasma T_4 over T_3 on BAT function *in vivo* may reflect an action of T_4 in the central nervous system, where plasma T_4 is the main source of intracellular T_3 (14, 15), and the generated T_3 might enhance the neuro-humoral stimulation of BAT. Alternatively, the *in vivo* observations may result from dynamic factors inherent to the *in vivo* condition. For example, a slow BAT-plasma T_3 exchange would be expected to reduce the uptake of plasma T_3, while it would facilitate retention of the T_3 produced locally by 5′D-II without this enzyme having to generate large quantities of T_3. Such dynamic mechanisms were tentatively invoked to explain the importance of local T_3 generation to brain T_3 content (15), but because of the lack of an appropriate *in vitro* system, this hypothesis has remained untested.

Freshly dispersed brown adipocytes respond to adrenergic stimulation in a variety of ways, among others by increasing respiration, fatty acid release, cAMP generation (16, 17), and 5′D-II activity (18–21). They, thus, appear as a potentially good experimental model to examine these questions left by *in vivo* studies and to further our knowledge of BAT physiology. Consequently, we examined the effect of thyroid hormone on the adrenergic stimulation of UCP mRNA in freshly isolated brown adipocytes.

Materials and Methods

Isolation of brown adipocytes

Our method was based on that reported by Fain *et al.* (22), as subsequently modified by Petterson and Vallin (23), Sundin *et al.* (16), and ourselves (19, 20). Further minor modifications, described below, were introduced to improve the responsiveness of the cells and optimize the recovery of BAT RNA. BAT was obtained from either euthyroid or hypothyroid rats. Handling of the animals was performed according to protocol 191–128–02, approved by the institutional Animal Care and Use Committee. Hypothyroidism was induced by thyroidectomy, followed by the administration of 0.03% methimazole in the drinking water. Animals were used not earlier than 3 weeks after thyroidectomy. Previous experiments showed that this regimen promptly brings serum T_4 and T_3 to undetectable levels, with complete arrest of growth in 2–3 weeks (24).

We employed two media for handling the tissue and cells, the dispersion medium, and the incubation medium. They only differed in the fatty acid content of the albumin. The basic medium was Eagle's Minimum Essential Medium buffered with 24 mM sodium bicarbonate. Before adjusting the pH, the medium was supplemented with (final concentrations): glutamine, 2 mM; glucose, 10 mM; fructose, 10 mM; and pyruvic acid, 1 mM. The pH was then brought down to 7.4 with a few drops of 0.1 N HCl. The cell dispersion medium had 4% fatty acid-poor BSA, and the incubation medium had 4% fatty acid-free BSA. The basic medium was stored at 4 C for up to 2 weeks, but BSA and the supplements listed above were added immediately before the experiments.

Rats were killed by decapitation without anesthesia, and interscapular brown fat was rapidly dissected and placed in dispersion medium kept at room temperature. Six to 10 rats were killed to obtain cells for one experiment. After rapidly removing connective tissue and white fat, the tissue was finely minced with scissors, blotted, and resuspended in 1 ml fresh dispersion medium per rat containing 2 mg/ml collagenase. The tissue suspension was then transferred to a 30-ml Nalgene bottle, which was gassed with 5% CO_2-95% O_2, capped, and incubated for 10 min at 37 C in a rotary shaker at 140 rpm. After 10 min, the fragments of tissue were recovered in a chiffon nylon membrane, and the filtrate, containing cell debris and fat, was discarded. The cleaned fragments of tissue were then harvested with a spatula onto a piece of parafilm and transferred to another Nalgene bottle containing an equal volume of fresh dispersion buffer with the same collagenase concentration. Flasks were gassed as described above, tightly capped, returned to the incubator, and shaken as before. In addition, every 10–15 min the tubes were vigorously shaken for 10–15 sec. The end of digestion was determined by the appearance of homogeneous turbidity in the medium and the disappearance of most visible tissue fragments. This usually took 35–40 min, but it may vary with the quality of the collagenase. When the digestion was completed, 1 vol dispersion medium was added, and the digested tissue was filtered through a chiffon nylon membrane into one or two plastic graduated centrifuged tubes. The undigested tissue fragments were discarded. These tubes were centrifuged for 2 min at 50 × *g* to float the fat-laden brown adipocytes. At the end of centrifugation, a 100-μl disposable glass micropipette or a lumbar tap needle was carefully slid by the side of the tube into its bottom, and the infranatant was carefully aspirated. Cells were then washed with 10 ml dispersion buffer without collagenase, finally resuspended in incubation buffer in a volume 2–3 times the volume of the cell

pellet, and transferred into a Nalgene bottle, where the cell suspension was gently mixed to a homogeneous suspension. A 1:20 dilution of this dispersion was counted. Yield was usually 5×10^6 cells/BAT pad or more.

Cells were appropriately diluted in incubation buffer and transferred to capped 100 × 13-mm plastic tubes for the experimental incubations. These usually lasted 6 h, during which tubes were gassed with a 95% O_2-5% CO_2 mixture three times. The viability of the cells was greater then 90%, as judged by trypan blue exclusion in albumin-free medium. Cells remained viable and responsive to various stimuli for at least 8 h. No more than 250,000 cells/tube were incubated in a 1-ml volume. Pilot experiments showed that the responses of UCP mRNA to stimulation ceases to be linearly related to cell number when there are over 300,000 cells/tube.

RNA preparation and analysis

At the end of the incubations, cells from two or three individual incubations were pooled and transferred to 40-ml tubes. The volume was made 40 ml with albumin-free medium, representing a greater than 10-fold dilution of BSA. This allowed the cells to sediment when centrifuged at 30,000 × *g* for 10 min at 15 C. As judged by DNA measurements, recovery with this harvesting method is complete, and recovery of UCP mRNA is usually greater than 90% (see *Results* and Fig. 1). After this step, then, supernatant was carefully aspirated and discarded, and the cell pellet was collected in 1–2 ml guanidinium isothiocyanate containing the standard additions recommended for RNA extraction (25). The cell lysates were further dispersed with a Brinkmann sonicator-homogenizer (Westbury, NY) for 6 sec at setting 3, layered atop a 2.4-M CsCl cushion, and centrifuged following exactly the procedure previously described (10). Six to eight individual incubations were carried out for each experimental condition, which, with the pooling described above, generated two to four samples of RNA, each RNA sample representing 0.6–1 million cells.

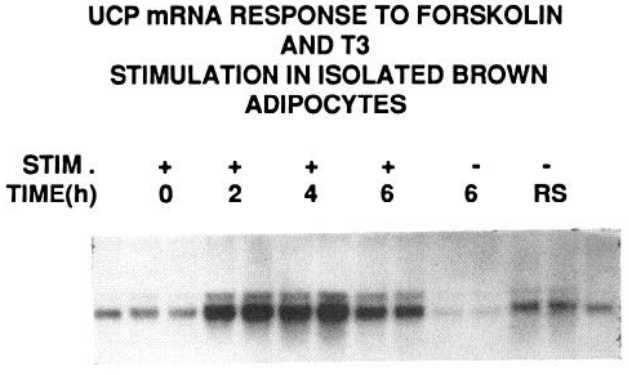

FIG. 1. Stimulation of UCP mRNA levels by forskolin and T_3 in dispersed brown adipocytes obtained from euthyroid rats. Northern blot of brown adipocyte RNA probed with ^{32}P-labeled UCP cDNA. Cells were obtained from euthyroid rats, as described in *Materials and Methods*, and incubated for the indicated times with 1 μM forskolin and 50 ng T_3/ml. Cells from triplicate incubations (total, ~9×10^5) were pooled and subjected to the RNA extraction procedure described in *Materials and Methods*. Each lane was loaded with 2 μg total RNA. The extreme *left* and *right lanes* were loaded with 2 μg total BAT RNA from euthyroid rats exposed to cold overnight. RS, Recovery standard; each of these lanes were also loaded with 2 μg RNA from an identical number of cells that were directly lysed in guanidinium thiocyanate without being submitted to any of the manipulations of the cells undergoing the incubation. Densitometric data are given in *Results*.

RNA was analyzed by Northern or dot blot analysis. Northern blot was used initially to insure that the various manipulations did not affect the size or relative amounts of the 1.9- and 1.6-kilobase UCP mRNA species, known now to correspond to two sites of polyadenylation (26). Since neither the size nor the relative proportion of the two UCP mRNA species was affected by the experimental manipulations, most of the studies reported here are from dot blot analysis. Two to 5 μg total brown adipocyte RNA were loaded per lane or dot. The probe for UCP mRNA was a full-length rat UCP cDNA kindly given to us by Dr. K. Freeman (Hamilton, Ontario, Canada) (27), labeled with [^{32}P]deoxy-ATP by random priming (28). For quantification, dots were punched out of the blotting membrane and counted in a β-scintillation counter. Known amounts of UCP mRNA transcribed *in vitro* from full-length UCP cDNA cloned in pSP64 were used as standard (7). For the most part, results are expressed as nanograms of this *in vitro*-synthesized UCP mRNA. Northern blots were analyzed by scanning densitometry.

Hormones and drug additions

Additions were made in volumes ranging 10–100 μl. Whenever the volume was larger than 50 μl, the substance was dissolved in incubation medium. Forskolin, an stimulator of adenylate cyclase, was dissolved to a concentration of 10 mM in dimethylsulfoxide and diluted subsequently in water to a concentration of 100 μM and added to the incubations in 10 μl. 8-Bromo-cAMP was added as a final concentration of 3 mM dissolved in 100 μl whole medium. Isoproterenol and NE were added in 20-μl aliquots to obtain final concentrations of 10 and 1 μM, respectively, and phenylephrine in 50 μl to final concentration of 5 μM. Ten microliters of 17.6 mg/ml ascorbic acid were added to each tube to protect these substances from oxidation (1 mM final). T_4 and T_3, in the free acid form, were dissolved to a concentration of 1 mg/ml in 40 mM NaOH and appropriately diluted in normal saline to deliver the desired concentration in volumes of 10 μl. In the presence of 4% BSA, the dialyzable fractions of T_4 and T_3 were, respectively, $0.9 \pm 0.2 \times 10^{-3}$ and $5.6 \pm 0.2 \times 10^{-3}$ (n = 3), in agreement with values obtained with the same concentration of human serum albumin (29). These values are 1.3- to 1.4-fold the dialyzable fractions of T_4 and T_3 in rat plasma (30); thus, total concentrations in medium are readily comparable to *in vivo* situations.

Statistical analyses

Data are presented as the mean ± SEM. Statistical significance was assessed by Student's *t* test or Neuman-Keuls test, when multiple comparisons were made.

Results

Total RNA and UCP mRNA recovery: responses to adrenergic and thyroid hormone stimulation

The yield of total RNA was 13–18 μg/million brown adipocytes. Northern blot analysis consistently showed

the two UCP mRNA species identified *in vivo*, 1.9 and 1.6 kilobases long, and in a ratio of 0.2 to 0.3, as found *in vivo*. The relative abundance of UCP mRNA, as judged from standards made with full-length UCP mRNA obtained by *in vitro* transcription ranged from 4–6 ng/μg total RNA, 2- to 3-fold the abundance found in fresh BAT (7, 31). This is in all likelihood caused by the significant proportion in the tissue of cells that do not express UCP, namely immature brown adipocytes (32, 33) and non-BAT cells (34). Figure 1 shows a Northern blot of RNA obtained from euthyroid brown adipocytes. Cells were stimulated with 50 ng T_3/ml (77 nM) and 1 μM forskolin and harvested after 2, 4, or 6 h of incubation. The free concentration of T_3 obtained in these conditions (0.43 nM = 77 nM × 5.6 × 10^{-3}), about 125-fold the euthyroid rat plasma free T_3 concentration (30), causes maximal UCP response to cold or NE in hypothyroid rats *in vivo* (8, 9) and is maximally stimulatory *in vitro* (as shown and discussed below). The baseline was 3.9 ± 0.3 (arbitrary densitometric units; n = 2). The response was maximal by 2 h (12.4 ± 0.8), stayed the same at 4 h (12.9 ± 1.8), and declined by 6 h (8.1 ± 0.8). This response is similar in timing and magnitude to that observed *in vivo* after maximal doses of NE or cold exposure (10). Similar results have been obtained with 3 mM 8-bromo-cAMP and 50 ng/ml T_3 (not shown). Note that in cells incubated for 6 h in the absence of stimulation, there was substantial loss of UCP mRNA (to 1.3 ± 0.02) and that the recovery of UCP mRNA after harvesting the cells was virtually complete (recovery standard, 3.7 ± 0.15).

T_3 stabilizes mature UCP mRNA and amplifies the effect of forskolin in euthyroid brown adipocytes

As shown in Fig. 1, in the absence of adrenergic stimulation or thyroid hormone, UCP mRNA disappears rapidly. In Fig. 2, we show that 50 ng/ml T_3 can prevent the disappearance of this mRNA. However, this concentration of thyroid hormone does not increase the level of UCP mRNA significantly over the basal value (11.7 ± 0.7 *vs.* 9.8 ± 0.9 ng). Forskolin (1 μM) significantly increased UCP mRNA abundance (27.3 ± 1.4 ng), but in the presence of T_3, the stimulation was significantly greater (40.1 ± 0.8 ng; $P < 0.001$). These results are consistent with stabilization of the mature UCP mRNA by T_3, and this interpretation is further supported by the data in Table 1. In this experiment, UCP mRNA disappeared with an apparent $t_{1/2}$ of 7 h in untreated cells. Actinomycin (2 μM), added about 30 min before time zero, did not cause a faster disappearance of this RNA, suggesting a low rate of UCP gene transcription in unstimulated cells. At this concentration, actinomycin causes greater than 95% inhibition of [^{3}H]uridine incorporation into RNA within 0.5 h (19). The addition of T_3 prevented the rapid decline of UCP mRNA, but as in the previous experiment, it did not significantly increase the abundance of this mRNA. Moreover, the effect of T_3 was equal in the presence or absence of actinomycin, suggesting that on-going transcription is not required for this effect of T_3 and confirming the *in vivo* finding that T_3 does not increase transcription in the absence of adrenergic stimulation (7). We have previously reported

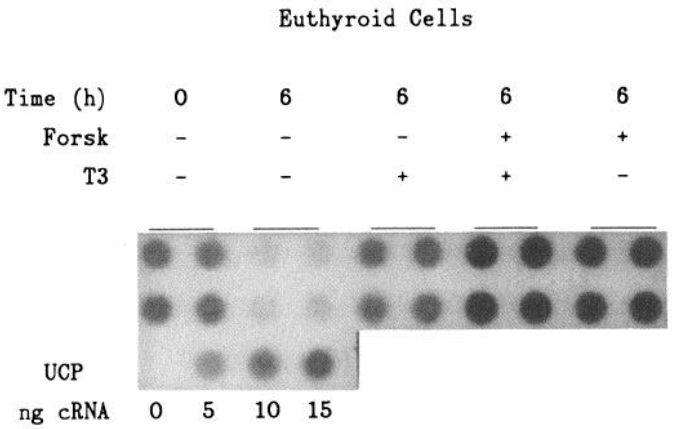

FIG. 2. Effect T_3 on UCP mRNA stability and response to forskolin (Forsk) in dispersed brown adipocytes from euthyroid rats. Dot blot analysis of total RNA obtained from euthyroid rat brown adipocytes. Each *dot* represents 3 μg total RNA obtained from pooled triplicate incubations containing 250,000 cells each. The indicated nanogram of *in vitro*-synthesized UCP mRNA served as the standard. Cells were incubated for 6 h, during which they were treated as indicated. The forskolin concentration was 1 μM, and the T_3 concentration was 50 ng/ml (77 nM). Quantitative data are given in *Results*.

TABLE 1. Effect of T_3 on UCP mRNA disappearance in euthyroid dispersed brown adipocytes

Condition	UCP [^{32}P]cDNA hybridized (cpm)		
	0 h	3 h	6 h
Control	1412	1062	982
	1519	1200	957
Actinomycin		1064	865
		1037	847
T_3		1676	1615
		1515	1390
Actinomycin + T_3		1618	1669
		1578	1578
Paper background	303		
	233		

Total RNA from brown adipocytes of euthryoid rats was obtained and dot blotted as described in *Materials and Methods*. Results are expressed as raw counts per min of the ^{32}P-labeled UCP cDNA probe hybridized by the blotted dots punched from the nitrocellulose paper; paper background refers to counts nonspecifically adsorbed to identical areas of the paper without RNA. The actinomycin-D concentration was 2 μM, and the T_3 concentration was 50 ng/ml (77 nM).

stabilization of mature and precursor UCP mRNA by T_3 (31), in addition to its amplification of the transcriptional effect of NE (7).

Hypothyroid brown adipocytes do not respond to adrenergic stimulation, and this is promptly corrected by T_3

In contrast to euthyroid cells, hypothyroid brown fat cells do not undergo an increase in UCP mRNA abundance after adrenergic stimulation, as depicted in Fig. 3. Two separate experiments are summarized in this figure. The *upper panel* simply shows the contrast between the vigorous response of euthyroid cells to forskolin and the absence of response in hypothyroid cells. This defect is fully corrected within 3 h of adding T_3 to the medium, as illustrated in the *lower panel.* In this experiment, cells were challenged with forskolin after 3 h of incubation. No T_3 was added to the euthyroid cells, while half of the hypothyroid cells were incubated from the start with 50 ng T_3/ml. Note that the response of UCP mRNA to forskolin in hypothyroid cells exposed to T_3 was identical to that of euthyroid brown adipocytes (~2.5-fold increase).

Response of UCP mRNA to adrenergic stimulation in hypothyroid brown adipocytes is readily restored by T_4 by a mechanisms requiring its conversion to T_3

In an experimental format analogous to that shown in Fig. 3, the results depicted in Fig. 4 show that hypothyroid brown adipocytes also failed to respond to a combination of phenylephrine and isoproterenol, as they failed to respond to forskolin. This combination of phenylephrine and isoproterenol (5 and 10 μM) caused maximal stimulation of respiration euthyroid brown adipocytes and 5′D-II in hypothyroid cells (16, 19); the latter is important for the experiments to be described. The addition of T_4 (35 ng/ml) at the outset of the incubation restored the response as effectively as 50 ng/ml T_3. This total concentration of T_4 in the medium (45 nM) created a free concentration of approximately 40 pM, virtually identical to the free T_4 concentration in the euthyroid rat (30). This is reminiscent of the *in vivo* situation, where replacement of T_4 suffices to fully restore the UCP response to adrenergic stimulation in hypothyroid rats (8–10), and suggests that under appropriate adrenergic stimulation of 5′D-II activity (17), isolated intact brown

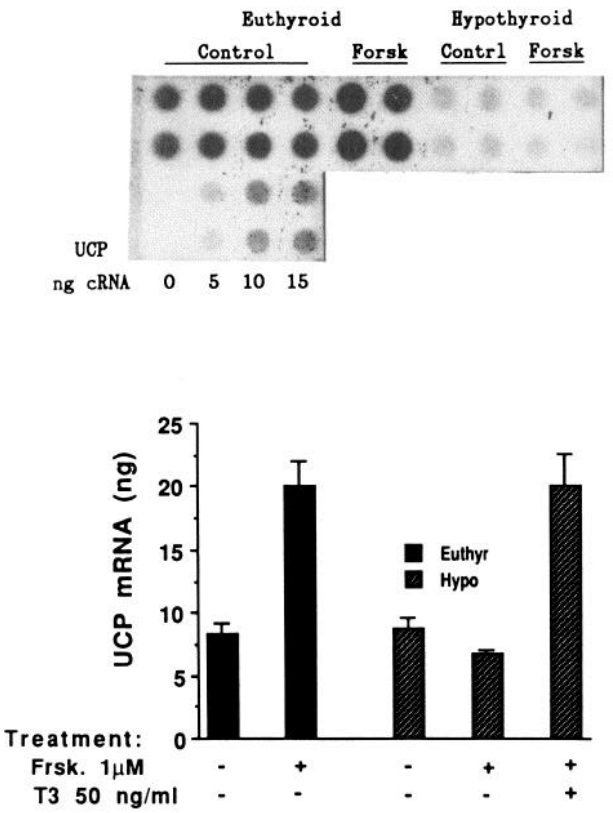

FIG. 3. Effects of thyroid status of the rats and of thyroid hormone added *in vitro* on the responsiveness of UCP mRNA to forskolin (Forsk or Frsk.) stimulation of dispersed brown adipocytes. In these experiments, forskolin (1 μM) was added after 3 h of incubation, while T_3 (50 ng/ml; 77 nM) was present, when indicated, for the 6 h of incubation. UCP mRNA was quantified against mRNA produced by *in vitro* transcription, as indicated in *Materials and Methods. Upper panel,* Dot blot analysis of 5 μg euthyroid (Euthyr) or hypothyroid (Hypo) rat brown adipocytes. *Lower panel,* A similar experiment depicted in graphic form, except that only 3 μg total RNA were blotted. Contrl, Control.

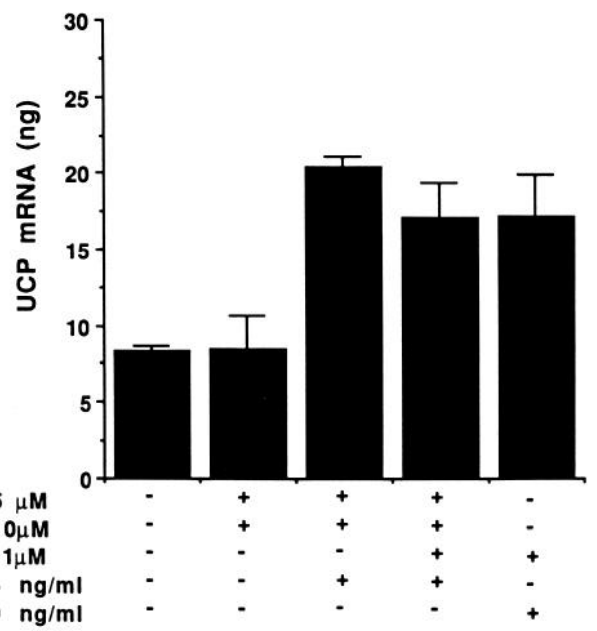

FIG. 4. Effect of T_4 or T_3 on UCP mRNA responses to isoproterenol (Iso.) and phenylephrine (Phe.) in dispersed brown adipocytes from hypothyroid rats. In a setting similar to that in Fig. 3, 3 μg total RNA from duplicate incubations were quantified against *in vitro*-produced UCP mRNA. Adrenergic agonists and thyroid hormones were added in the concentrations indicated for the 6 h of incubation. Fsk., Forskolin.

adipocytes actively convert T_4 to T_3; hence, the increase in deiodinase activity under these circumstances is physiologically relevant.

In agreement with this view, when 5′D-II was blocked with 10 μM iopanoic acid (IOP; Fig. 5), the effect of T_4 was abolished. However, when T_3 (50 ng/ml) was added along with IOP, UCP mRNA responded vigorously as in the cells not blocked with IOP or in those treated with T_3 alone (Figs. 3 and 4). This indicates that IOP does not interfere with the action of T_3. Also note that the cells treated with IOP underwent a reduction in UCP mRNA below the basal level, which probably reflects the rapid UCP mRNA disappearance in the absence of T_3, described above. At this concentration, T_4 probably does not provide enough thyromimetic potency to stabilize UCP mRNA.

As in vivo, T_4 is a better source of T_3 for the stimulated brown adipocyte than extracellular T_3

To explore further the relative potencies of T_3 and T_4 under these circumstances, hypothyroid cells were stimulated with forskolin in the absence and presence of various concentrations of T_3 or 35 ng/ml T_4. The results are shown in Fig. 6. Cells were exposed to the hormones and forskolin for 6 h from the onset of the incubation. The raw data are shown on the *left*, with the mean ± SEM counts per min of [^{32}P]UCP cDNA hybridized indicated on the *side*. In the *right panel*, the UCP mRNA increments over baseline are depicted graphically, untransformed on the *top* and linearized on the *bottom*. One-way analysis of variance indicated highly significant effects ($F = 216$; $P < 0.001$). Neuman-Keuls test indicated that the effect of 15 ng/ml T_3 was significantly less than that of 25 or 50 ng/ml. The effects of these two

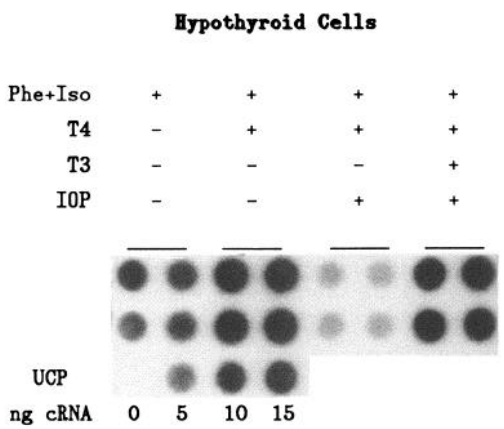

FIG. 5. Effect of IOP on the T_4-induced restoration of UCP mRNA responsiveness to forskolin in hypothyroid rat brown adipocytes. Three micrograms of total brown adipocyte RNA from triplicate incubations were loaded per dot. The concentrations of phenylephrine (Phe), isoproterenol (Iso), T_4, and T_3 were the same as those in Fig. 4. The IOP concentration was 10 μM. All additions were present throughout the 6 h of incubation.

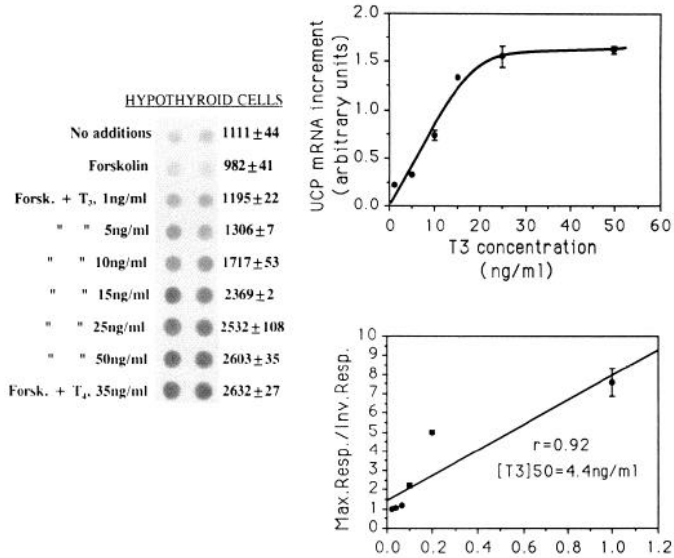

FIG. 6. Effects of various concentrations of T_3 on UCP mRNA responsiveness to forskolin in hypothyroid rat brown adipocytes. Two micrograms of total RNA from triplicate incubations were loaded per dot. T_3 or T_4, in the indicated concentrations, and forskolin (1 μM) were present throughout the 6 h of incubation. *Left panel*, Raw data. Numbers on the *right* show the mean ± SEM counts per min of ^{32}P-labeled UCP cDNA hybridized. *Right panel*, Data presented in graphic form after subtracting the zero time value, *i.e.* responses represent the increment over the baseline level. On the *bottom*, the same data have been rearranged for the purpose of linearization. When no SEM bar is evident, it is because its size is smaller than the dot symbols. $[T_3]_{50}$ is the calculated T_3 concentration that would cause half the maximal UCP mRNA increment. One-way analyses of variance and Neuman-Keuls test indicate that a plateau is reached with 25 ng/ml T_3, *i.e.* the response of UCP mRNA is not significantly different from that to 50 ng/ml, but both responses are significantly higher than that to 15 ng T_3/ml. The response to 35 ng/ml T_4 is not different from the maximal response to T_3. See text for details.

concentrations are not significantly different, defining a plateau, and do not differ from the effect of 35 ng/ml T_4. In 4% albumin, 25 ng/ml T_3 (38 nM) generates a free concentration of 215 pM, while, as indicated above, the free concentration of T_4 at 35 ng/ml is about 40 pM in this medium (29). The calculated total T_3 concentration to obtain a half-maximal effect ($[T_3]_{50}$; Fig. 6, *bottom right*) is 4.4 ng/ml, which represents a free T_3 concentration of about 38 pM. Thus, at 40 pM, the concentration of T_4 that causes maximal UCP mRNA stimulation, T_3 causes only half of the maximal response.

Discussion

We undertook the present studies to see whether we could reproduce *in vitro* observations made *in vivo*. This would not only provide us a model to further our understanding of the cellular and molecular bases of facultative thermogenesis, but would also answer specific questions left by *in vivo* studies. Floating cells from dispersed interscapular BAT have been demonstrated to be mature

brown adipocytes that respond to adrenergic stimulation with increases in cAMP, lipolysis, respiration (16), and 5′D-II activity (17, 19, 20). We have further shown here that these cells contain UCP mRNA and that the relative abundance of this is markedly and rapidly increased by the stimulation of the cAMP adrenergic cascade. The UCP mRNA species in these cells are the same as those demonstrated *in vivo*, and as in this condition, their relative abundance does not appear to be affected by the experimental manipulations. We also demonstrated that thyroid hormone is essential for the adrenergic stimulation of UCP mRNA. The effect of thyroid hormone is concentration dependent. Furthermore, as *in vivo*, a maximal response of UCP mRNA to adrenergic stimulation can be obtained with a physiological concentration of free T_4, while supraphysiological concentrations of free T_3 are required to obtain that level of response. Notably, the effect of physiological concentrations of T_4 can be abolished by blocking 5′D-II with IOP. We have also presented evidence that thyroid hormone prolongs the half-life of UCP mRNA and that this probably contributes to the maintenance of UCP mRNA levels under sustained stimulation, as anticipated from *in vivo* studies (31). These results are important from several standpoints, as discusssed below.

Isolated mature brown adipocytes respond vigorously to stimulation of adenylate cyclase with forskolin in the presence of T_3 with an elevation of UCP mRNA levels. The cellular conditions created by forskolin and T_3 are probably similar to those occurring under maximal stimulation of the tissue by acute cold exposure or NE injection, since under these circumstances, there is stimulation of adenylate cyclase by NE (35) and of 5′D-II (36), which leads to the virtual saturation of the nuclear T_3 receptors (11). The time course and magnitude of the UCP mRNA response are nearly identical to those observed *in vivo* (7, 31, 37). In addition, our results suggest that the activation of the α_1-adrenergic pathway appears not to be important for maximal UCP mRNA stimulation, since the response obtained with a combination of isoproterenol (β-agonist) plus phenylephrine (α_1-agonist) was not greater than that with forskolin alone, which selectively stimulates adenylate cyclase. This observation is consistent with *in vivo* results in rats (37, 38) and seemingly at variance with observations made in mice (39) or mouse brown adipocytes differentiated in culture (13), where the α_1 pathway appears important for a full UCP response to adrenergic stimulation. Certainly, further studies are required to define the role of the α-adrenergic pathway in the stimulation of UCP expression and BAT thermogenesis in different species, for which isolated mature brown adipocytes seem to be a suitable model.

As mentioned in the introduction, the adrenergic stimulation of both respiration and UCP concentration has been documented *in vitro*, the former in freshly dispersed brown adipocytes (16) and the latter in brown adipocytes differentiated in primary cultures (13, 33), but to date it has not been possible to reproduce *in vitro* the effects of thyroid hormone observed *in vivo*. Even though thyroid hormone appears necessary for NE stimulation in primary cultures maintained in serum-free medium (13), the effect is not nearly as dramatic as that *in vivo*, and the deiodinase does not appear to play a significant role in these cells. Thus, in brown adipocytes differentiated in primary cultures, T_3 appears far more potent than T_4, and supraphysiological concentrations of the latter are required to see an effect, much as one would anticipate from their intrinsic thyromimetic potency (13). This raises the possibility that at least part of the *in vivo* effects of T_4 are the result of actions of the hormone elsewhere, for example at the level of the central nervous system, where T_4 is also the main source of cellular T_3 (14). Although unlikely, this possibility remained viable until the present studies. The results shown here demonstrate that the *in vivo* observations are the result of a direct effect of thyroid hormone on the brown adipocyte. The similarities with the *in vivo* condition are both qualitative and quantitative. Thus, the half-maximal T_3 concentration is approximately 4.4 ng/ml, and the maximal response is approached with concentrations in excess of 20 ng/ml. One can estimate the nuclear T_3 receptor occupancy at various concentrations of T_3 knowing the concentration of T_3 required for half-maximal occupancy at equilibrium (9, 40). This concentration is 1.16 ng/ml (9, 41), corresponding to a free T_3 concentration of 7.4 pM, with which one can estimate that at 4.4 ng/ml of T_3 in the medium, nuclear occupancy is approximately 84%[1], nearly identical to the occupancy required *in vivo* for the half-maximal UCP response (9). At 20–25 ng T_3/ml, the concentration of T_3 required for a maximal response, one can likewise calculate a nuclear occupancy of 97–98%, coinciding with the concept that near saturation of these receptors with T_3 is necessary for a full response of UCP to adrenergic stimulation (9).

The present results also show that BAT 5′D-II plays a critical role in the physiology of these cells. As *in vivo*, T_4 appears to be a better source of T_3 for the brown fat

[1] This was estimated from the following relation (9, 40): NT_3 occupancy = T_3 conc. ÷ (T_3 conc. + $[T_3]_{50}$), where nuclear T_3 receptor occupancy (NT_3) is expressed as a fraction of the maximal binding capacity, $[T_3]_{50}$ is the half-saturating T_3 concentration, as determined by saturation analysis (9, 41), and T_3 conc. is the medium T_3 level. In this case, we did all of the calculations using free hormone concentrations. For $[T_3]_{50}$, we used 7.4 pM obtained from *in vivo* total $[T_3]_{50}$ (1.16 ng/ml = 1.78 nM) (9, 41) times the dialyzable fraction of T_3 in rat serum (4.15×10^{-3}) (30). The free fraction of T_3 in 4% BSA-containing medium is 5.6×10^{-3}, so that at 4.4 ng/ml the estimated free T_3 level is 38 pM. By substituting these values in the equation: $NT_3 = 38 \div (38 + 7.4) = 0.837$ or approximately 84%.

cell nuclear receptors. Thus, at a molar concentration at which medium T_3 barely induces half-maximal stimulation of UCP mRNA, T_4 induces maximal stimulation. Since the T_4 effect is obliterated by blocking 5'D-II with IOP, it is logical to conclude that the effect of T_4 is not the result of its action as such, but, rather, of the T_3 that generates inside the cell. Recall that IOP did not decrease the effect of extracellular T_3 at the concentration used, and that the intrinsic potency of T_4, *i.e.* when its conversion to T_3 is prevented, is about one tenth that of T_3 (42, 43). The reproduction *in vitro* of the *in vivo* findings in this regard is very important. As mentioned in the introduction, the question remained of whether T_4 was a better source of T_3 than plasma T_3 because of the participation of dynamic factors inherent to the *in vivo* condition, rather than to factors inherent to the cell biology of 5'D-II. If such dynamic factors were important, relative to T_4, T_3 would have been more potent *in vitro* than *in vivo*, and T_4 and T_3 would have shown apparent relative potencies more in accord with their intrinsic thyromimetic power. The present results indicate that dynamic factors have minimal participation, if any, in the *in vivo* observations.

The effect of T_3 on UCP mRNA expression seems to be fast, as fast as that *in vivo*, since within 3 h of exposing brown adipocytes from hypothyroid rats to T_3, they fully recover their responsiveness to adrenergic stimulation. Moreover, the data confirm the concept that T_3 has to be present inside the brown adipocyte for maximal responsiveness of UCP to adrenergic stimulation; that is, T_3 does not appear to generate a long-lived intermediate. Thus, in the 2–3 h that it takes to obtain cells, cells from euthyroid rats loose thyroid hormone and do not respond maximally to adrenergic stimulation unless T_3 is added. This is in agreement with the concept that in euthyroid rats the activation of 5'D-II is necessary to provide T_3 rapidly and in sufficient quantities for a maximal response of UCP mRNA to acute stimulation (8, 9, 37). The rapidity of the effect of T_3 on UCP responsiveness and the *in vivo* observation that ongoing protein synthesis is not required for this T_3 effect (7) are consistent with the idea that the stimulation of the UCP gene is directly mediated by the T_3-receptor complex.

The present results also show an equally blunted UCP mRNA response in hypothyroid cells to isoproterenol, which operates through the β-adrenergic receptor, and to forskolin, which directly activates adenylate cyclase. These observations make it unlikely that a reduction in the number of β-adrenergic receptors previously reported in hypothyroid BAT (44) plays a major role in the blunted hypothyroid response of UCP mRNA to NE, but leaves open the possibility of a defect in the generation of cAMP that is rapidly corrected by T_3. In this regard, Sundin *et al.* (16) have reported normal accumulation of cAMP by hypothyroid brown adipocytes 15 min after isoproterenol or forskolin stimulation (16), but more recent reports show reduced cAMP accumulation 2 h after stimulation with isoproterenol or NE in brown adipocytes from hypothyroid rats (17). The *in vitro* model described here will be helpful to define a defect at the level of cAMP accumulation; if confirmed, the model will also be helpful to define its implications for the global thermogenic responses (of which UCP is just one part) and to investigate its mechanism.

We have previously reported that the initial accumulation of UCP mRNA after cold or NE stimulation of rats can be fully accounted for by an increase in transcription initiation of the gene and that T_3 amplifies this effect of NE (7). Under more prolonged stimulation, however, the participation of other T_3-dependent mechanisms in the elevation of UCP mRNA became evident, namely increased efficiency of processing of UCP gene transcripts and stabilization of mature UCP mRNA (31). The present results provide direct evidence of stabilization of UCP mRNA by T_3, but show, in addition, that this effect of T_3 is not sufficient to elevate the levels of this mRNA in the absence of significant levels of transcription, explaining why T_3 cannot increase UCP mRNA levels *in vivo* in the absence of adrenergic stimulation (7, 37).

Altogether, the present results show that freshly dispersed, mature brown adipocytes appear to be a powerful model system to study the cell biology of BAT. The products of the UCP gene are not only of great biological importance for the physiology of cold adaptation and energy balance, but, given their abundance and rapid changes, they provide excellent end points to study molecular and cellular aspects of the interaction between the sympathetic nervous system and thyroid hormone. Lastly, the present results ratify a critical role for thyroid hormone in facultative thermogenesis.

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