Decrease in mitochondrial energy coupling by thyroid hormones: a physiological effect rather than a pathological hyperthyroidism consequence

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Abstract The effect of the in vivo thyroid status on mitochondrial membrane potential ($\Delta\Psi_m$) in isolated rat hepatocytes was studies by means of a cytofluorimetric technique and the $\Delta\Psi_m$-specific probe JC-1. It is shown that the $\Delta\Psi_m$ level decreases in the order hypothyroid > euthyroid > hyperthyroid. Polarographic measurement of the hepatocyte respiratory rates revealed an opposite trend of values: the highest respiratory rate in hepatocytes from hyperthyroid animals, the lowest in those from hypothyroid ones. This means that mitochondrial energy coupling is highest in hypothyroid hepatocytes and lowest in hyperthyroid hepatocytes. 6-Ketocholestanol added to hepatocytes failed to counterbalance the uncoupling effect of thyroid hormones on $\Delta\Psi_m$ and respiration rate. Under the same conditions, 6-ketocholestanol appeared to be effective in recoupling of respiration uncoupled by low concentrations of the artificial protonophore FCCP. The mechanism and possible physiological functions of the thyroid hormone-induced decrease in mitochondrial energy coupling are discussed.

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Key words: Thyroid hormone; Mitochondrion; Hepatocyte; Uncoupling; Membrane potential

1. Introduction

Stimulation of metabolic rate and decrease in its efficiency by thyroid hormones has been known for many years. The calorigenic effect of these hormones was first described as early as 1895 [1]. Martius and Hess [2] postulated that uncoupling of respiration and phosphorylation is involved in this effect and showed that thyroxine added to mitochondria in vitro caused uncoupling. Unfortunately, the hormone concentrations required for the effect were many orders of magnitude higher than those present in vivo (see also [3]). As for mitochondria isolated from hyperthyroid animals, they showed, as a rule, lowered energy coupling which could be explained by a lower resistance of these mitochondria to damage during organelle isolation. In 1956 Beyer et al. concluded that in vitro aging of mitochondria "reveals that the difference between the thyxine-treated and normal animals is the degree of lability of the oxidation-phosphorylation system" [4].

The discussion on whether thyroid hormone-induced uncoupling is (i) a physiological phenomenon, (ii) a pathology related to hyperproduction of the hormones, or (iii) an in vitro artifact has continued for almost half a century. Only quite recently, some observations were published that cannot be explained by an artifact. Lanni et al. [5] and Masaki et al. [6] have shown that thyroid hormones in vivo strongly increase the level of mRNA of uncoupling protein 2 in some tissues. Uncoupling protein 2 (UCP2) [7–9] has a similar (but not identical) sequence to uncoupling protein 1 (thermogenin) found in brown fat where it is responsible for thermoregulatory uncoupling (for review, see [10]). The UCP-2 mRNA level in rat heart, skeletal muscle and brown fat increases during 48 h exposure to 6°C [11]. This points to the involvement of UCP2 in thermoregulatory uncoupling occurring not only in brown fat but also in muscle tissues [12–14]. Independently, Gong et al. [15] and Larkin et al. [16] reported that the mRNA level of another member of the UCP family, UCP3 [8,17], is strongly increased by thyroid hormones in brown fat and skeletal muscles. Cold exposure was also effective in brown fat but not in muscle [17]. The very fact that thyroid hormones induce UCP2 and UCP3 strongly suggests that uncoupling is inherent in their in vivo effects.

UCP2 mRNA is found in many tissues except in hepatocytes [7–9,18]. As for UCP3 mRNA, it is specific for brown fat and muscle. Nonetheless, Gregory and Berry have observed a decrease in $\Delta\Psi_m$ in hepatocytes from hyperthyroid rats [19]. This observation was confirmed by Harper and Brand [20]. According to Brand and coworkers [21], proton conductance of the mitochondrial membrane in hepatocytes from hypothyroid animals is decreased compared with euthyroid controls. The same group failed to observe any $\Delta\Psi_m$ increase in hypothyroid hepatocytes although hyperthyroid ones showed an obvious $\Delta\Psi_m$ decrease [20].

In the present paper, a cytofluorimetric method developed to follow $\Delta\Psi_m$ [22–27] was used to study isolated living hepatocytes. The method employs the lipophilic penetrating cation JC-1 as a $\Delta\Psi_m$ probe. The cation accumulates electrophoretically in the $\Delta\Psi_m$-bearing mitochondria. When the intramitochondrial concentration of JC-1 increases, the cation forms J-aggregates. This results in a large shift of fluorescence emission (from 527 nm to 590 nm) [25–27]. The experiments revealed that euthyroid hepatocytes show a lower mitochondrial membrane potential and a higher respiration rate than

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Abbreviations: $\Delta\Psi_m$, mitochondrial transmembrane electric potential difference; FCCP, *p*-trifluoro-methoxycarbonylcyanide phenylhydrazone; JC-1, 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazolylcarbocyan iodine; kCh, 6-ketocholestanol (5α-cholestan-3β-ol-6-one); T_3, 3,5,3′-triiodothyronine; UCP, uncoupling protein

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hypothyroid ones. Hyperthyroidism was found to entail further membrane potential decrease and respiration increase.

2. Materials and methods

Male rats, Sprague-Dawley strain, pathogen germ free, weighing 180–200 g were used. Animals were maintained on a 12-h light-dark cycle at 21°C and were provided with standard rat chow diet and water ad libitum. Rats were killed by decapitation for preparation of mitochondria, or operated under Ketalar (Parke-Davis, Italy) anesthesia (0.2 ml/100 g wet weight) for hepatocyte isolation. Hyperthyroidism was induced by subcutaneous injections for 2–3 days of 0.5 mg/100 g 3,5,3′-triiodothyronine (T_3) dissolved in a minimal amount of 0.01 N NaOH and diluted with saline to 1 mg/ml. Control rats were injected with 0.9% NaCl solution. On the day of the experiment, the rats were given the last dose of T_3 at 9.00 h and were killed 1 h later. Hypothyroidism was induced with 0.02% methimazole added to the drinking water for 8–10 days [28]. In order to check whether the treated rats changed their thyroid state, the rate of mitochondrial respiration was measured using α-glycerol phosphate as respiratory substrate since the activity of mitochondrial α-glycerol phosphate dehydrogenase (EC 1.1.99.5) is known to strictly correlate with the thyroid state of animals [29]. Mitochondria were prepared as described by Bobyleva et al. [30] from 10% total liver homogenate. Mitochondrial respiration was measured at 30°C in a medium of the following composition: 100 mM KCl, 10 mM $MgCl_2$, 10 mM Tris-HCl, 10 mM Na,K phosphate buffer, pH 7.4, 10 mM DL-α-glycerol phosphate and 3 μM rotenone. It was shown that mitochondria from methimazole-treated, non-treated, and T_3-treated rats oxidized DL-α-glycerol phosphate in state 3 at rates of 1.8, 6.6 and 17.7 ng atom O/(mg protein × min), respectively.

Hepatocytes were prepared according to Krebs [31] using in situ liver perfusion with collagenase. 10 mM glucose was added to the perfusion medium. Isolated and washed hepatocytes were suspended ($3–5\times10^7$ cells/ml) in Krebs-Henseleit medium supplemented with 33 mM HEPES and 10 mM glucose, pH 7.4 (suspension medium). As distinguished from the Krebs protocol, 2.5% dialyzed albumin was added to cells only during their washing, and the final suspension was kept in the medium without albumin. Hepatocytes were kept in small portions (1–2 ml) in tubes on ice. At the end of the preparation, cell viability was checked with the trypan blue (0.3% w/v) exclusion test. Only cells with viability higher than 90% were used for experiments.

Oxygen consumption was recorded with a Clark-type oxygen electrode and ESI polarograph (Italy). Hepatocytes were incubated in the suspension medium supplemented with 1.3 μM oligomycin, 10 mM lactate and 1 mM pyruvate. Hepatocyte concentration was 1×10^6 cells/ml. The total volume of the flask was 3 ml. The measurements were done at 37°C.

The electrical potential difference across inner mitochondrial membrane in isolated hepatocytes ($\Delta\Psi_m$) was monitored using cytofluorimetry and the $\Delta\Psi_m$-specific fluorescent probe JC-1. 10^7 fresh hepatocytes were suspended in the suspension medium at a concentration of 10^6 cells/ml and incubated for 10 min at room temperature in the dark with 10 μM JC-1. Cells were washed twice with the suspension medium and finally resuspended in a total volume of 10 ml. At this stage, the viability of cells decreased slightly, but was always higher than 85%. Hepatocytes obtained were stored on ice. A portion of cells (5 ml) was treated with 1.3 μM oligomycin, 10 mM lactate and 1 mM pyruvate in a shaking bath at 37°C. After 3 min treatment, 1 ml hepatocyte suspension was collected and analyzed. The analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA), equipped with a single 488 nm argon laser. The values of the photomultiplier (PMT) detecting the signal on fluorescence channel 1 (FL1) and channel 2 (FL2) were set at 250 and 240 V, respectively; the FL1-FL2 compensation was 1.0%, the FL2-FL1 compensation was 44.5%. A minimum of 10 000 cells per sample was acquired in list mode and analyzed with Lysys II software (Becton Dickinson), as described by Cossarizza et al. [32] and Polla et al. [33]. Fluorescent histograms are shown in a linear scale to allow the correct detection of the median fluorescence channel. Cells with a high $\Delta\Psi_m$ are those showing high red fluorescence due to the formation of J-aggregates.

Protein concentration was measured by the biuret method [34] with bovine serum albumin as the reference.

Enzymes and coenzymes were purchased from Boehringer-Mannheim (Germany); rotenone, oligomycin, methimazole, 6-ketocholestanol, and FCCP were from Sigma. JC-1 was from Molecular Probes (Eugene, OR, USA). FCCP, rotenone, oligomycin, 6-ketocholestanol were dissolved in 95% ethanol. JC-1 was dissolved in DMPA (Sigma).

3. Results and discussion

Table 1 shows the respiration rates of isolated rat liver mitochondria and hepatocytes treated with oligomycin (so-called state 4 respiration, left column). These rates increased with the increase of thyroid status (hypothyroid < euthyroid < hyperthyroid). The recoupler 6-ketocholestanol (kCh) failed to abolish the thyroid hormone-induced increase in the respiration rate. Addition of a low concentration of the protonophorous uncoupler FCCP was stimulatory in all cases. In line with previous observations [35,36], this stimulation was abolished by the recoupler kCh; in mitochondria, a higher FCCP concentration completely released the kCh inhibition.

Fig. 1 demonstrates the results of the experiments where $\Delta\Psi_m$ was measured by cytofluorimetry using the JC-1 probe. The left shift of the peak indicates a $\Delta\Psi_m$ decrease [24–26]. It is shown that hypothyroidism enhanced and hyperthyroidism lowered the $\Delta\Psi_m$ level. Addition of kCh failed to prevent the thyroid hormone-induced $\Delta\Psi_m$ lowering. As for high FCCP, kCh was more efficient in the $\Delta\Psi_m$ decrease in euthyroid hepatocytes compared with hypothyroid ones.

Fig. 2 shows that kCh was competent in recoupling of mitochondria in the euthyroid hepatocytes treated with low FCCP. This means that the lack of a kCh effect on the thyroid hormone-induced uncoupling in the euthyroid hepatocytes cannot be accounted for by inability of kCh to reach mitochondria in intact cells.

In both hypo- and hyperthyroid hepatocytes, uncoupling by

Table 1
Effect of thyroid status, 6-ketocholestanol, and FCCP on isolated mitochondria and hepatocytes in the presence of oligomycin

Subject	Thyroid state	No addition	kCh	Low FCCP	Low FCCP+kCh	High FCCP+kCh
Mitochondria	Hypothyroid	10.9 ± 3.6		29.2 ± 3.9	12.6 ± 3.9	42.1 ± 15.8
	Euthyroid	22.5 ± 6.0		47.8 ± 12.8	24.6 ± 6.6	54.6 ± 11.2
	Hyperthyroid	31.3 ± 2.1		59.9 ± 7.0	31.0 ± 3.4	64.1 ± 11.9
Hepatocytes	Hypothyroid	18.4 ± 2.0	15.1 ± 3.0	24.9 ± 1.1	17.9 ± 2.3	22.9 ± 3.8
	Euthyroid	25.8 ± 2.3	25.0 ± 2.6	35.7 ± 3.3	26.8 ± 2.5	30.8 ± 3.5
	Hyperthyroid	33.3 ± 1.9	29.6 ± 4.6	49.3 ± 5.9	31.8 ± 3.2	29.8 ± 2.8

Figures represent means ± S.D.
Mitochondria were incubated with 5 mM succinate, 3×10^{-6} M rotenone and 1.3×10^{-6} M oligomycin. For other conditions, see Section 2. Additions: low FCCP, 40 nM for mitochondria and 60 nM for cells; 0.08 mM kCh; high FCCP, 180 nM for mitochondria, 240 nM for hypo- and euthyroid cells, 120 nM for hyperthyroid cells. Respiration rates are expressed as ng atom O/(mg protein × min) or ng atom O/(10^6 cells × min) for mitochondria or cells, respectively.

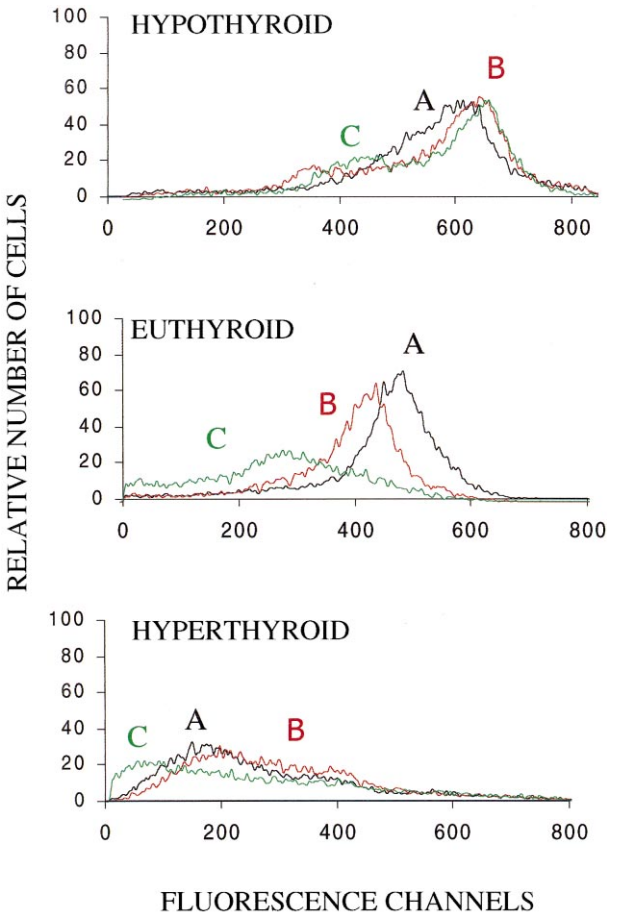


Fig. 1. Thyroid hormones induce a decrease of $\Delta\Psi_m$ in hepatocytes in a 6-ketocholestanol-resistant fashion. Representative fluorescence distribution from histogram of JC-1-stained hepatocytes treated with oligomycin. One experiment out of five for each thyroid state is shown. A, no additions; B, 80 μM kCh; C, 80 μM kCh and 240 nM FCCP.

low FCCP and, hence, recoupling by kCh were less clearcut than in the euthyroid ones. This is probably due to the facts that (i) thyroid hormones were shown to facilitate uncoupling by FCCP [37] so under hypothyroid conditions the effect of low FCCP is small, and (ii) hyperthyroidism per se results in such a strong uncoupling that the JC-1 probe responds only slightly to low FCCP.

The above data clearly show that some decrease in the mitochondrial energy coupling is inherent not only in the hyper- but also in the euthyroid state. In fact, in euthyroid hepatocytes the respiration rate is *higher* but $\Delta\Psi_m$ is *lower* than in hypothyroid hepatocytes. This means that a certain lowering of the energy coupling by thyroid hormones should be considered a physiological effect rather than a pathology caused by hormone overproduction. In this context, it should be mentioned that Harper and Brand [20] failed to observe any $\Delta\Psi_m$ decrease in euthyroid hepatocytes compared with hypothyroid ones. This discrepancy is probably a result of the different methods employed to measure $\Delta\Psi_m$ (JC-1 and TPP^+ in our and Harper and Brand's experiments, respectively). An investigation carried out in our group [22] showed that JC-1 monitors the $\Delta\Psi_m$ level more adequately in intact cells, although in isolated mitochondria the TPP^+ probe is quite good [38].

In cells other than hepatocytes, the thyroid hormone-induced decrease in the energy coupling may be due to induction of proteins belonging to the UCP family [5,6,15,16]. Apparently UCPs operate as transmembrane carriers of fatty acid anions, facilitating in this way the H^+-conducting fatty acid cycle: protonated fatty acids diffuse to the mitochondrial matrix via lipid parts of the membrane whereas their anions are electrophoretically expelled in the opposite direction via UCP [10,39]. However, UCPs are absent from hepatocytes [7,8,18] so other mechanisms to decrease the energy coupling should be considered.

One of them may be that in hepatocytes, the ATP/ADP antiporter substitutes for UCP as the fatty acid anion translocator. In this group, it was shown that the antiporter mediates the uncoupling effect of fatty acids in mitochondria [14,39,40] and proteoliposomes [41] (see also [42]). On the other hand, it is known that thyroid hormones increase the level of the ATP/ADP antiporter in hepatocytes [43–45]. A correlation of the uncoupling efficiency of fatty acids and the amount of the ATP/ADP antiporter was revealed by Schunfeld when mitochondria from various tissues differing in antiporter content were compared [46]. Thus the thyroid hormone-induced uncoupling in liver can be explained by a

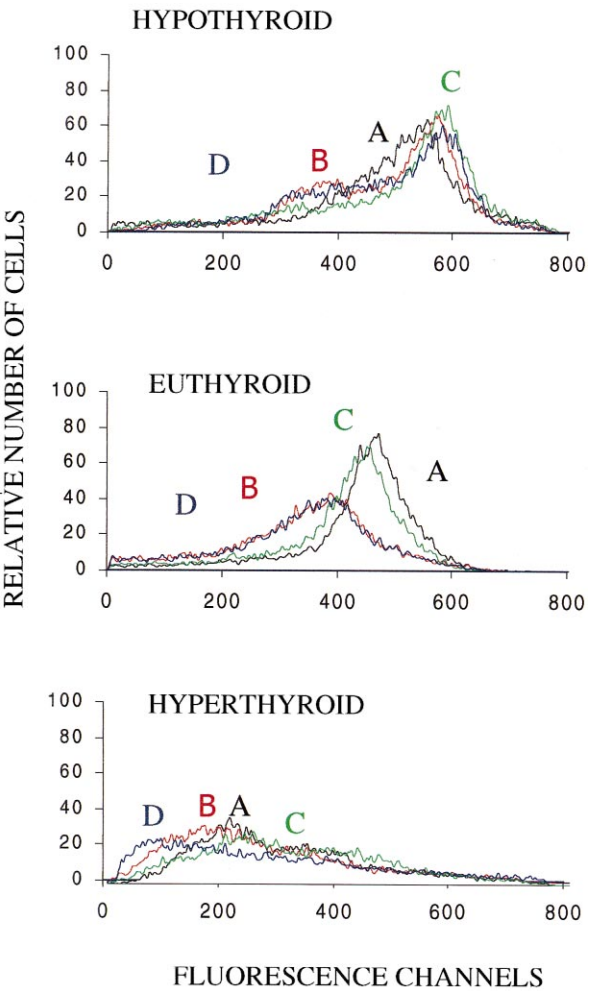


Fig. 2. 6-Ketocholestanol recouples hepatocytes uncoupled with low concentrations of FCCP. For conditions, see Fig. 1. A, no additions; B, 60 nM FCCP; C, 60 nM FCCP and 80 μM kCh; D, 240 nM FCCP and 80 μM kCh.

hormone-induced increase in the ATP/ADP antiporter concentration, entailing elevation of mitochondrial proton conductance mediated by free fatty acids. In fact, there are indications of an in vivo increase in H^+ conductance of the mitochondrial membrane by thyroid hormones [21,47,48]. Recently, Schunfeld et al. [49] reported that expression of the ATP/ADP antiporter in liver mitochondria parallels the increase in their sensitivity to uncoupling effect of fatty acids.

It should be emphasized that the thyroid hormone-linked uncoupling in hepatocytes proved to be kCh-resistant like uncoupling caused by fatty acids and in contrast to that caused by low concentrations of FCCP-like uncouplers [36]. Thus one can conclude that this uncoupling is mediated mainly by fatty acids or by another endogenous uncoupler which is also kCh-resistant. As for a thyroid hormone-related uncoupling mechanism which can be abolished by kCh [37], if it exists, it plays only a minor role in hepatocytes.

It is not excluded that a decrease in the energy coupling by thyroid hormones in the liver tissue is related, to some degree, to the so called decoupling, i.e. a decrease in H^+ pump efficiency of the respiratory chain, rather than to an increase in dissipation of the proton motive force due to elevated H^+ conductance. The molecular mechanism of decoupling is obscure. One can find in the literature some speculations that it is decoupling that is responsible for some effects of thyroid hormones on mitochondria [50] (see, however, [51]).

The physiological role of a decrease in energy coupling caused by thyroid hormones in euthyroid animals may be related to (i) thermoregulation [10,14], (ii) metabolic functions of respiration (i.e. respiration-linked interconversions of some substances) [10,14], and (iii) antioxidant defence [18,52]. In all these cases, tight coupling may be undesirable (for discussion, see [14]).

Another effect of thyroid hormones consists in an increase in amount and activity of the respiratory chain enzymes (reviewed by Soboll [48], see also [53]). However, this phenomenon is not sufficient to explain the $\Delta\Psi_m$ decrease caused by thyroid hormones.

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