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Expression of uncoupling protein-3 and mitochondrial activity in the transition from hypothyroid to hyperthyroid state in rat skeletal muscle

A. Lanni[a,b], L. Beneduce[b], A. Lombardi[b], M. Moreno[b,c], O. Boss[d], P. Muzzin[d], J.P. Giacobino[d], F. Goglia[b,*]

[a] *Dipartimento di Scienze della Vita, Seconda Università degli Studi di Napoli, Caserta, Italy*
[b] *Dipartimento di Fisiologia Generale ed Ambientale, Via Mezzocannone 8, I-80134, Napoli, Italy*
[c] *Facoltà di Scienze, Università degli Studi del Sannio, Benevento, Italy*
[d] *Department of Medical Biochemistry, Faculty of Medicine, University of Geneva 1, Geneva, Switzerland*

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Abstract We sought a correlation between rat skeletal muscle triiodothyronine (T3)-mediated regulation of uncoupling protein-3 (UCP3) expression and mitochondrial activity. UCP3 mRNA expression increased strongly during the hypothyroid-hyperthyroid transition. The rank order of mitochondrial State 3 and State 4 respiration rates was hypothyroid < euthyroid < hyperthyroid. The State 4 increase may have been due to the increased UCP3 expression, as the proton leak kinetic was stimulated in the hypothyroid-hyperthyroid transition and a good correlation exists between the State 4 and UCP3 mRNA level. As a significant proportion of an organism's resting oxygen consumption is dedicated to opposing the proton leak, skeletal muscle mitochondrial UCP3 may mediate part of T3's effect on energy metabolism.

Key words: Uncoupling protein; Thyroid hormone; Skeletal muscle; Mitochondrion; Membrane potential

1. Introduction

Thyroid hormones (THs) play important roles in differentiation, growth, cognitive development and metabolism. In adults, the most important effect attributed to THs, in particular triiodothyronine (T3) and possibly diiodothyronine (T2), is their influence on metabolic rate and the oxygen consumption of nearly all tissues [1,2].

At the cellular level, T2 and T3 appear to have different mechanisms of action.

T2 seems to act principally via mitochondria, while one of the most important effects attributed to T3 is the transcriptional or post-transcriptional regulation of those target genes encoding components of the mitochondrial energy-transducing apparatus (for reviews, see [3] and [4]). In this context, effects of particular interest are those exerted by T3 on the regulation of the synthesis of uncoupling proteins (UCPs). Apart from the well-known regulation of UCP1 by T3 [5], it has recently been reported that T3 stimulates expression of the mRNAs for both UCP2 (in several organs) [6,7] and UCP3 (in skeletal muscle) [8,9]. This is particularly interesting in view of the old hypothesis that an uncoupling of respiration and phosphorylation is involved in the effects of thyroid hormones. Although this idea has been under discussion for many years, it is still not clear whether the reported uncoupling effects of thyroid hormones reflect physiological events, or whether they are artifactual.

UCPs are differently distributed and regulated in different species, suggesting a variety of physiological roles.

1. UCP1 is present in very low amounts in adult humans, and it is restricted to brown adipose tissue in newborns as well as in small mammals, in whom it contributes to the regulation of body temperature and to energy balance [10,11].
2. UCP2 is widely expressed in adult tissues in rodents and humans, and it may play a role in the regulation of body weight and energy balance, in fever and in the defence against the generation of reactive oxygen species [12]
3. UCP3 is highly expressed in skeletal muscle in rodents and humans where its expression is modulated by food intake [13], thyroid hormones [8,9] and cold exposure [14].

So far, a decrease in the mitochondrial electrochemical potential due to UCP2 [15,16] or UCP3 [9,13] has been demonstrated only in transformed yeast cells or in transfected C2C12 mouse myoblasts, and it would be important to show this effect in vivo. As thyroid hormones have a well-known fundamental role in the regulation of energy metabolism, and as the contribution made by skeletal muscle to the rat's metabolic rate has been estimated to be about 30% [17], we thought it interesting to measure the mitochondrial energy coupling and the expression of UCP3 mRNA in skeletal muscle from rats in different thyroid states. These experiments for the first time could clearly demonstrate a correlation between the levels of UCP3 expression and mitochondria energy coupling in one and the same animals. Since the maintenance of a proton-motive force against the proton leak may account for a significant proportion of the resting oxygen consumption of an organism [18], we also examined the relationship between resting respiration rate $\Delta\Psi$ in oligomycin inhibited mitochondria obtained from tissues underexpressing UCP3 mRNA (hypothyroid mitochondria) and those overexpressing UCP3 mRNA (hyperthyroid mitochondria). Our results lead us to hypothesize a possible role for mitochondrial skeletal muscle UCP3 as one of the mediators of the effect of T3 on energy metabolism.

*Corresponding author. Fax: +39 (81) 5526027/55236.

Abbreviations: UCP1, uncoupling protein-1; UCP2, uncoupling protein-2; UCP3, uncoupling protein-3; T3, triiodothyronine; PTU, 6-*n*-propylthiouracil; IOP, iopanoic acid; $\Delta\Psi$, mitochondrial transmembrane electric potential difference; State 3, respiratory state in which ATP synthesis is at maximal rate; State 4, respiratory state in which there is no ATP synthesis; RCR, respiratory control ratio

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2. Materials and methods

2.1. Rat treatment regimes and tissue sampling

Male Wistar rats (220–230 g) were kept, one per cage, under an artificial lighting regime of 12 h light:12 h darkness in a temperature-controlled room at 28°C. A commercial mash was available ad libitum and the animals had free access to water. Hypothyroidism was produced in rats by a daily i.p. injection of 6-*n*-propylthiouracil (PTU) (1 mg/100 g body weight) for 3 weeks, together with a weekly i.p. injection of iopanoic acid (IOP) (6 mg/100 g body weight). Euthyroid controls were sham-injected with saline.

Chronic hyperthyroidism was induced in euthyroid rats by seven daily i.p. injections of 15 μg T_3/100 g body weight, while control rats (euthyroid and hypothyroid) received saline injections. The iodothyronine doses and the treatment duration were chosen so as to obtain a change in the levels of the thyroid hormones without significantly changing the body weight of the animals. At the end of the treatment (24 h after the last dose of T3), rats were anaesthetized by i.p. administration of chloral hydrate (40 mg/100 g body weight) and killed by decapitation. Skeletal muscles (hind-limb muscles) were isolated and either immediately processed for the preparation of the mitochondria or immediately frozen in liquid nitrogen, then stored at −80°C until further processing. All experiments were performed in accordance with local and national guidelines covering animal experiments.

2.2. Mitochondrial preparations

Skeletal muscle fragments (1 g) were homogenized using a Potter Evehjem homogenizer in 10 vol of an isolation medium consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, 5 mM EGTA and protease (1 mg/g tissue). In order not to damage the mitochondria, the homogenization was carried out very gently and 5 min were allowed for the protease to act. In some experiments, the isolation medium was supplemented with 0.5% (w/v) fatty acid-free BSA. The homogenates were centrifuged at 500×*g* for 10 min at 4°C, the resulting supernatant was centrifuged at 8000×*g* and the pellet collected was washed twice in the same isolation medium. The final mitochondrial pellet was resuspended in a minimal volume and kept on ice.

2.3. Measurement of membrane potential (ΔΨ) and respiration rate

ΔΨ 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 [19]. A Ph_3MeP^+-binding correction of 0.4 was applied, as recommended by Rolfe et al. [20]. ΔΨ was measured in the presence of nigericine so that the whole proton-motive force could be expressed as ΔΨ. Respiration rate was determined simultaneously using a Clark-type oxygen electrode.

Mitochondrial membrane potential and respiration rate were measured in a mitochondrial suspension (0.5 mg/ml) at 37°C in standard incubation medium (80 mM KCl, 50 mM HEPES (pH 7), 1 mM EGTA, 5 mM K_2HPO_4, 4 μM rotenone, 80 ng/ml nigericine using a saturating amount of succinate as substrate (5 mM). The State 4 and 3 of respiration were measured in the same incubation medium without nigericine. State 3 respiration was initiated by addition of ADP (300 μM) and RCR was calculated according to Estabrook [21]. In some experiments, the incubation medium was supplemented with 0.5% w/v fatty acid-free BSA.

The protein concentration was determined by the method of Hartree [22].

2.4. Determination of the kinetic response of the proton leak to changes in membrane potential in skeletal muscle mitochondria

In resting mitochondria, the respiration rate has to account principally to the leak of protons across the inner membrane. For the evaluation of the kinetic response of the mitochondrial proton leak to a change in ΔΨ, the incubation medium was supplemented by oligomycin (1 μg/ml). The respiration rate was inhibited by sequential addition of malonate (normally up to 2.5 mM, but up to 5 mM in mitochondria obtained in presence of BSA). We chose an assay temperature of 37°C in order to be able to attribute the non-linear relationship between respiration rate and proton-motive force, obtained when non-phosphorylating mitochondria are titrated with malonate, to an increase in the proton leak conductance of the inner membrane at high proton-motive force, instead of to a variation in the number of protons pumped by the respiratory chain per oxygen atom consumed [23,24].

2.5. RNA isolation and Northern blotting

Total RNAs from skeletal muscle were isolated by the method of Chomczynski and Sacchi [25]. For Northern blots, 20 μg of total RNA was separated on a 1% (w/v) agarose/formaldehyde gel and transferred onto nylon membranes with 20×SSC (1×SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0). To detect specific mRNA, we used a 769 bp probe derived from rat UCP3 cDNA [26] (GenBank accession number U92069) which was labelled with ^{32}P using a random priming system.

Hybridization and washing were carried out as described by Church and Gilbert [27]. A 28S-derived oligonucleotide was used to normalize the amount of RNA for each line.

3. Results

As shown in Fig. 1 (upper panel), the level of UCP3 mRNA in the hind-limb muscle was much lower in hypothyroid rats than in normal euthyroid animals, while in hyperthyroid rats the UCP3 mRNA expression was strongly increased. The differences were of the order of 5 times between euthyroid and hypothyroid states and 5 times between hyperthyroid and euthyroid states (lower panel).

As shown in Table 1, when respiring in the absence of BSA, the mitochondria obtained from hyperthyroid rats had a respiratory rate in both State 3 and State 4 that was significantly higher than the corresponding value for mitochondria from hypothyroid animals. The percentage difference between States 4 and 3 was comparable for the three groups of rats and the transition hypothyroid-euthyroid produced a greater increase for State 4 and for State 3 than the transition euthyroid-hyperthyroid. The result of all this was that RCR (respiratory control ratio) values were not significantly different

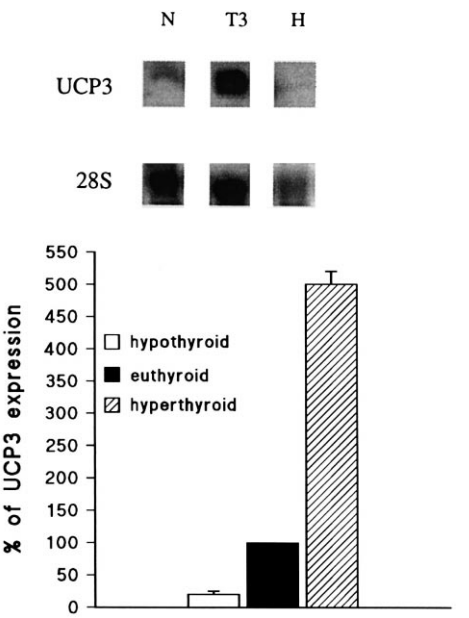

Fig. 1. Upper panel: Expression levels of UCP3 mRNA in skeletal muscle of hypothyroid, euthyroid and hyperthyroid rats. Lines: N = euthyroid rats, T3 = hyperthyroid rats, H = hypothyroid rats. Lower panel: UCP3 mRNA expression in hypothyroid and hyperthyroid skeletal muscle expressed as a percentage of the euthyroid level (Molecular Image, Bio-Rad). Values shown are the mean ± S.E.M. of three different determinations.

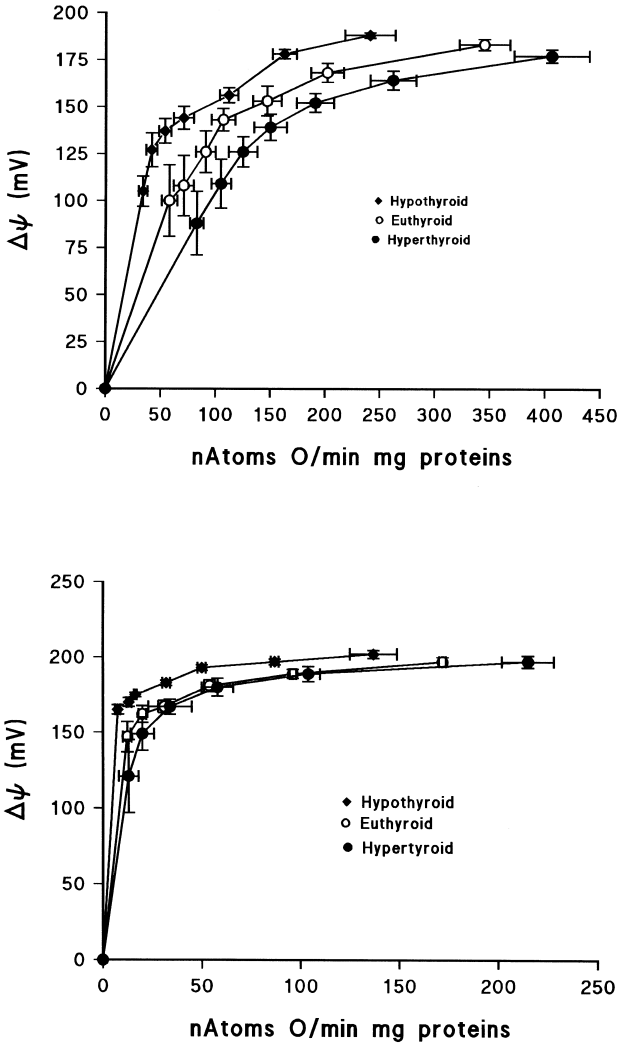

Fig. 2. Kinetic responses of proton leak to a change in ΔΨ in skeletal muscle mitochondria: comparison between hypothyroid, euthyroid and hyperthyroid rats. Panels show the kinetics of the proton leak in mitochondria prepared in the absence (upper panel) or presence (lower panel) of 0.5% BSA. The respiration medium was also supplemented with 0.5% BSA. Each point represents the mean ± S.E.M. of duplicate determinations in four different mitochondrial preparations.

among the three thyroid states. Similar results were obtained when mitochondria were incubated in the presence of BSA, except that State 4 respiration rates were lower and RCR values were about 5 (data not shown).

Table 1
State 4 and State 3 respiration rates (nAtoms O/min × mg protein) and RCR in skeletal muscle mitochondria from hypothyroid, euthyroid and hyperthyroid rats

	Thyroid state		
	Hypothyroid	Euthyroid	Hyperthyroid
State 4	255 ± 4	311 ± 8*	351 ± 28*
State 3	507 ± 23	674 ± 44*	695 ± 34*
RCR	1.99	2.17	1.98

Values are the mean ± S.E. of four different mitochondrial preparations. Differences were considered significant at $P < 0.05$ (Student-Newman-Keuls test).
*$P < 0.05$ vs. hypothyroid state.

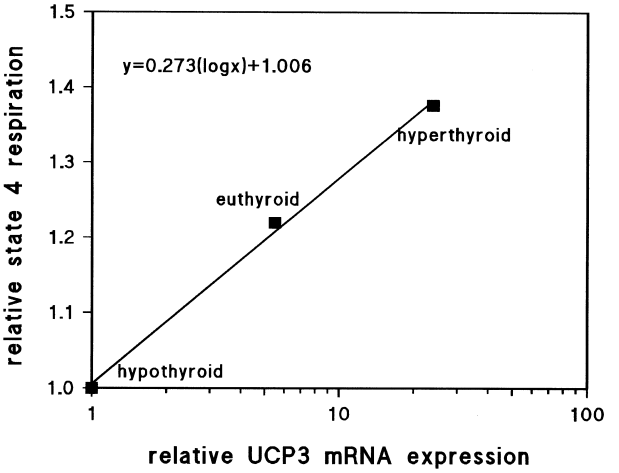

Fig. 3. Correlation analysis of UCP3 mRNA expression and State 4 respiration rate in different thyroid states. The relative values for UCP3 mRNA expression and for State 4 respiration rate were each obtained by dividing the value in a given thyroid state by the value obtained for the hypothyroid state. The correlation parameters were $r = 0.999$, $r_2 = 0.997$; $t = 18.26$, $P = 0.035$.

Fig. 2 shows the kinetic response of the proton leak to a change in ΔΨ in skeletal muscle mitochondria from hypothyroid, euthyroid and hyperthyroid rats in the absence (upper panel) or presence (lower panel) of fatty acid-free BSA. The kinetic response of the proton leak to a change in ΔΨ was influenced by the thyroid state. A stimulation of the above kinetics occurred in the hypothyroid-euthyroid-hyperthyroid transition. In other words, at any given mitochondrial membrane potential, respiration rate increased in the order hypothyroid < euthyroid < hyperthyroid.

Since at an assay temperature of 37°C there would be no change in the efficiency of the mitochondrial electron pump, and assuming that the ratio H^+/O remains constant whatever the thyroid states (as already shown in liver mitochondria [28]), the difference observed here between hypothyroid, euthyroid and hyperthyroid mitochondria could be attributed to a variation in the proton leak. As the present measurements have been performed on skeletal muscle mitochondria, we cannot exclude that an increase in conductance for ions other than H^+ and/or of a decrease in the H^+/e ratio of the respiratory chain pumps could, at least in part, explain our results. When the same analysis was performed on mitochondria prepared in the presence of fatty acid-free BSA (Fig. 2 lower panel) only a slight difference in the kinetics of the proton leak response was observed across the three thyroid states.

4. Discussion

The calorigenic effect of thyroid hormones has been known for many years and one of the oldest hypotheses put forward to explain this effect was that of Martius and Hess. These authors obtained the first evidence that the in vitro addition of thyroxine to mitochondria caused uncoupling, as measured by a decrease in the RCR [28,29]. This hypothesis was subsequently discarded because of the large doses required for the effect in vitro and the lack of similar observations in vivo.

Actually, mitochondria from animals in different thyroid states do not show any difference in RCR. This absence of a change in RCR, however, does not exclude the possibility

that changes in State 3 and/or State 4 may occur. In fact, an increase in both proton leak and phosphorylating machinery (ATP synthase, adenine nucleotide translocator, phosphate transporter) kinetics can lead to an unchanged set-point for the coupling ratio [30]. Quite recently, it has been shown that in cells from hyperthyroid animals the increased respiration is caused by alterations in both the proton leak and phosphorylating system [30,31] without significant alterations in RCR. The data presented in this report are in line with these observations. In the transition from a hypothyroid to a hyperthyroid state, we did not observe any change in RCR although the values for State 4 increased significantly in the transition from hypothyroid to euthyroid and from euthyroid to hyperthyroid state. The progressive increase of UCP3 expression in the hypothyroid-euthyroid-hyperthyroid transition might be the cause of the increase in State 4 respiration with which a good correlation is observed (see Fig. 3).

As previously shown, UCP2 expression also shows an increase in skeletal muscle in the transition hypothyroid-hyperthyroid [7]. However, the changes in UCP3 expression reported here are of the order of 5 times, whereas the change in UCP2 expression was by only about 60% [6,7]. It should be pointed out that the changes in the State 4 respiration rate and in the kinetic response of proton leak to $\Delta\Psi$ were not of the same order of magnitude as those in UCP3 mRNA expression. The greatest changes in mitochondrial respiration rate and in the kinetic response of proton leak to a change in $\Delta\Psi$ were observed in the hypothyroid-euthyroid transition. This shows that changes in mitochondrial respiration rate can be observed in a range of physiological thyroid hormone values.

Taken together, the results of this study, showing a correlation between UCP3 mRNA expression and mitochondrial energy coupling, may explain the differences observed among muscle samples from animals in different thyroid states. Similar changes in mitochondrial respiration rates have been observed by Bobyleva et al. [32] in isolated rat hepatocytes and by Hafner et al. [33] in rat liver mitochondria. Even if UCPs are absent from hepatocytes (UCP2 is present only in Kupffer cells in the liver) suggesting that in this case another mechanism has to be considered (the ATP/ADP antiporter could mediate the mitochondrial proton conductance [32]), the above changes in mitochondrial functions may represent a typical cellular response, involving most tissues, to the thyroid status of the animal.

The result of this study showing that no clear cut change in proton leak kinetics is seen across the three thyroid states when fatty acid-free BSA is present in the medium indicates that free fatty acids may play a role in the observed effects of thyroid hormones supporting the hypothesis that UCP3 operates as carrier for fatty acid anions [34]. This is in agreement with the results of Gonzales-Barroso et al. [35] who showed that fatty acid may stimulate UCP or a non-specific carrier depending on the concentration of free fatty acids. In addition it has already been shown that at least for UCP1 function free fatty acids are requested [36].

Since it has been shown that (i) the ATP/ADP antiporter, like UCP, also mediates the uncoupling effects of fatty acids [37] and (ii) there is a positive correlation between the thyroid state and the ATP/ADP antiporter level [38], an involvement of the ATP/ADP antiporter, in the observed mitochondrial uncoupling, cannot be excluded.

It has been shown that thyroid hormones may also influence the amount and the activity of the respiratory chain components. However, these effects seem not to be related to changes in energy coupling.

In conclusion, the present results seem to support the idea that uncoupling proteins (where present) may mediate the effects that thyroid hormones (in particular T3) exert at the mitochondrial level and their general effects on energy metabolism.

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