

# Skeletal muscle mitochondrial free-fatty-acid content and membrane potential sensitivity in different thyroid states: involvement of uncoupling protein-3 and adenine nucleotide translocase

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**Abstract** The effect of triiodothyronine (T3) on mitochondrial efficiency could be related to an increase in the concentrations of some proteins, such as uncoupling proteins (UCPs). Free fatty acids (FFA) seem to be a cofactor essential for the uncoupling activity of UCP3. In this paper, we report that the hypothyroidism–hyperthyroidism transition is accompanied by increases: (i) in the endogenous levels of mitochondrial FFA and (ii) in the sensitivity to FFA shown by the mitochondrial respiration rate and membrane potential, which correlated with the level of UCP3 protein. The level of the mRNA for adenine-nucleotide translocase-1 (ANT) was not affected by the thyroid state, while the ANT contribution to FFA-induced changes in mitochondrial uncoupling was low in the hypothyroid and euthyroid states but became more relevant in the hyperthyroid state at the highest concentration of FFA.

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**Key words:** Mitochondrion; Thyroid hormone; Uncoupling protein; Adenine-nucleotide translocase; Fatty acid

## 1. Introduction

The idea that triiodothyronine (T3) is able to change mitochondrial efficiency can be traced back to the studies by Lardy and Feldcott in the early 1950s. They showed that the addition of thyroxine to isolated mitochondria *in vitro* led to a reduction in the respiratory control ratio (RCR) [1]. On the basis of such data, they suggested that the major cellular mechanism underlying the calorogenic effect of thyroid hormones might be an uncoupling of oxidative phosphorylation. This hypothesis was subsequently discarded as not really physiologically relevant. Recently, however, new results have led to revival of interest in the ‘uncoupling hypothesis’ as applied to thyroid hormones. It has been shown that: (i) hyperthyroidism is associated with an increased leak of protons through the inner mitochondrial membrane, whereas the opposite occurs in hypothyroid mitochondria [2] and (ii) that uncoupling proteins (UCPs) are present in almost all tissues,

not just in brown adipose tissue. Several findings pointed strongly towards a role for UCP3 in the mechanisms underlying T3-dependent energy expenditure, namely: (i) UCP3 is almost exclusively present in skeletal muscle [3], (ii) the skeletal-muscle proton leak accounts for a significant fraction of the resting metabolic rate in the whole animal [4], (iii) mice overexpressing UCP3 are hyperphagic and lean, with a decreased mitochondrial efficiency [5], and (iv) in skeletal muscle and heart, T3 is able to increase the UCP3 mRNA [6–8] and protein [9,10] levels.

Recently, we showed that the expression of UCP3 mRNA in skeletal muscle increases during the hypothyroidism–hyperthyroidism transition and that mitochondria isolated from the skeletal muscle of euthyroid and hyperthyroid rats show a proton leak higher than that of their hypothyroid counterparts [8]. The latter result has been confirmed by Jucker et al. [10] who, while assessing mitochondrial energy coupling in skeletal muscle *in vivo* by a non-invasive method (nuclear magnetic resonance spectroscopy), found that injection of T3 into hypothyroid rats led to mitochondrial uncoupling in skeletal muscle. More recently, by time-course studies, we have shown that UCP3 has the potential to be a molecular determinant in the regulation of resting metabolic rate by thyroid hormone [9].

In the above study on rat skeletal muscle mitochondria [8], the changes in proton-leak kinetics that accompanied the hypothyroidism–hyperthyroidism transition disappeared when fatty-acid-free bovine serum albumin (BSA) was included in the incubation medium. An explanation for these results could be that the difference in proton leak between the hypo- and hyperthyroid states is related to a difference in the endogenous content of mitochondrial free fatty acids (FFA) capable of affecting uncoupled respiration, and that BSA, by chelating FFA, abolished their effect. However, the same results could also be explained on the basis of a crucial involvement of FFA as cofactors for UCP3-uncoupling activity. The latter hypothesis seems to be supported by the recent findings of Echtay et al., who found FFA to be important cofactors for the activation by coenzyme Q (CoQ) of UCP2 and UCP3 (expressed in *Escherichia coli*), and by the more recent ones of Echtay and Brand, who found CoQ, FFA and reactive oxygen species (ROS) to be involved in the variations in the UCP-mediated proton leak [11,12]. Because of this, it seemed important to study the differences in endogenous mitochondrial FFA levels among different thyroid states and also the

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sensitivity to FFA shown by the mitochondrial respiration rate (Jo) and membrane potential (mp). In the present study, we measured the endogenous FFA content in skeletal muscle mitochondria from animals in different thyroid states, as well as the sensitivity shown by Jo and mp to exogenous FFA. Knowing that in the liver, T3 is able to affect ANT (adenine-nucleotide translocase, a carrier sensitive to FFA and able to uncouple mitochondria) we also evaluated the contribution made by ANT to the T3-induced changes in the above parameters in skeletal muscle.

## 2. Materials and methods

### 2.1. Materials

T3 was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). A polyclonal antibody raised against the C-terminal region of the human UCP3 protein (AB3046) was purchased from Chemicon International (Temecula, CA, USA).

### 2.2. Animal treatment

All animal studies were conducted in accordance with the National Guide for the Care and Use of Laboratory Animals. Three different groups of Male Wistar rats (250–300 g) were used throughout: hypothyroid, euthyroid and hyperthyroid groups.

Hypothyroidism was induced by the i.p. administration of propylthiouracil (1 mg/100 g bw) for 4 weeks together with a weekly i.p. injection of iopanoic acid (6 mg/100 g bw). Hyperthyroidism was induced by the daily i.p. administration of T3 (15 µg/100 g bw) for 1 week.

In the euthyroid group, the rats were injected with saline.

### 2.3. Isolation of mitochondria

Mitochondria from gastrocnemius and tibialis anterior muscles were isolated after homogenization in an incubation medium consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA and 5 mM EGTA, pH 7.4. After homogenization, each sample was centrifuged at  $700\times g$ , with the resulting supernatant being centrifuged at  $8000\times g$ . The mitochondrial pellet was washed twice, resuspended in a minimal volume and kept on ice.

### 2.4. Western blot analysis

Analyses were performed using mitochondria suspended in isolation medium supplemented with the following protease inhibitors: 1 mM benzamide, 4 µg/ml aprotinin, 1 µg/ml pepstatin, 2 µg/ml leupeptin, 5 µg/ml betastatin, 50 µg/ml N-tosyl-L-phenylalanine-chloromethyl ketone and 0.1 mM phenylmethylsulfonylfluoride (all from Sigma-Aldrich Corp.).

Mitochondrial lysate was prepared by resuspending the mitochondria in sodium dodecyl sulfate (SDS) loading buffer, as described by Laemmli [13], followed by heating for 3 min at 95°C. Mitochondrial lysates containing 30 µg protein were loaded in each lane and electrophoresed on a 13% SDS-PAGE (polyacrylamide gel electrophoresis) gel. A polyclonal antibody against UCP3 and an anti-rabbit antibody were used as primary and secondary antibodies, respectively, on duplicate filters in a chemiluminescence protein-detection method based on a protocol from a commercially available kit (NEN, Boston, MA, USA). The protein concentration was determined by the method of Hartree [14]. UCP3 protein levels were determined and quantified in separate preparations from three rats.

### 2.5. Determination of respiration rate

Respiratory parameters were determined using a Clark-type oxygen electrode. They were measured in a mitochondrial suspension (0.5 mg/ml) at 37°C in standard incubation medium A [80 mM KCl/50 mM HEPES (pH 7)/1 mM EGTA/5 mM  $K_2HPO_4$ /5 mM  $MgCl_2$ ] both in the presence and in the absence of 1% (w/v) BSA, using a saturating amount of succinate as substrate (5 mM). State 3 respiration was initiated by the addition of ADP (200 µM).

### 2.6. Evaluation of mitochondrial FFA content in different thyroid states

Mitochondria (0.1–0.15 mg) were incubated in 1 ml of standard incubation medium A. The respiration in the presence of endogenous FFA (State 4<sub>-BSA</sub>) was initiated by energizing mitochondria with

succinate and subsequently inhibited by the addition of 1% BSA (State 4<sub>+BSA</sub>). At this concentration, BSA is the predominant protein and FFA can therefore be considered to be buffered by the albumin. State 4<sub>+BSA</sub> was successively stimulated by adding increasing amounts of oleate. We chose oleate since the binding-affinity constant of BSA for oleate is known and, by using the equation of Richieri et al. [15], it is possible to estimate the real amount of oleate that is not bound to BSA, and therefore free to interact with mitochondria (free oleate).

For each mitochondrial preparation we determined the amount of free oleate that, when added to mitochondria respiring in State 4<sub>+BSA</sub>, was able to restore the value of State 4<sub>-BSA</sub>; we used this value as an index of the mitochondrial FFA content. Although this method does not give a real value for mitochondrial endogenous FFA content, it is useful for the present study because: (i) it gives an indication of the levels of FFA able to affect State 4 respiration and (ii) our aim was to estimate the change occurring in the level of FFA during the hypothyroidism–hyperthyroidism transition.

In the approach we used, we assumed that in each mitochondrial preparation, the endogenous pool of fatty acid behaves as oleate in stimulating respiration.

### 2.7. Measurement of proton-motive force

Proton-motive force is the sum of membrane potential (mp) and the  $H^+$  gradient across the mitochondrial membrane. To simplify measurements, the pH gradient was abolished by the addition of nigericin so that mitochondria increase mp to compensate and the whole of the proton-motive force was expressed as  $\Delta\psi$ . The magnitude of mp was determined from the distribution of the lipophilic cation triphenylmethylphosphonium ( $Ph_3MeP^+$ ), which was measured using a  $Ph_3MeP^+$ -sensitive electrode, as described by Brown and Brand [16]. A  $Ph_3MeP^+$ -binding correction of 0.4 was applied for mitochondria from each group.

### 2.8. Effect of oleate on mitochondria

The responses shown by mitochondrial membrane potential and respiration rate to oleate were measured simultaneously in standard incubation medium A supplemented with 1% BSA, nigericin (80 ng/ml) and oligomycin (1 µM), measurement being made in the presence and in the absence of carboxyatractilide (15 µg/ml). The presence of EGTA in the incubation medium allowed us to exclude the eventual opening of permeability transition pores. Respiration was initiated by the addition of succinate to the mitochondrial suspension and then stimulated by increasing the concentration of oleate (final concentrations 0.25, 0.5 and 0.75 mM, corresponding to calculated free concentrations of 11, 33 and 172 nM, respectively). For the measurements described, oleate was dissolved in 50% ethanol; the ethanol did not in itself have any effect on the parameters measured and so is represented herein as a zero concentration of oleate.

### 2.9. Statistical analysis

The statistical significance of differences between groups was determined by use of a one-way analysis of variance followed by a Student–Newman–Keuls test.

## 3. Results

### 3.1. Respiratory parameters for skeletal muscle mitochondria from euthyroid, hypothyroid and hyperthyroid rats

Table 1 shows the effect of thyroid state on mitochondrial respiratory parameters determined in the presence and in the absence of BSA. In the presence of BSA, the values obtained for States 4 and 3 were significantly higher for euthyroid and hyperthyroid mitochondria than for the hypothyroid ones. As the percentage increases during the hypothyroid–hyperthyroid transition were similar for States 3 and 4, the RCR value remained unchanged.

The State 4 respiration rate was higher in euthyroid mitochondria than in hypothyroid mitochondria by a similar percentage whether measured in the absence or presence of BSA (29% and 27%, respectively). However, the corresponding value for hyperthyroid versus hypothyroid mitochondria differed

Table 1

Respiration rates for skeletal muscle mitochondria from hypothyroid, euthyroid and hyperthyroid rats

	Hypothyroid	Euthyroid	Hyperthyroid
State 4 <sub>+BSA</sub>	92.3 ± 9.8	117.5 ± 6.4*	111.3 ± 3.8*
State 3 <sub>+BSA</sub>	378.4 ± 20	470 ± 15*	480 ± 18*
RCR	4.1 ± 0.2	4.0 ± 0.3	4.3 ± 0.3
State 4 <sub>-BSA</sub>	225.4 ± 18.3	292.2 ± 17.8*	336.7 ± 25.8*
Endogenous FFA concentration (nM)	23.2 ± 3.6	31.2 ± 3.3*	60.8 ± 13.4*°

Calculated respiration rates (nA oxygen/(min mg proteins)) are for incubation with BSA either present in the incubation medium (State 4<sub>+BSA</sub> and State 3<sub>+BSA</sub>) or absent from it (State 4<sub>-BSA</sub>). Values represent the mean ± S.E. from five different experiments.

\* $P < 0.05$  vs. hypothyroid state, ° $P < 0.05$  vs. euthyroid state.

markedly between the absence of BSA (49%) and its presence (20%).

The above results could mean that hyperthyroid mitochondria have a higher FFA content and/or that hyperthyroidism makes mitochondria more sensitive to FFA.

To try to distinguish between these possibilities, we determined the mitochondrial endogenous FFA concentration that was responsible for the observed differences between the State 4 respiration rates measured in the absence and presence of BSA. To this end, we determined the amount of oleate that, when progressively added to mitochondria respiring in State 4 in the presence of BSA, was able to restore the respiration rate

to the value observed in the absence of BSA. On this basis, our data (Table 1) show that the FFA content of euthyroid mitochondria was 34% higher than that of hypothyroid ones, while that of hyperthyroid mitochondria was 95% and 162% higher than that of euthyroid and hypothyroid mitochondria, respectively.

### 3.2. Effect of increasing the concentration of oleate on respiration rate and membrane potential in skeletal muscle mitochondria from hypothyroid, euthyroid and hyperthyroid rats

Fig. 1 illustrates the effect of oleate on respiration rate and

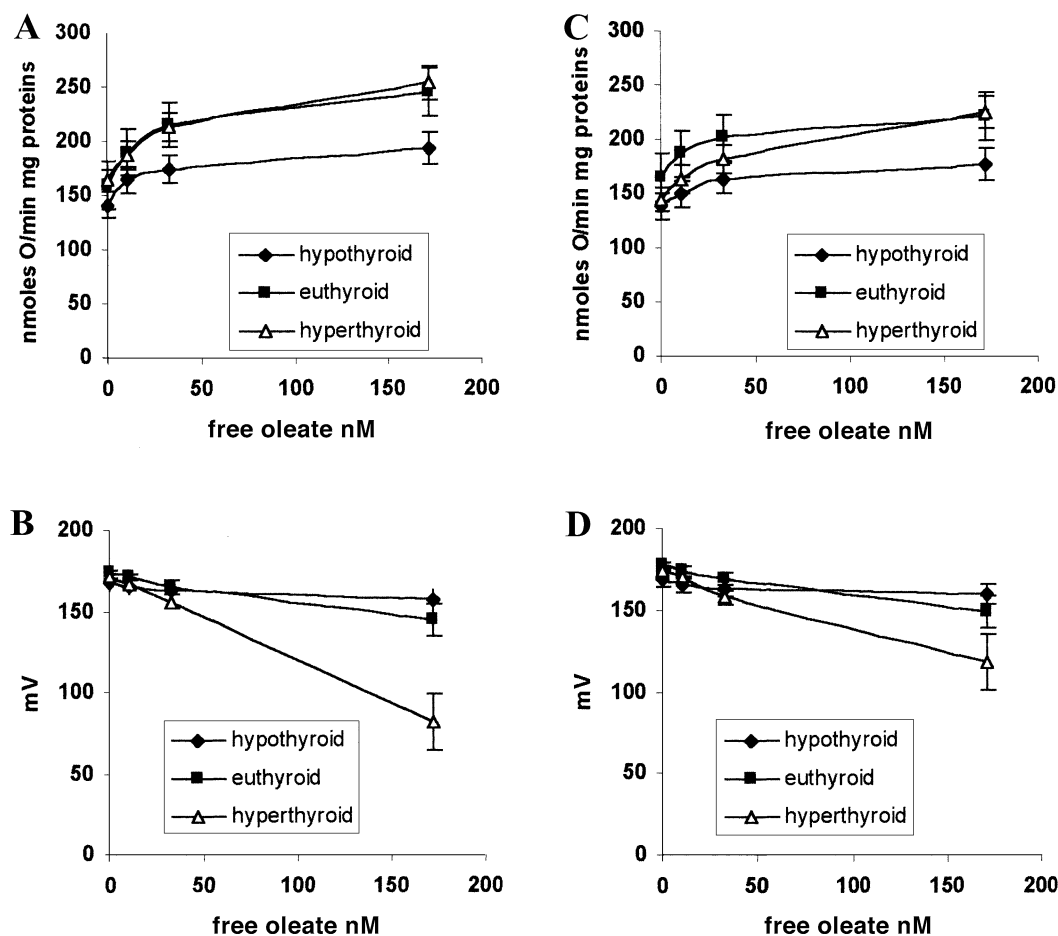


Fig. 1. Effects of oleate titration on respiration rate (panels A and C) and membrane potential (panels B and D) in skeletal muscle mitochondria isolated from hypothyroid, euthyroid and hyperthyroid rats. Panels C and D depict titrations performed in the presence of carboxyatractylide (15 µg/ml), panels A and B those performed in its absence. The concentrations of oleate shown represent those which are not bound to BSA and are therefore able to interact with mitochondria. Each value represents the mean ± S.E. from four different mitochondrial preparations.

membrane potential in skeletal muscle mitochondria in different thyroid states when measured in the absence (panels A and B) or presence (panels C and D) of carboxyatractilide (CAT), a specific inhibitor of ANT activity. In each thyroid state, oleate induced simultaneously an increase in the oligomycin-inhibited respiration rate and a decrease in membrane potential (which is diagnostic of mitochondrial uncoupling). However, the FFA sensitivity of mitochondria increased in the order hypothyroid–euthyroid–hyperthyroid state. Hypothyroid mitochondria showed the lowest respiration rate and the highest membrane potential, while the opposite was true for hyperthyroid mitochondria (see Fig. 1). Over the range of oleate concentrations we used (0–172 nM free oleate), the slope of the curves relating membrane potential to the free oleate concentration increased in the order hypothyroid–euthyroid–hyperthyroid both in the presence (panel D) and in the absence of CAT (panel B). The involvement of ANT in the effect of oleate on mitochondrial respiration and membrane potential seemed to be low (compare panels C and D with A and B), the only noteworthy effect of ANT inhibition being seen on membrane potential in the hyperthyroid state and then only in the presence of the highest concentration of FFA. To be specific, we found 172 nM oleate induced a decrease in membrane potential of about 3%, 6% and 53% in hypothyroid, euthyroid and hyperthyroid mitochondria, respectively (panel B). When the ANT contribution was excluded, the decrease in membrane potential was found to be 4%, 6% and 33% for hypothyroid, euthyroid, and hyperthyroid mitochondria (panel D).

### 3.3. UCP3 mitochondrial protein content and *ANT1* mRNA levels

Fig. 2 shows the results of Western blot analysis of the UCP3 content of skeletal muscle mitochondria. Quantification revealed that in hyperthyroid mitochondria, the UCP3 level was about 3.5 times that seen in the euthyroid state and seven times that in the hypothyroid state.

In addition, skeletal muscle *ANT1* mRNA was evaluated in hypothyroid, euthyroid and hyperthyroid rats by reverse-transcription (RT)-PCR, but its level was found not to be influenced by the thyroid state (data not shown).

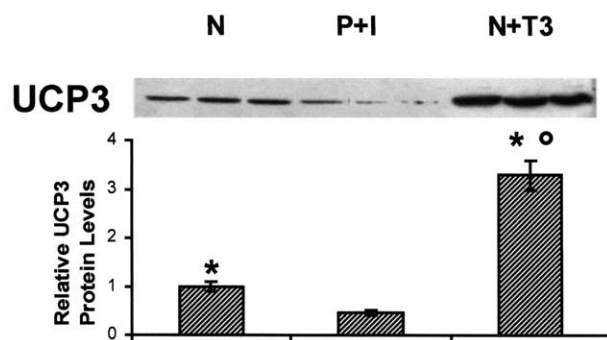


Fig. 2. UCP3 level in skeletal muscle mitochondria isolated from euthyroid (N), hypothyroid (P+I) and hyperthyroid (N+T3) animals. The upper panel shows Western immunoblot analysis. The lower panel, quantification of the data shown in the upper panel. Data are relative to the value for control (N) muscle (taken as 100). \* $P < 0.05$  vs. hypothyroid state, ° $P < 0.05$  vs. euthyroid state.

## 4. Discussion

Mitochondrial respiration is not fully coupled to ADP phosphorylation and some of the energy of the reduced substrate is lost as heat. Most of the uncoupling is due to a leak of protons across the mitochondrial inner membrane, the major process controlling mitochondrial oxygen consumption when cellular ATP requirements are low. This non-productive proton-conductance pathway is physiologically relevant, as it accounts for a significant portion of cellular metabolic rate at rest [4].

Thyroid hormone affects mitochondrial efficiency, and UCP3 could mediate this effect. As FFA seem to be important cofactors for UCP3 activity, it was important to estimate the changes in FFA content and/or sensitivity associated with alterations in thyroid state.

During the hypothyroidism–hyperthyroidism transition we showed: (i) an increase in State 4 respiration (a state of respiration controlled principally by proton-leak reactions [17]) that showed a high correlation with the increase in UCP3 protein levels only when FFA were present ( $r = 0.8$ ), (ii) a progressive increase in the endogenous mitochondrial content of FFA able to affect uncoupled respiration that correlates strictly with the increase in UCP3 protein level ( $r = 0.998$ ), and (iii) a progressive increase in the uncoupling effect of oleate (as evaluated by measuring the increase in respiration rate that occurred concomitant with a decrease in membrane potential) that was associated with an increase in the UCP3 level.

The data we obtained showing a higher FFA content in hyperthyroid mitochondria and a higher sensitivity of such mitochondria to added FFA are in accord with data published by Simonyan et al. [18], who obtained similar results in a different physiological situation (when the UCP3 level increased due to a cold exposure). In these conditions, in fact, they reported an increased recoupling effect of BSA and a higher sensitivity of skeletal muscle mitochondria to the added FFA. They observed similar effects after an *in vivo* injection of thyroxine.

ANT is a mitochondrial carrier able to mediate fatty-acid-induced uncoupling by carrying deprotonated FFA from the matrix to the intermembrane space, as first proposed by Skulachev [19]. Its activity could affect the estimation of the amount of FFA-mediated uncoupling attributed to UCP3, leading to possible errors. In rats, two isoforms of ANT are expressed in a tissue-specific manner (the muscle-specific form ANT1 and the ubiquitous form ANT2) [20]. In rat liver, ANT2 is the main isoform and its expression is regulated by T3 [21,22]. By contrast, ANT1 is not responsive to thyroid hormones since injection of T3 into hypothyroid rats does not affect the ANT1 mRNA level. The involvement of the ANT antiporter in thyroid-hormone-induced uncoupling has been demonstrated by Schönfeld et al. [23] in mitochondria from rat liver, a tissue lacking UCPS (with the exception of Kupffer cells, which express UCP2 [24]). They reported a two-fold increase in mitochondrial ANT content and an almost four-fold increase in its activity after the administration of T3 to hypothyroid rats, suggesting that mitochondrial enrichment with ANT protein does not account for all of the increased activity. Other factors, such as the mitochondrial lipid environment or ATP-ADP pool, both of which are influenced by the thyroid state [23,25], can affect both ANT activity and



ANT-mediated uncoupling. For this reason, the lack of response shown by ANT1 mRNA to T3 injection [21] does not exclude the involvement of this protein in mitochondrial T3-induced uncoupling.

In this context, we confirmed that gastrocnemius ANT1 mRNA is not regulated by T3; in fact, in this muscle no changes in its level were evident whatever the thyroid state (data not shown). Despite this, we showed that in skeletal muscle, ANT is, to some extent, involved in the uncoupling effect of fatty acids in mitochondria and that it mediates a part of the T3-induced uncoupling. In fact, the contribution made by ANT to the changes in mitochondrial uncoupling was evident only in the hyperthyroid state and then only at the highest FFA concentration used.

We should point out that we cannot exclude a contribution to T3-mediated FFA-dependent uncoupling by other mitochondrial anion carriers able to transport fatty acid anions (glutamate/aspartate antiporter, dicarboxylate carrier and phosphate carrier).

In conclusion, our data indicate that in the euthyroid state, FFA influence UCP3 activity, which is presumably mainly responsible for FFA-mediated uncoupling, but the ANT contribution is much less affected. However, in the hyperthyroid state (in the presence of the highest concentration of FFA we used) the ANT contribution became more relevant. On the basis of these and previous results [2,27] we might predict a tissue specificity in thyroid-hormone-induced mitochondrial uncoupling: in the liver, the effect may be principally mediated by ANT2 (or another, as yet unknown, UCP), while in skeletal muscle the effect may be principally due to UCP3.

On the basis of the results reported here we can speculate that hyperthyroid rats may be a physiological animal model 'analogous' to genetically manipulated UCP3-overexpressing mice. In fact, both hyperthyroid and UCP3-overexpressing animals show a higher level of UCP3 than normal ones. Moreover, both have an increased metabolic rate, use lipid as their preferential substrate and have skeletal muscle mitochondria that are more uncoupled than those in normal animals [5,26].

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