

Evidence of UCP1-independent regulation of norepinephrine-induced thermogenesis in brown fat

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Ribeiro, Miriam O., Fabiana L. A. S. Lebrun, Marcelo A. Christoffolete, Marcelo Branco, Alessandra Crescenzi, Suzy D. Carvalho, Núbio Negrão, and Antonio C. Bianco. Evidence of UCP1-independent regulation of norepinephrine-induced thermogenesis in brown fat. *Am J Physiol Endocrinol Metab* 279: E314–E322, 2000.—To study the thermal response of interscapular brown fat (IBF) to norepinephrine (NE), urethane-anesthetized rats (1.2 g/kg ip) maintained at 28–30°C received a constant venous infusion of NE ($0\text{--}2 \times 10^4$ pmol/min) over a period of 60 min. IBF temperatures (T_{IBF}) were recorded with a small thermistor fixed under the IBF pad. Data were plotted against time and expressed as maximal variation ($\Delta T^\circ\text{C}$). Saline-injected rats showed a decrease in T_{IBF} of $\sim 0.6^\circ\text{C}$. NE infusion increased T_{IBF} by a maximum of $\sim 3.0^\circ\text{C}$ at a dose of 10^4 pmol $\cdot$ min $^{-1}$ $\cdot$ 100 g body wt $^{-1}$. Surgically thyroidectomized (Tx) rats kept on 0.05% methimazole showed a flat response to NE. Treatment with thyroxine (T_4 , 0.8 $\mu\text{g} \cdot 100$ g body wt $^{-1} \cdot$ day $^{-1}$) for 2–15 days normalized mitochondrial UCP1 (Western blotting) and IBF thermal response to NE, whereas iopanoic acid (5 mg $\cdot$ 100 g body wt $^{-1} \cdot$ day $^{-1}$) blocked the effects of T_4 . Treatment with 3,5,3'-triiodothyronine (T_3 , 0.6 $\mu\text{g} \cdot 100$ g body wt $^{-1} \cdot$ day $^{-1}$) for up to 15 days did not normalize UCP1 levels. However, these animals showed a normal IBF thermal response to NE. Cold exposure for 5 days or feeding a cafeteria diet for 20 days increased UCP1 levels by ~ 3.5 -fold. Nevertheless, the IBF thermal response was only greater than that of controls when maximal doses of NE (2×10^4 pmol/min and higher) were used. Conclusions: 1) hypothyroidism is associated with a blunted IBF thermal response to NE; 2) two- to fourfold changes in mitochondrial UCP1 concentration are not necessarily translated into heat production during NE infusion.

brown adipose tissue; thermal response; thyroid hormones; uncoupling protein; cold exposure; cafeteria diet

BROWN FAT IS AN IMPORTANT SITE of nonshivering thermogenesis that is activated to supplement shivering and rapidly warms the body through arousal of hibernating or torpid mammals, or during cold exposure (9, 17). Its thermogenic capacity is due to a mitochondrial uncoupling protein (UCP1) that short-circuits the proton gradient across the inner mitochondrial membrane, bypassing the less

abundant ATP synthase and thereby uncoupling fuel oxidation from the phosphorylation of ADP. The other members of the UCP group, namely UCP2 and UCP3, which are also capable of mitochondrial uncoupling, are present in brown fat as well as other tissues, but their physiological role is still controversial (5, 18, 32). Brown fat thermogenesis is triggered by norepinephrine (NE), which leads to a powerful stimulation of local lipolysis. Increased cytosolic fatty acids ultimately start the uncoupling process (21).

Facultative thermogenesis is positively influenced by thyroid hormones. Accordingly, it is well known that hypothyroid rats have decreased cold resistance and die within hours of being placed at 4°C. Even if injected with high doses of NE, they fail to increase oxygen consumption (38). Previous studies (24, 35), however, attributed only a permissive role to thyroxine (T_4) in the NE-induced brown fat thermogenesis. The finding of type 2 deiodinase (D2) in brown fat (23) and the fact that it is stimulated severalfold by NE (33) have led to a better understanding of the active role of thyroid hormones in brown fat thermogenesis. Shortly after cold exposure is initiated, brown fat 3,5,3'-triiodothyronine (T_3) concentration increases by about threefold (34), and nuclear T_3 receptor occupancy reaches virtual saturation (3). Consequently, processes in brown fat that are primarily dependent on thyroid hormone are also activated during sympathetic stimulation of the tissue, and physiological changes that take place during cold and/or NE stimulation of the brown fat reflect a composite interaction between NE- and thyroid hormone-generated signals that eventually leads to sustained heat liberation. The major thyroid hormone-dependent pathways that have been characterized in brown fat so far are 1) adrenergic responsiveness, 2) UCP1 expression, and 3) lipogenesis (31).

The brown fat of thyroidectomized (Tx) rats is thermogenically inactive. cAMP generation in response to NE, mitochondrial content, and UCP1 expression are all greatly decreased. As a result, its metabolic re-

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sponse is greatly reduced (31). Because local D2 is increased severalfold during hypothyroidism, a single subphysiological dose of T_4 is sufficient to rapidly normalize UCP1 mRNA levels. Indeed, the quick normalization of body temperature after a few hours of T_4 administration was attributed to the restoration of UCP1 content in brown fat. However, we have recently found that the thermal response of interscapular brown fat (IBF) to NE infusion was normalized in T_3 -treated Tx rats even when its UCP1 content was only one-half of that found in intact euthyroid rats (7). More remarkably, when UCP1 was normalized in these T_3 -treated Tx rats, the thermal response of IBF was twice as high as that found in intact rats. These findings prompted us to expand our investigation concerning the relationship between brown fat mitochondrial UCP1 content and thermal response to NE infusion in various thyroid states and conditions known to affect UCP1 content, namely cold exposure and ingestion of a cafeteria diet.

MATERIALS AND METHODS

Animals and diets. Unless otherwise specified, all drugs and reagents were purchased from Sigma Chemical (St. Louis, MO). Experiments were performed on male Wistar rats weighing 150–200 g that were obtained from our breeding colony. Studies were conducted in accordance with the highest standards of humane animal care. Animals were maintained on a 12:12-h dark-light cycle at 28–30°C and had free access to standard animal chow and water. When exposed to cold (4°C for 5 days), the animals were kept in individual cages with bedding. Surgical thyroidectomy (Tx) was carried out under light ether anesthesia; immediately after surgery, the animals were placed on 0.05% methimazole in the drinking water and then were used 4 wk later. Body weight was monitored at all times to check the arrest of growth in hypothyroid animals. Some rats were implanted subcutaneously with a continuous-release pellet (Innovative Research of America, Sarasota, FL) of T_4 ($0.8 \mu\text{g} \cdot 100 \text{ g body wt}^{-1} \cdot \text{day}^{-1}$) or T_3 ($0.15\text{--}1.0 \mu\text{g} \cdot 100 \text{ g body wt}^{-1} \cdot \text{day}^{-1}$), as indicated in the text. Iopanoic acid (IOP) was used at $5 \text{ mg} \cdot 100 \text{ g body wt}^{-1} \cdot \text{day}^{-1}$ when indicated.

The cafeteria dietary regimen used was described previously (12); it lasted 20 days. Essentially, rats were offered large amounts of various palatable food items in addition to standard chow. The diet consisted largely of chocolate, cookies, biscuits, cakes, peanuts, ham, and cheese, totaling $\sim 3.8 \text{ kcal/g}$, as opposed to the $\sim 1.8 \text{ kcal/g}$ of the chow diet. Coca-Cola and Guaraná Antartica, a local soda brand, were alternated daily. Enough food was offered daily so that, despite increased consumption, rats could not eat all of it. Each day leftovers were collected and replaced with new and different items.

Timing, length, and combination of the different treatments are described along with each experiment.

IBF thermal response to NE infusion. The response was determined as previously described in detail (7). Briefly, all animals were anesthetized with urethan (1.2 g/kg ip) on the morning (9–10 h) of the experiment. A polyethylene (P-50) cannula was inserted into the left jugular vein and later used for NE infusion. IBF temperatures (T_{IBF}) were measured using a precalibrated thermistor probe secured under the brown fat pad. Core temperature (T_{C}) was measured with a colonic probe. T_{IBF} was measured during a period of $\sim 15 \text{ min}$

to obtain a stable baseline, and then NE infusion was started. NE infusion ($0\text{--}2 \times 10^4 \text{ pmol/min}$) was performed with an infusion pump (Harvard model 2274) at a rate of $0.643 \mu\text{l/min}$ for 20–90 min. NE doses are expressed per 100 g body wt throughout the experiments. Raw data were plotted over time and expressed in terms of maximal changes in temperature over time: ΔT_{IBF} (°C) vs. time (min).

Analytical procedures. At the end of the experimental period, rats were killed by decapitation, and the IBF was rapidly removed and processed for mitochondrial isolation (7). Procedures were performed at 4°C or in an ice-cold water bath as needed. Protein measurement was by the Bradford method (6). UCP1 was quantified after mitochondrial proteins were size-fractionated by 12% SDS-PAGE and identified by Western blotting, as previously described (2, 7). Serum concentrations of T_4 and T_3 were measured by RIA as described previously (7). For determination of plasma NE, venous blood samples were collected while the rats were still alive $\sim 5 \text{ min}$ before NE infusion was stopped. NE was measured by HPLC as previously described (8).

Statistical analysis. Results are expressed as means $\pm$ SD throughout the text and in Tables 1 and 2 and Figs. 1–5. Multiple comparisons were performed by one-way ANOVA followed by the Student-Newman-Keuls test.

RESULTS

IBF thermal profile during NE infusion in intact rats. After stabilization, T_{C} and T_{IBF} were $36.4 \pm 0.2^\circ\text{C}$ and $35.4 \pm 0.3^\circ\text{C}$, respectively. The difference was probably due to a more superficial localization of IBF. Figure 1A displays the profile of a typical IBF thermal response to a 20-min infusion of three different NE doses. T_{C} is also shown as a reference. At the lowest NE infusion rate ($1 \times 10^3 \text{ pmol/min}$), T_{IBF} increased rapidly, up to 0.5°C , and T_{C} increased less, only to 0.1°C . In the next set of animals, NE was infused at a higher rate ($2 \times 10^3 \text{ pmol/min}$), and the rise in T_{IBF} and T_{C} was steeper and reached higher maximal values, ~ 0.9 and 0.3°C , respectively. The highest NE infusion rate ($5 \times 10^3 \text{ pmol/min}$) triggered the maximum temperature variation of $\sim 1.2^\circ\text{C}$ in IBF and of 0.40°C for T_{C} . Regardless of the NE dose used, once infusion ended, IBF temperatures decreased rapidly during the next 20 min, whereas T_{C} normalization was much slower. Next we performed a time-response curve using the NE dose of $5 \times 10^3 \text{ pmol/min}$. The results are shown in Fig. 1B. There was a progressively higher IBF thermal response to NE with time, up to 90 min, when a plateau was reached at $\sim 2.2^\circ\text{C}$. Similar results were observed with T_{C} except that the plateau was $\sim 0.7^\circ\text{C}$.

Effects of thyroid status on IBF responsiveness to NE. On the basis of conditions discussed above, we used intact rats to perform a dose-response curve to NE by plotting the maximal change in T_{IBF} (ΔT_{IBF}) vs. the NE infusion rate, which varied from 0 (saline) to 10^4 pmol/min and lasted 60 min. As shown in Fig. 2A, plasma NE concentration increased progressively at every increase in NE infusion rate regardless of the thyroid status of the animals. The corresponding IBF thermal responses are presented in Fig. 2B. Infusion of saline into anesthetized rats resulted in a progressively lower T_{IBF} that reached $-0.6 \pm 0.2^\circ\text{C}$ at the end of the infusion period. A positive IBF thermal response was

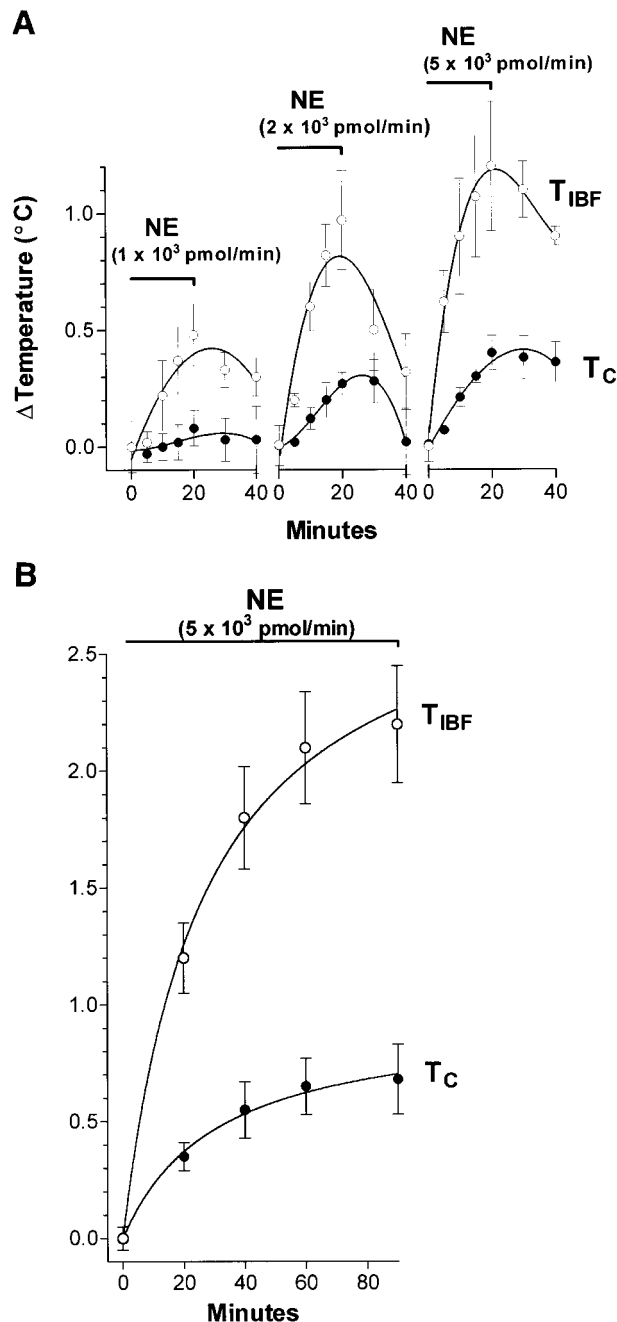


Fig. 1. Interscapular brown fat (IBF) temperature (T_{IBF}) and core temperature (T_{C}) profiles during norepinephrine (NE) infusion in intact urethane-anesthetized rats. A: intact rats received NE infusion at rates of 1×10^3 , 2×10^3 , or 5×10^3 pmol/min over a period of 20 min. Both temperatures were measured for an additional 20-min period after NE infusion was terminated. B: same conditions as A, except that NE (5×10^3 pmol/min) infusion lasted 90 min. The NE infusion period is indicated as a horizontal line. Values are means $\pm$ SD of 3–5 rats.

only detected in rats infused with 5×10^2 pmol/min NE. From that dose on, the IBF thermal response was progressively greater with the increase in NE infusion rate, and the curve obtained had a typical sigmoid shape (Fig. 2B). The maximum ΔT_{IBF} obtained was $\sim 3^{\circ}\text{C}$ at an NE infusion rate of $\sim 10^4$ pmol/min. A

plateau could not be reached, because higher NE infusion rates caused the rats to have difficulty in breathing and to experience sudden death. Infusion of selective adrenergic agonists, i.e., isoproterenol and phenylephrine, over an equivalent dose range, gave similar results to the NE infusion, and all thermal responses were blocked by propranolol (50 mg/kg) given 30 min before the experiment (data not shown).

Next, we used a similar approach in Tx rats to investigate the IBF thermal responses to NE infusion. Figure 2B shows that, regardless of the NE infusion rate used, the T_{IBF} of these animals did not increase during infusion. This response was very consistent and reproducible in several separate and independent experiments. Table 1 shows that serum concentration of thyroid hormones was undetectable in Tx rats. In addition, mitochondrial UCP1 concentration and UCP1 content in the IBF pad were reduced to 25–35% in Tx rats.

The next set of experiments was designed to investigate the potency of T_4 in reversing these Tx-induced changes in the IBF thermal response to NE. When the doses and the schedule of administration were planned, special emphasis was placed on the role of the local D_2 . Results of T_4 replacement are presented in Fig. 2C. The dose of T_4 employed ($0.8 \mu\text{g} \cdot 100 \text{ g body wt}^{-1} \cdot \text{day}^{-1}$) is equivalent to the daily T_4 production for the rat (22). It is remarkable that treatment with T_4 for only 2 days almost normalized mitochondrial UCP1 concentration in IBF (Table 1) and the thermal response to NE infusion, which was essentially not different from that of intact rats. Note that this treatment was not long enough to normalize serum concentrations of either T_4 or T_3 (Table 1). An additional important finding was that the effects of T_4 administration on mitochondrial UCP1 concentration or IBF thermal response to NE were completely blocked by pretreatment with IOP, a competitive D_2 inhibitor.

Time frame of the T_4 effects in Tx rats. Correlation with UCP1 concentration. On the basis of these results, we decided to investigate further the time frame involved in the normalization of the IBF thermal response to NE in Tx rats when T_4 is used and to correlate the results with UCP1 concentration. We did the same thing with T_3 , and the results are shown in the next section. Therefore, Tx rats were treated with a physiological replacement dose of T_4 ($0.8 \mu\text{g} \cdot 100 \text{ g body wt}^{-1} \cdot \text{day}^{-1}$) for up to 15 days. The results are shown in Table 2 and Fig. 3A. Serum concentrations of both iodothyronines increased progressively with time and were normalized by 5 days (Table 2). As above, the administration of T_4 was rapidly associated with a restoration of the IBF thermal response. Basically by 1 day the IBF response was half-maximum, and by 2 days it was normal. Longer periods on T_4 did not result in greater IBF thermal response.

Next, the IBF of these same animals was processed for UCP1 measurement. The results of mitochondrial UCP1 concentration (ng/ μg) or IBF UCP1 content (mg/pad) were then plotted against the maximal IBF thermal response, which is shown in Fig. 3, B and C. Note

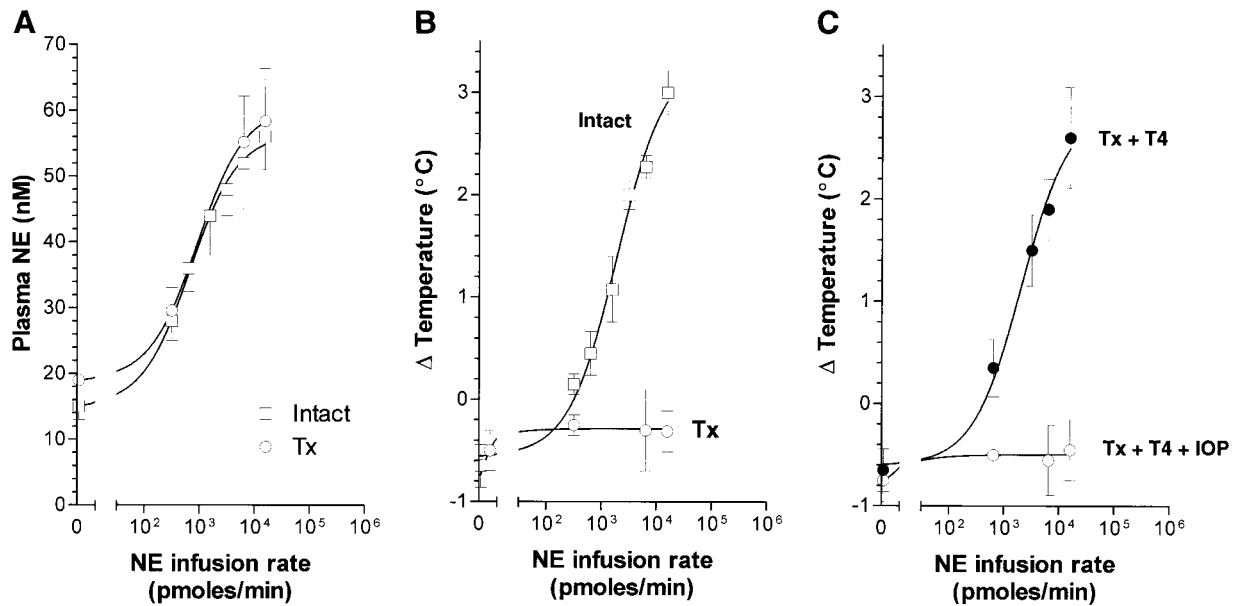


Fig. 2. Plasma NE concentration and IBF thermal response during NE infusion in intact or thyroidectomized (Tx) rats. NE plasma concentration (A) in intact ($\square$) or Tx ($\circ$) rats at the end of a 60-min NE infusion. Temperature profile obtained for (B) intact ($\square$) or Tx ($\circ$) rats, or (C) Tx rats treated for 2 days with thyroxine (T_4 , $0.8 \mu\text{g} \cdot 100 \text{ g body wt}^{-1} \cdot \text{day}^{-1}$; $\bullet$) $\pm$ iopanoic acid (IOP, $5 \text{ mg} \cdot 100 \text{ g body wt}^{-1} \cdot \text{day}^{-1}$; $\circ$) during NE infusion at the indicated rates. Each entry is the maximal Δ in temperature observed during the 1-h infusion period. Serum concentrations of T_4 and 3,5,3'-iodothyronine (T_3), as well as brown fat mitochondrial uncoupling protein (UCP)1 concentration and content, are indicated in Table 1. Values are means $\pm$ SD of 3–5 rats. Note that the abscissa is interrupted.

that the results were grouped according to the NE dose used. The values obtained in intact rats are also shown for easy comparison. Mitochondrial UCP1 concentration and IBF pad content increased rapidly during T_4 treatment, and by 2–3 days they were almost normalized. The resulting maximal IBF thermal responses for each NE dose increased with UCP1 concentration and IBF UCP1 content, and after 3 days of T_4 treatment they were not different from those of intact animals.

Time frame of the T_3 effects in Tx rats. Correlation with UCP1 concentration. A similar strategy was used with T_3 administration. In this case, however, we used

Table 1. Serum concentrations of T_4 and T_3 and brown fat mitochondrial UCP1 concentration and content in intact, Tx, and thyroid hormone-treated Tx rats

Groups	Serum, ng/ml		UCP1	
	T_4	T_3	Concn, ng/ μg protein	Content, mg/IBF pad
Intact	44 ± 3.7	0.54 ± 0.06	43 ± 6	7.2 ± 1.5
Tx	$<5^*$	$<0.05^*$	$16 \pm 3^*$	$1.9 \pm 0.3^*$
Tx + T_4	$27 \pm 3.1^*$	$0.31 \pm 0.04^*$	38 ± 6	$5.5 \pm 1.0^*$
Tx + T_4 + IOP	$35 \pm 4.1^{*\dagger}$	$0.11 \pm 0.03^{*\dagger}$	$18 \pm 4^{*\dagger}$	$2.5 \pm 0.5^{*\dagger}$

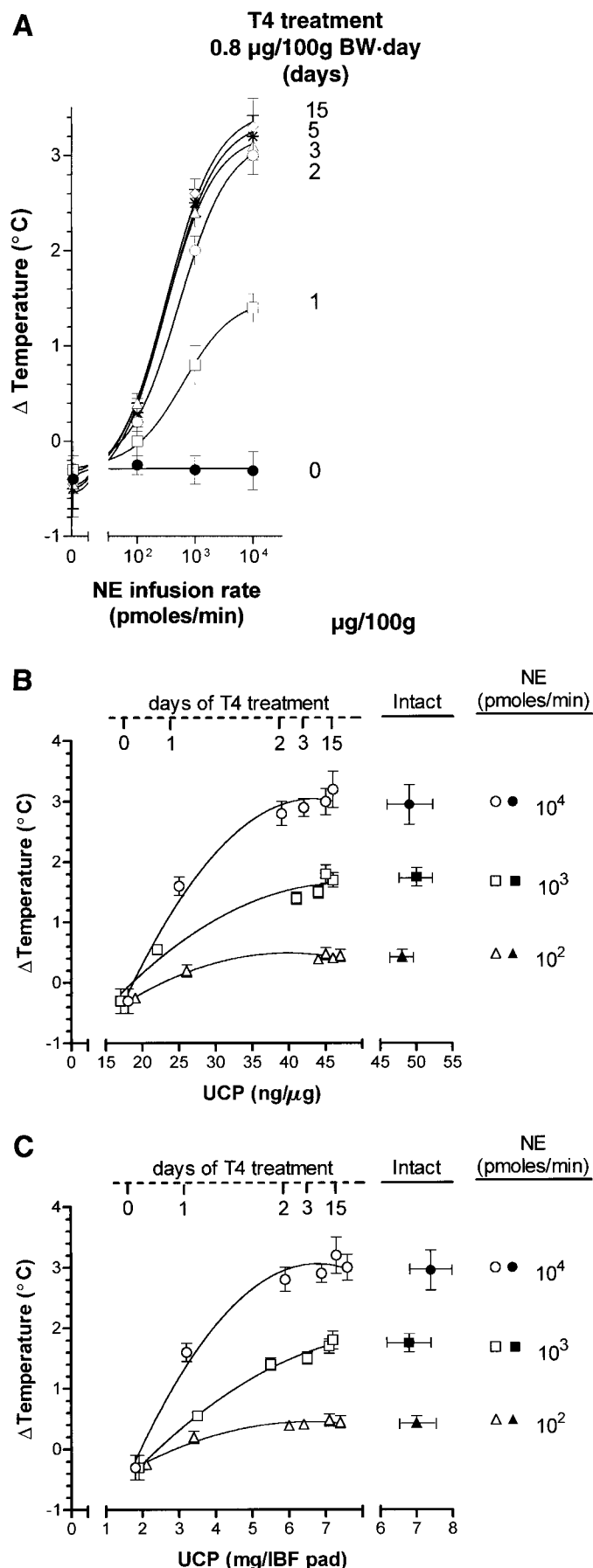
Values are means $\pm$ SD of 3–5 rats. Rats were implanted subcutaneously with a continuous release pellet of thyroxine (T_4 , $0.80 \mu\text{g} \cdot 100 \text{ g}^{-1} \cdot \text{day}^{-1}$). Iopanoic acid (IOP) was injected ip at a dose of $5 \text{ mg} \cdot 100 \text{ g body wt}^{-1} \cdot \text{day}^{-1}$. All treatments lasted 48 h. The interscapular brown fat (IBF) thermal response of these animals to norepinephrine (NE) infusion is shown in Fig. 2. T_3 , 3,5,3'-iodothyronine; UCP1, uncoupling protein 1; Tx, thyroidectomized. $^*P < 0.05$ vs. Intact; $^{\dagger}P < 0.05$ vs. Tx + T_4 .

twice the physiological replacement dose. Therefore, Tx rats were treated with T_3 ($0.6 \mu\text{g} \cdot 100 \text{ g body wt}^{-1} \cdot \text{day}^{-1}$) for 1–15 days. At the end of each period, we measured the IBF thermal response to NE infusion at various rates. The results are shown in Fig. 4A. As before, Tx rats showed a flat response to NE infusion regardless of the dose administered. On the other hand, 1 day of treatment with T_3 caused a significant thermal response that increased with the duration of treatment. By 15 days of T_3 administration, the IBF thermal response was not significantly different from that of intact rats. It is interesting to note that the highest effects of T_3 treatment were detected in the animals infused with the highest dose of NE (Fig. 4A). Next, the IBF thermal responses were plotted against UCP1 concentration (ng/ μg ; Fig. 4B) and IBF UCP1 content (mg/pad). First, it is notable that, regardless of the duration of the T_3 treatment, the mitochondrial

Table 2. Serum concentrations of T_4 and T_3 in T_4 -treated Tx rats

Days	Serum, ng/ml	
	T_4	T_3
0	<5	<0.05
1	7.1 ± 1	0.19 ± 0.04
2	15 ± 2.2	0.29 ± 0.33
3	37 ± 3.8	0.41 ± 0.06
5	43 ± 5.2	0.59 ± 0.07
15	45 ± 4.1	0.55 ± 1.0

Values are means $\pm$ SD of 3 rats. Rats were implanted sc with a continuous release pellet of T_4 ($0.80 \mu\text{g} \cdot 100 \text{ g}^{-1} \cdot \text{day}^{-1}$). The IBF thermal response of these animals to NE infusion is shown in Fig. 3.



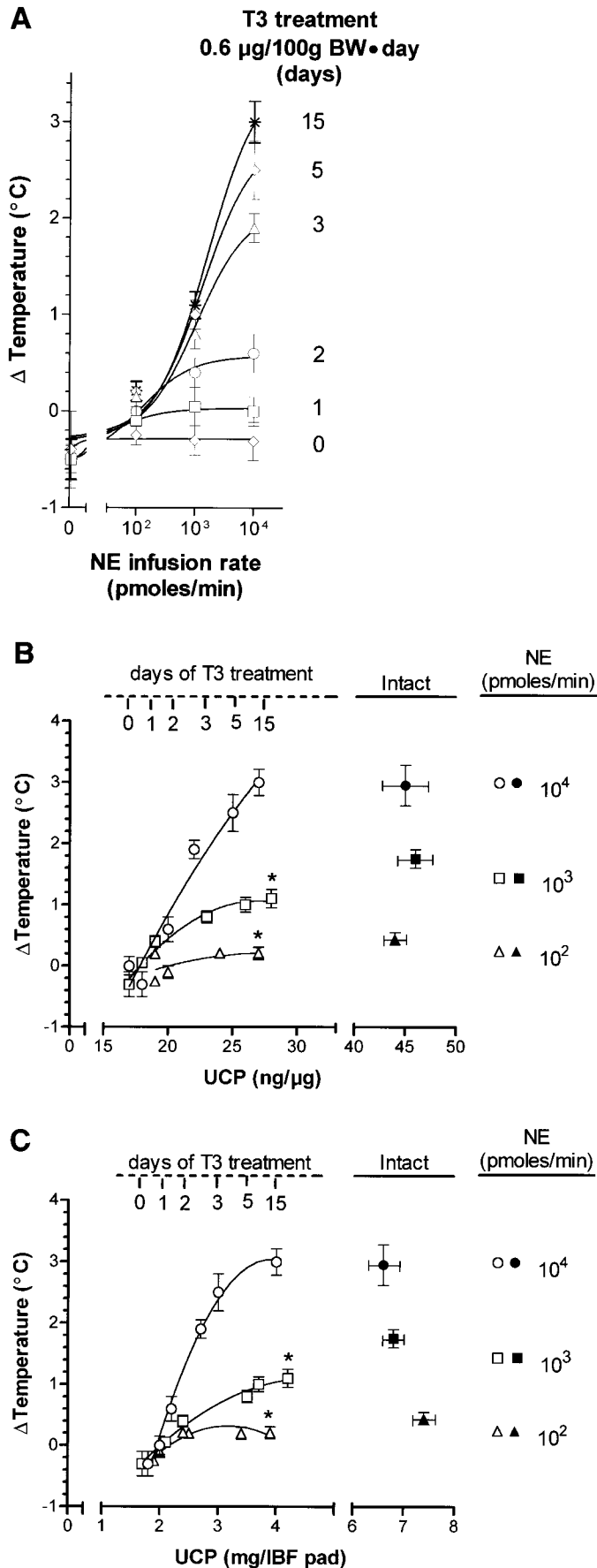
UCP1 concentration or the IBF UCP1 content was never normalized, staying always ~40% below the value seen in intact rats. Regardless of the NE dose used, there was a positive correlation between UCP1 concentration and IBF UCP1 content vs. IBF thermal response. It is interesting, however, that with the lower (10^2 pmoles/min) and the intermediate (10^3 pmoles/min) NE doses, the maximum IBF thermal response did not reach the levels seen in intact rats. It was only with the highest NE dose (10^4 pmoles/min) that the IBF response was normalized, even though the mitochondrial UCP1 concentration and UCP1 IBF content were equivalent in all three groups of rats. What is remarkable is that a normal IBF thermal response was obtained in rats that had only ~60% of the UCP1 found in intact rats.

Effects of cold exposure or feeding a cafeteria diet on the IBF thermal profile during NE infusion in intact rats. The next set of experiments was designed to investigate further the correlation between IBF UCP1 concentration and its thermal response to NE infusion. Therefore, we studied the IBF thermal response of cold-exposed rats (5 days) or rats fed a cafeteria diet (20 days), two conditions known to increase UCP1 concentration. In fact, UCP1 concentration was found to be 154 ± 23 ng/µg protein in the cold-exposed rats and 165 ± 25 ng/µg protein in the rats fed a cafeteria diet, respectively 3.4- and 3.6-fold higher than in intact rats. Figure 5A shows that the IBF NE-stimulated thermal profile of cold-exposed rats was very similar to that of intact rats, except that with the higher NE doses the maximal T_{IBF} was $>6^\circ\text{C}$ higher than that in the rats infused with saline, exceeding by $\sim 3^\circ\text{C}$ the response observed in intact rats. A similar finding was detected in the rats fed a cafeteria diet, which is shown in Fig. 5B, except that in this case a small difference between the two curves was observed with the lower doses of NE as well.

DISCUSSION

Brown fat has an enormous capacity to generate heat, which is illustrated here by the $\sim 3^\circ\text{C}$ rise in T_{IBF} of intact rats during maximal NE infusion and by similar reports in the literature (8, 16, 20). The brown fat of Tx rats, however, is known to present biochemical characteristics of impaired thermogenesis (2, 4). Accordingly, in the present study we found that the T_{IBF} response to NE in Tx rats was remarkably flat. Indeed, the IBF thermal response was not only decreased, as other important markers of brown fat thermogenesis, but was abolished. As an example, even

Fig. 3. IBF thermal response during NE infusion in T_4 -treated Tx rats. T_4 -treated Tx rats received NE infusion at various rates over a period of 1 h. A: thermal response was evaluated as described in the legend to Fig. 2. The T_4 dose is indicated, and treatment lasted 0–15 days. Data shown in A are plotted against mitochondrial UCP1 concentration (ng/µg; B) or IBF UCP1 content (mg/pad; C) for the 3 NE doses used. The 3 curves correspond to the indicated NE infusion rates (open symbols). Note that the abscissa is interrupted. For comparison, correlation values obtained for intact rats are shown as closed symbols. Values are means $\pm$ SD of 3 rats.



though the brown fat mitochondrial UCP1 content of Tx rats was reduced compared with intact rats, it was clearly detectable and alone could not explain the flat thermal response. It is likely, therefore, that rather than an isolated UCP1 insufficiency, the lack of thermal responsiveness to NE in Tx rats is a combination of multiple deficiencies, including decreased adrenergic responsiveness (10, 27–30).

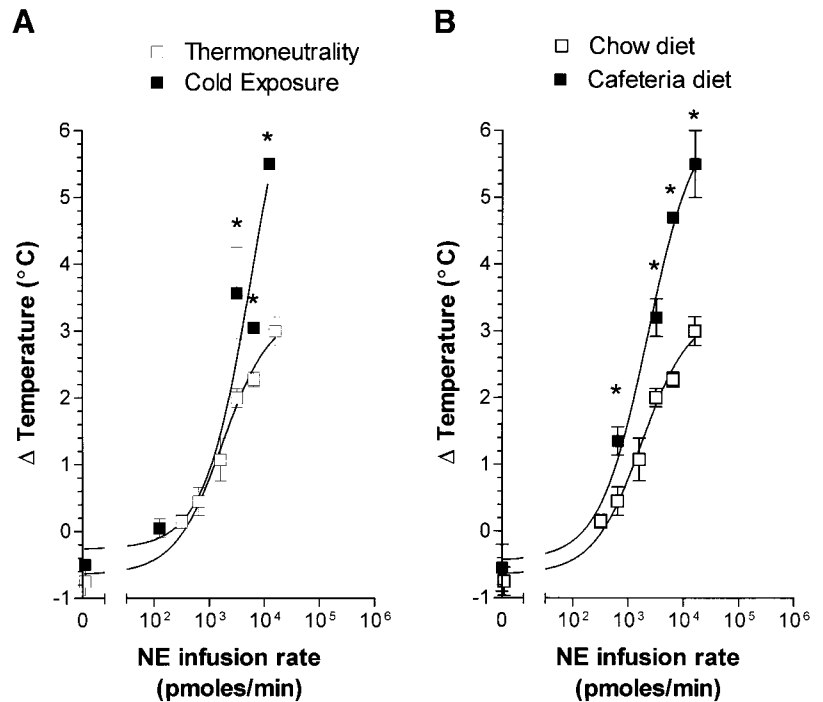
In the present study, we showed that the lack of IBF thermal response to NE in Tx rats was improved after 24 h and completely restored after 48 h of physiological replacement with T_4 . So whatever shortcomings had developed during hypothyroidism were rapidly reversed by T_4 administration. In previous studies, we found that within 24–48 h of physiological replacement with T_4 , UCP1 gene transcription (1, 26) mRNA levels (2) and mitochondrial concentration (2, 4) were restored in Tx rats. However, the physiological significance of those changes in terms of capacity of heat production has not been ascertained. Nevertheless, it is not clear how much of the brown fat thermal responsiveness to NE in intact or T_4 -treated Tx rats is determined or limited by UCP1 content.

To address this issue, we set out to study mitochondrial UCP1 and NE-induced IBF thermogenesis during treatment with thyroid hormones. Replacement with T_4 , at a dose equal to the daily production rate, rapidly normalized both UCP1 and thermal responsiveness to NE. Treatment with IOP, a D2 inhibitor, blocked the effect of T_4 , confirming our findings that T_3 concentration in brown fat is strongly influenced by the local T_4 -to- T_3 conversion (3, 11). By virtue of being several-fold stimulated in Tx rats, the brown fat D2 minimizes the effects of hypothyroidism and restores brown fat euthyroidism earlier than in most tissues (4).

To study the effects of T_3 , we purposely chose a dose (~ 2 times its daily production rate; $0.6 \mu\text{g} \cdot 100 \text{ g body wt}^{-1} \cdot \text{day}^{-1}$) that would cause systemic hyperthyroidism but not be high enough to restore euthyroidism to the brown fat. This dose of T_3 results in a nuclear T_3 receptor occupancy of $\sim 50\%$ in brown fat, which is similar to that of most tissues but low for brown fat (4). This is because in intact rats, the brown fat D2 sustains a higher degree of nuclear T_3 receptor occupancy [$\sim 70\%$; (3)], a situation that can only be reproduced in Tx rats if they receive replacement treatment with T_4 , or if T_3 is given at a dose of $1.2 \mu\text{g} \cdot 100 \text{ g body wt}^{-1} \cdot \text{day}^{-1}$, ~ 4 times the daily production rate (4). The

Fig. 4. IBF thermal response during NE infusion in T_3 -treated Tx rats. T_3 -treated Tx rats received NE infusion at various rates over a period of 1 h. A: the thermal response was evaluated as described in the legend to Fig. 2. The T_3 dose is indicated, and treatment lasted 0–15 days. Data shown in A are plotted against mitochondrial UCP1 concentration (ng/ μg ; B) or IBF UCP1 content (mg/pad; C) as described in the legend to Fig. 3. Note that the abscissa is interrupted. For comparison, correlation values obtained for intact rats are shown as closed symbols. Serum T_3 concentrations (ng/ml) were, for 0 days, <5 ; for 1 day, 0.12 ± 0.02 ; for 2 days, 0.20 ± 0.03 ; for 3 days, 0.33 ± 0.06 ; for 5 days, 0.49 ± 0.07 ; and for 15 days, 0.57 ± 0.09 . Serum T_3 concentration in intact rats: 0.54 ± 0.06 ng/ml, as shown in Table 1. Values are means $\pm$ SD of 3 rats. * $P < 0.05$ vs. intact rats.

Fig. 5. IBF thermal response during NE infusion in intact rats fed a cafeteria diet or exposed to cold. Intact rats received NE infusion at various rates over a period of 1 h. In *A*, rats had been previously exposed to cold for 5 days (■) or kept at 28–30°C (□) before receiving NE infusion. In *B*, intact rats had been fed a cafeteria diet for 20 days (■) or a chow diet (□) before receiving the NE infusion. The thermal response was evaluated as described in the legend to Fig. 2. Values are means $\pm$ SD of 2–3 rats. * $P < 0.05$ vs. control rats.



strategy led us to picture a unique condition in which the thermogenic potential of UCP1 is clearly amplified by other factor(s). These T_3 -treated Tx animals presented a time-dependent increase in UCP1 levels that reached only 25–30 ng/ μ g protein after 15 days, still $\sim 40\%$ lower than intact rats. Nevertheless, there was a detectable increase in the brown fat thermal response at every change in UCP1 level, even when these increments in UCP1 were only minimal and barely significant. In addition, despite having much lower mitochondrial UCP1 levels, these T_3 -treated Tx rats reached an IBF thermal response to NE equivalent to the intact or T_4 -treated Tx rats. It is remarkable that IBF pads containing nearly a twofold difference in their UCP1 pools (T_4 -treated vs. T_3 -treated Tx rats) could have equivalent thermal responses to NE infusion.

A closer look at our findings, however, reveals that with lower doses of NE (10^3 or 10^2 pmol/min), the IBF thermal response of T_3 -treated Tx rats was not normalized, remaining significantly below that observed in intact or T_4 -treated rats. Only during maximal NE stimulation (10^4 pmol/min) was the IBF thermogenesis normalized, raising the logical suspicion that brown fat adrenergic responsiveness plays a regulatory role under these conditions. Although there are coexisting β_1 -, β_2 -, and β_3 -adrenergic receptor subtypes in brown fat, activation of the β_3 -subtype is predominantly responsible for the thermogenic responses (15, 41). These receptors have a lower affinity for NE and, unlike β_1 and β_2 , are not sensitive to homologous-mediated desensitization.

Brown fat adrenergic responsiveness is known to be negatively correlated with local sympathetic activity (31). Cold exposure, for example, decreases the adrenergic responsiveness of brown fat, an effect that has

been ascribed to downregulation of β_3 -receptors and to diminished G_s protein (14, 37). Thyroid status is also known to regulate brown fat sympathetic activity and, hence, its adrenergic responsiveness. Prolonged hypothyroidism is accompanied by increased brown fat sympathetic tone (39, 40) and decreased adrenergic responsiveness (27, 30), whereas the opposite is observed during hyperthyroidism (36). Work from Silva's laboratory (10, 28, 29) and others (25) has indicated the existence of a postreceptor defect that leads to a drastic reduction of cAMP generation in brown fat of hypothyroid rats in response to NE. Combined with the low UCP1, this defect helps to explain our first finding, i.e., the flat IBF thermogenic response to NE.

To understand the differences of IBF thermal responsiveness between T_4 - and T_3 -treated rats, it is important to keep in mind that the sympathetic activity and adrenergic responsiveness in the brown fat of these animals were different. T_4 -replaced Tx animals eventually restored their euthyroid status (systemically and at the brown fat level) so that UCP1 increase was followed by a decrease in brown fat sympathetic activity and normalization of its adrenergic responsiveness. On the other hand, the T_3 -treated Tx rats had, in fact, systemic hyperthyroidism (the T_3 dose was twice the physiological replacement), which decreases brown fat sympathetic activity. It is therefore likely that the resulting increased adrenergic responsiveness allowed a normal thermogenic response to NE, even though the UCP1 levels were 50% lower.

To identify other conditions in which the IBF thermal response could be modulated at a pre-UCP1 level, we set out to evaluate NE-induced brown fat thermogenesis in rats exposed to cold or fed a cafeteria diet, both conditions in which the UCP1 and the sympa-

thetic tone of brown fat are increased. As expected, both experimental groups had a ~3.5-fold increase in brown fat UCP1. However, particularly in the cold-exposed rats, the IBF thermal profile in response to NE infusion was very similar to that in warm-acclimated rats, except when high doses of NE were used. It is therefore clear that, within most of the NE dose range used, the thermal responsiveness of the IBF was limited to the values found in the warm-acclimated rats. It is notable that this happened in brown fat that had ~3.5 times more UCP1. Then, as if an escape had occurred, the T_{IBF} increased ~3°C above what was observed in the warm-acclimated animals. A similar phenomenon was observed in the cafeteria-fed rats, except that T_{IBF} was slightly above control values throughout the thermal profile. Nevertheless, the major difference in thermal response was only seen when high doses of NE were used.

Given the fact that thermal responsiveness of brown fat to NE infusion was measured during a relatively short period of time (60 min), it is possible to speculate about the involvement of the masking/unmasking mechanism of UCP1 nucleotide binding sites (13, 19). Unmasking is triggered by fatty acids that interact with UCP1 so as to increase the conductance of protons. The concentration of fatty acids is regulated by the activity of hormone-sensitive lipase (HSL), itself activated by NE. It is possible that, under our experimental conditions, changes in brown fat adrenergic responsiveness influenced NE-induced HSL activity, modifying the UCP1 unmasking phenomenon and therefore contributing to the poor correlation between UCP1 levels and brown fat thermal responsiveness.

In conclusion, the present study exposed, for the first time, physiological situations in which there is a lack of correlation between UCP1 content and thermal responsiveness of brown fat to NE. Under conditions in which the thyroid status was modified or the brown fat was stimulated by cold exposure or feeding a cafeteria diet, there were substantial changes in UCP1 mitochondrial concentration that did not necessarily translate into heat production during NE infusion. Our data support the concept that brown fat adrenergic responsiveness is an important pre-UCP1 modulating step that can strongly up- or downregulate brown fat thermogenesis and help the achievement of thermal homeostasis.

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