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3,5-Diiodo-L-Thyronine increases F_oF_1-ATP synthase activity and cardiolipin level in liver mitochondria of hypothyroid rats

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Abstract Short-term effects of 3,5-L-diiodothyronine (T_2) administration to hypothyroid rats on F_oF_1-ATP synthase activity were investigated in liver mitochondria. One hour after T_2 injection, state 4 and state 3 respiration rates were noticeably stimulated in mitochondria subsequently isolated. F_oF_1-ATP synthase activity, which was reduced in mitochondria from hypothyroid rats as compared to mitochondria from euthyroid rats, was significantly increased by T_2 administration in both the ATP-synthesis and hydrolysis direction. No change in β-subunit mRNA accumulation and protein amount of the α-β subunit of F_oF_1-ATP synthase was found, ruling out a T_2 genomic effect. In T_2-treated rats, changes in the composition of mitochondrial phospholipids were observed, cardiolipin (CL) showing the greatest alteration. In mitochondria isolated from hypothyroid rats the decrease in the amount of CL was accompanied by an increase in the level of peroxidised CL. T_2 administration to hypothyroid rats enhanced the level of CL and decreased the amount of peroxidised CL in subsequently isolated mitochondria, tending to restore the CL value to the euthyroid level. Minor T_2-induced changes in mitochondrial fatty acid composition were detected. Overall, the enhanced F_oF_1-ATP synthase activity observed following injection of T_2 to hypothyroid rats may be ascribed, at least in part, to an increased level of mitochondrial CL associated with decreased peroxidation of CL.

Keywords Cardiolipin · Cardiolipin hydroperoxides · 3,5-Diiodo-L-Thyronine · F_oF_1-ATP synthase · Hypothyroidism · Liver · Mitochondria · Rat

Abbreviations

ANT	Adenine nucleotide translocator
CL	Cardiolipin
ESMP	EDTA sub-mitochondrial particles
FAME	Fatty acid methyl esters
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
13-HPODE	13-hydroperoxy-9,11-octadecanedienoyl acid
IOP	Iopanoic acid
PTU	6-n-propyl-2-thiouracil
RCI	Respiratory control index
T_2	3,5-diiodo-L-thyronine
T_3	3,3′,5-triiodo-L-thyronine

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Introduction

The thyroid hormone 3,3′,5-triiodo-L-thyronine (T_3) is known to play an important role in the regulation of hepatic metabolism, and mitochondria are considered to be likely subcellular targets of T_3 action (Enrìquez et al. 1999; Goglia et al. 1999; Mrácek et al. 2005). Extensive biochemical and morphological changes occur in mitochondria as the thyroid state is modified with age, by exposure to cold as well as by thyroidectomy and administration of T_3 (Goglia et al. 1999). This hormone regulates mitochondrial respiration having increased rates in hyperthyroidism and decreased rates in hypothyroidism (Paradies and Ruggiero 1988; Paradies et al. 1991; Nogueira et al. 2002). Changes in lipid content, lipid composition and lipid-protein interaction in mitochondria from hypothyroid and T_3-treated animals have been documented (Paradies et al. 1991; Nogueira et al. 2002; Giudetti et al. 2006).

It is well known that the activities of several mitochondrial proteins are greatly influenced by their lipid environment. Among membrane lipids, a special role is played by cardiolipin (CL), a phospholipid predominantly found in the mitochondrial inner membrane (Chicco and Sparagna 2007). In vitro studies showed that CL interacts with a large number of mitochondrial proteins and it is required for the optimal activity of several enzymes in the mitochondrial respiratory chain, such as NADH dehydrogenase (Complex I), ubiquinol: cytochrome c oxidoreductase (Complex III), cytochrome c oxidase (Complex IV) and ATP synthase (Complex V) (for review see Chicco and Sparagna 2007). CL is also required for optimal activity of several mitochondrial carriers, including the phosphate carrier and the adenine nucleotide translocator (ANT) (Paradies et al. 1991; Nogueira et al. 2002), both involved in ATP formation. Alterations of the structure and content of CL are responsible for mitochondrial dysfunction in a variety of pathological settings (Paradies et al. 1991; Chicco and Sparagna 2007; Petrosillo et al. 2007).

A growing number of studies have indicated that, in addition to T_3, diiodothyronines such as 3,5-diiodo-L-thyronine (T_2), produced in vivo by the action of 5′-iodothyronine deiodynase on T_3, has relevant physiological properties (Moreno et al. 1997; Goglia 2005). T_2, previously considered only as a T_3 catabolite, is able to mimic the effects of T_3 on energy metabolism (Goglia 2005). T_2 stimulates mitochondrial respiratory capacity in humans and in some rat tissues (Horst et al. 1989; Ball et al. 1997). Administration of T_2 to hypothyroid rats rapidly enhances the rate of mitochondrial fatty acid oxidation and thermogenesis as well as respiration rates in mitochondria from rat-skeletal muscle (Lombardi et al. 2009).

While T_3 is generally considered to play genomic actions, T_2 effects are more rapid than those of T_3 and occur by direct interaction of the hormone with mitochondria (Goglia 2005). T_2 was found to stimulate directly cytochrome–c–oxidase (COX) activity in rat-liver homogenates and specific binding of T_2 to subunit Va of COX from bovine heart has been reported (Arnold et al. 1998).

The F_oF_1-ATP synthase, mitochondrial complex V responsible for ATP synthesis, is composed of three parts: the catalytic sector F_1, consisting of five subunits (3α, 3β, γ, δ, ε), the H^+ translocating sector F_o and a stalk connecting F_1 to F_o. Although it has been demonstrated that mitochondrial ATP-synthase is one of the sites of thyroid hormone action (Hafner et al. 1990; Izquierdo and Cuezva 1993; Guerrieri et al. 1998), studies of T_2 effects on mitochondrial complex V are lacking (Mangiullo et al. 2010).

The present study provides the first evidence that T_2 administration to hypothyroid rats acutely (within 1 h) stimulates in liver mitochondria F_oF_1-ATP synthase activity in both the ATP-synthesis and hydrolysis direction. T_2 effects could be ascribed, at least in part, to changes in mitochondrial phospholipid composition. In particular, the content of CL, which was reduced in hypothyroid mitochondria due to its peroxidation, was noticeably increased in liver mitochondria of T_2-treated hypothyroid rats.

Materials and methods

Chemicals

6-n-propyl-2-thiouracil (PTU), iopanoic acid (IOP), oligomycin, T_2 (more than 99% pure) and all other reagents were from Sigma-Aldrich.

Animal treatment

Male Wistar rats (200–250 g) were housed one per cage in a temperature- (22±1 °C) and light- (light on 08:00 h–20:00 h) controlled room. A commercial mash and water were available for ad libitum consumption. T_2 and IOP were dissolved in 0.05 M NaOH and diluted with 0.09% NaCl. The rats were divided into three groups. Group 1, representing euthyroid rats, was treated with only vehicle for 3 weeks; group 2, rats were made hypothyroid by continuous administration of PTU in the drinking water (0.1% *w*/*v*) for 3 weeks together with a weekly i.p. injection of IOP (6 mg/100 g b.w.); group 3, rats treated as in group 2 but received a single i.p. injection of T_2 (150 μg/100 g b.w.) (hypothyroid+T_2). This pharmacological dose of T_2 was used because it produces a clear-cut, rapid effect on the energy-transduction apparatus of rat-liver mitochondria (Lombardi et al. 1998). Rats were killed 1 h after the injection of T_2. The experimental

design was in accordance with local and national guidelines regarding animal experiments.

Oxygraphic measurements

Rat livers were rapidly processed for mitochondria isolation using standard procedures. Freshly isolated mitochondria were assayed for oxygen consumption in a thermostatically oxygraph apparatus equipped with a Clark's electrode. Mitochondrial respiration (0.3 mg of mitochondrial protein/mL) was assayed in a medium consisting of 220 mM sucrose, 20 mM KCl, 2.5 mM KH_2PO_4, 1 mM EDTA, 20 mM Hepes, 5 mM $MgCl_2$, 0.1% bovine serum albumin (BSA) (fraction V, fatty acid-free from Sigma), pH 7.4.

Oxidation of succinate (5 mM), in the presence of rotenone (2 μg/mL), was used to determine the ability of T_2 to affect the mitochondrial respiration rate. State 3 respiration was initiated by the addition of 0.3 mM MgADP and followed until total consumption of ADP, then state 4 respiration was measured. Then, 4 mM FCCP was added to measure uncoupled respiration. State 3, state 4 and uncoupled respiration, respiratory control index (RCI) and ADP/O ratio were calculated as in Mangiullo et al. (2010).

Preparation of sub-mitochondrial particles

Inside-out sub-mitochondrial particles were prepared in the presence of EDTA (ESMP), by exposure of mitochondria to ultrasonic energy in a Branson Sonifier (Model W185, output 70 W for 60 s at pH 8.5). Protein concentrations were determined by the Bradford BioRad protein assay with BSA as a standard.

ATP Synthase and hydrolase activity

The rate of mitochondrial ATP synthesis was determined in freshly isolated mitochondria while the oligomycin-sensitive ATP hydrolase activity was measured in ESMP by an ATP-regenerating system (Zanotti et al. 2004).

Isolation of RNA and Real-Time qPCR analysis

Total RNA was extracted from rat liver using the SV Total RNA Isolation System kit (Promega) following the manufacturer's protocol. Only RNA without DNA contamination was used for subsequent preparation of cDNA synthesis. The reverse transcriptase (RT) reaction (20 μl) was carried out using 5 μg of total RNA, 100 ng of random hexamers and 200 units of SuperScript™ III RNase H-Reverse transcriptase (Invitrogen). Quantitative gene expression analysis was performed (SmartCycler System, Cepheid) using SYBR Green technology (FluoCycle, Euroclone) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization. The primers utilized in real-time PCR were the following: β-F1 forward: 5′-AGAGATGAGTGTTGAA CAGG-3′ and β-F1 reverse: 5′-TACAGAATAACCAC CATGGG-3′ (GenBank™ accession number NM_134364); GAPDH forward 5′-GCATGGCCTTCCGTGTTCCTACC-3′, GAPDH reverse 5′-GCCGCCTGCTTCACCACCTTCT-3′ (Gen-Bank™ accession number NM_017008.3).

Electrophoresis and immunoblotting procedures

Mitochondrial proteins were separated on 15% SDS-PAGE gels (Giudetti et al. 2006). The separated proteins were transferred to nitrocellulose membranes and incubated with primary antibodies against α/β subunit of F_oF_1-ATP synthase or porin diluted in 20 mM Tris/HCl, pH 7.6, 0.14 M NaCl, 0.5% Tween 20 (TBS-T). After washing with TBS-T, the membranes were incubated with HRP-conjugated secondary antibodies (Pierce) which were visualized using the chemiluminescence kit (GE Healthcare). The blots were then exposed to Hyperfilm and signals were quantified by densitometry. Porin, the mitochondrial outer membrane channel, was used as control because the amount of this protein is unaffected by the thyroid state of the animal (Giudetti et al. 2006).

Cholesterol, phospholipid and fatty acid analysis

Total lipids were extracted from mitochondria (10 mg protein) (Bligh and Dyer 1959) and the extracts, dried under N_2 flow, were resuspended in a proper volume of $CHCl_3$. The amount of cholesterol was assayed by High Performance Liquid Chromatography (HPLC) (Caputi Jambrenghi et al. 2007). Phospholipids were separated by HPLC (Giudetti et al. 2006), using a Beckman System Gold chromatograph equipped with an ultrasil-Si column (4.6×250 mm). The chromatographic system was programmed for gradient elution by using two mobile phases: solvent A, hexane/2-propanol 6:8 (*v*/*v*) and solvent B, hexane/2-propanol/water 6:8:1.4 (*v*/*v*/*v*). The percentage of solvent B in solvent A was increased in 15 min from 0% to 100%. Flow rate was 2 ml/min and detection was at 206 nm. Single phospholipids were identified by using known standards and quantitatively assayed by determining inorganic phosphorus (Nakamura 1952). For fatty acid analysis, liver mitochondria (10 mg protein) were saponified with ethanolic KOH for 90 min at 85–90 °C. Fatty acids were extracted with petroleum ether and their corresponding fatty acid methyl esters (FAME) were prepared by transesterification with methanolic boron trifluoride (17% BF3) at 65 °C for 30 min. FAME were then analyzed by gas–liquid chromatography (Giudetti et al. 2006). Peak identification was performed by using known standards and relative quantitation was automatically carried out by peak integration.

Preparation and analysis of peroxidised cardiolipin

Hydroperoxy-lynoleoyl cardiolipin was synthesized by an enzymatic reaction catalyzed by lipoxygenase type V from soybean according to Eskola and Laakso (1983). The reaction products were extracted from the mixture by the method of Bligh and Dyer (1959) and the products were purified by reverse-phase preparative column chromatography using $CH_3OH/(C_2H_5)_2O$ 95:5 (*v*/*v*). Quantitative phosphorus analysis was performed according to Nakamura (1952). The amount of hydroperoxy-lynoleoyl cardiolipin was measured using ferrous ion oxidation in the presence of xylenol orange according to the method of Gay and Gebiki (2003). The extent of lipid peroxidation was determined by the generation of conjugated dienes as described by Buege and Aust (1978). The absorption spectrum of the extracted cardiolipin redissolved in methanol was recorded and the amount of conjugated dienes was calculated using ε_{234}= 25.2 mM^{-1} cm^{-1} (Buege and Aust 1978). The purified oxidized cardiolipin was analyzed by mass spectrometry showing that cardiolipin linoleoyl moieties were converted into 13-hydroperoxy-9,11-octadecanedienoyl acid (13-HPODE) and that the sample contained all four possible forms of cardiolipin hydroperoxides (mono-, di-, tri- and tetra-), with the mono-13-HPODE cardiolipin being the most prominent. The corresponding hydroxy-lynoleoyl cardiolipins were obtained by reduction with $NaBH_4$ according to Wratten et al. (1992), with the mono-13-hydroxy-9,11-octadecanedienoyl-cardiolipin being most distinct, as confirmed by mass spectrometry (mass spectra not shown).

Statistical analysis

Results shown represent means ± SD of the number of experiments indicated in every case. Statistical analysis was performed by Student's *t*-test. Differences were considered statistically significant at $P<0.05$.

Results

Preliminary experiments showed that incubation of isolated mitochondria from hypothyroid rats with T_2 did not affect mitochondrial coupled, uncoupled or phosphorylating respiration rates (data not shown), indicating that the rapid stimulation of respiration by T_2 requires cellular environment. Experiments were thus carried out where T_2 was i.p. injected to hypothyroid rats which were sacrificed 1 h later. The experimental design (PTU+IOP administration) we used (Moreno et al. 1997; Goglia 2005), allows the observed effects to be directly attributed to the iodothyronine administered (T_2).

Effect of T_2 on mitochondrial respiration rates and the ADP/O ratio

Consistent with previous data (Guerrieri et al. 1998; Nogueira et al. 2002; Mangiullo et al. 2010) the results in Table 1 show that state 4 and state 3 succinate-dependent oxygen consumption were significantly reduced in mitochondria isolated from hypothyroid compared to euthyroid animals. T_2 administration to hypothyroid rats noticeably increased respiratory rates in state 4, state 3 and FCCP-stimulated uncoupled respiration. Injection of T_2 in hypothyroid rats raised O_2 consumption in state 4 by 57% and in state 3 by 44%.

Neither the respiratory control index nor the ADP/O ratio were significantly altered in hypothyroid and in T_2–treated hypothyroid rats as compared to the euthyroid rats, thus indicating that the quality of mitochondrial preparations from control and treated rats was similar (Hinkle 2005).

Effect of T_2 on the ATP synthase activity

Figure 1a shows that in succinate-supplemented liver mitochondria, the rate of ATP synthesis, which was reduced in mitochondria from hypothyroid rats, was significantly increased in mitochondria of T_2-treated hypothyroid rats. In the latter, a stimulation of the rate of ATP synthesis was observed (230±20 nmoles ATP synthesised/min/mg protein of mitochondria) as compared to mitochondria from untreated hypothyroid rats (172±17 nmoles ATP synthesised/min/mg protein of mitochondria, $n=6$, $p<0.05$). Experiments with ESMP allow to study the effects of T_2 on ATP synthase, differentiating them from effects on ANT or the adenine nucleotide content (Hafner et al. 1990). To test whether the increased ATP synthesis observed upon T_2 treatment could be ascribed to a direct effect of the hormone on F_oF_1-ATP synthase, we measured the oligomycin-sensitive ATP hydrolase activity in ESMP, (Guerrieri et al. 1998). Figure 1b shows a decrease (−35%) of the oligomycin-sensitive ATP hydrolase activity in ESMP from hypothyroid compared to ESMP from euthyroid rats. In ESMP from T_2-treated hypothyroid rats the ATP hydrolase activity was significantly stimulated as compared to ESMP from untreated hypothyroid rats (1.99± 0.33 vs. 1.47±0.37 μmoles ATP hydrolysed/min/mg protein of mitochondria, $n=6$, $p<0.05$) (Fig. 1b).

T_2 effect on β-F_1 mRNA abundance and F_oF_1-ATP synthase expression

β-F_1 subunit is encoded by a single copy gene which is ubiquitously expressed in mammalian cells (Guerrieri et al. 1998). In order to see whether the observed changes in the F_oF_1-ATP synthase activity were associated with changes in

Table 1 Respiratory activities of hepatic mitochondria from euthyroid, hypothyroid and T_2-treated hypothyroid rats

Rats	State 4	State 3	FCCP-stimulated	RCI	ADP/O
Euthyroid	17.2±5[a]	115.5±12[a]	184.5±22	7.0±1	1.5±0.2
Hypothyroid	9.2±2[b]	58.0±9[b]	101.5±18	6.4±2	1.8±0.2
Hypothyroid+T_2	14.4±5[a]	83.5±9[a]	132.5±18	6.7±2	1.6±0.1

Succinate-dependent oxygen consumption was measured by a Clark oxygen electrode. Respiratory rates are expressed as natoms oxygen/min/mg of mitochondrial protein. Data represent the means ± SD of six different experiments each one performed in duplicate. RCI: respiratory control index, represents the ratio between state 3 and state 4 respiration rate

the transcription of the gene, the level of β-F_1 mRNA in liver from euthyroid and T_2-treated rats was measured. In agreement with previous data (Guerrieri et al. 1998; Mangiullo et al. 2010) we found that the abundance of the β-subunit mRNA strongly decreased in liver of hypothyroid rats. However, when compared to the latter, no significant change in mRNA level was observed in the case of T_2-treated hypothyroid rats (Fig. 2a). Analogously, immunoblot analysis (Fig. 2b) showed that the reduced protein amount of α-β subunit of ATP synthase observed in hypothyroid mitochondria was not affected by T_2 administration to hypothyroid rats. These findings indicate that in our experimental conditions T_2 has no effect on transcriptional and/or translational mechanisms of the β-subunit of the ATP synthase.

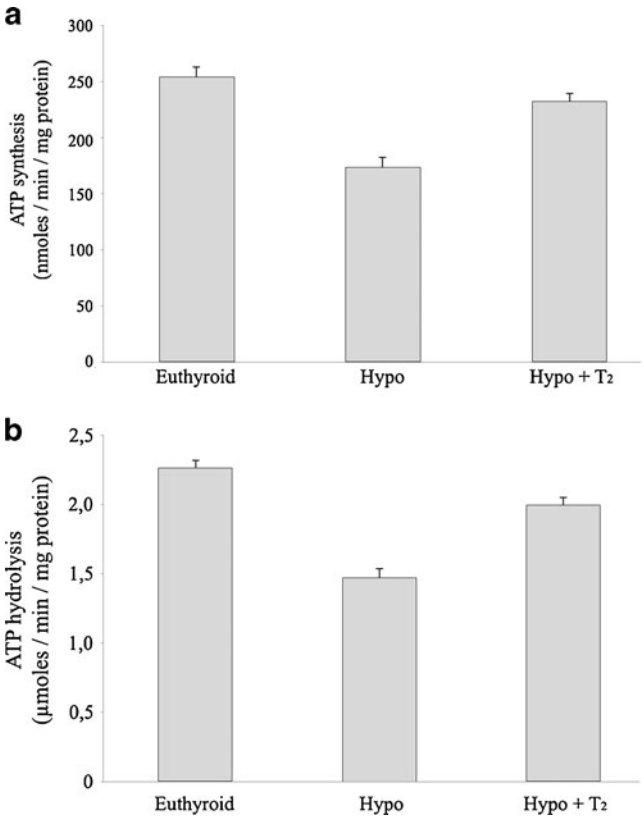

Fig. 1 F_oF_1-ATP synthase and hydrolase activities in rat-liver mitochondria and in sub-mitochondrial particles of hypothyroid and T_2-treated hypothyroid rats. **a** ATP synthesis was measured in freshly isolated liver mitochondria. Data represent the means ± SD of six different experiments performed with duplicate samples. **b** Oligomycin-sensitive ATP hydrolysis was measured in sub-mitochondrial particles prepared in the presence of EDTA (ESMP) and with ATP 1 mM using an ATP-regenerating system. Hypo = hypothyroid rats; Hypo+T_2=T_2-treated hypothyroid rats. Data represent the means ± SD of six different experiments performed with duplicate samples

Lipid composition in the mitochondrial membranes

Thyroid hormones have been shown to affect the mitochondrial membrane lipid composition (Hulbert 2000) and lipids influence many aspects of mitochondrial functions, including enzyme activity, coupling of respiration, and susceptibility to oxidative damage (Paradies et al. 2010). Therefore, the major phospholipid fractions of mitochondrial membranes in different thyroid states were quantified by HPLC analysis (Table 2). The main changes observed were in the mitochondrial level of CL. In agreement with Paradies et al. (1991) we found that the amount of CL was reduced in mitochondria from hypothyroid rats as compared to mitochondria from euthyroid ones. When compared to hypothyroid rats, a 36% increase in the CL level was observed in liver mitochondria of T_2-treated hypothyroid rats. Smaller changes were detected in the percentage of phosphatidylethanolamine (PE) and phosphatidylcholine (PC). These changes led to a small but significant reduction of the PE/PC ratio in mitochondria from hypothyroid as compared to euthyroid ones. T_2 administration to hypothyroid rats increased slightly the PE and PC amount as well as their ratio without reaching values observed in euthyroid animals. No significant changes were found in the total amount of phospholipids as well as in the cholesterol content. Consequently, the cholesterol/phospholipids ratio remained almost unaltered.

It has been demonstrated that the H_2O_2 content of liver mitochondria increases in PTU-treated rats (Das and Chainy 2001; Venditti et al. 2003). In mitochondria from different rat tissues PTU-induced hypothyroidism decreased enzymatic and non-enzymatic antioxidant defenses and induced increased mitochondrial ROS generation (Das and

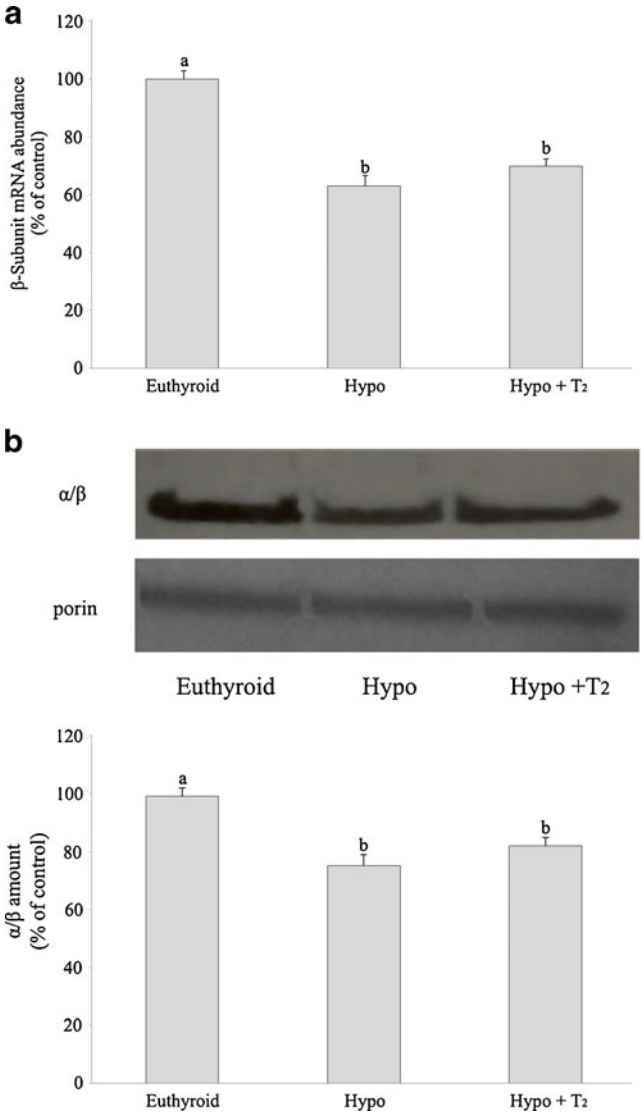

Fig. 2 Effect of T_2 treatment on β-subunit mRNA abundance and on expression of α/β subunits of ATP synthase in rat-liver mitochondria. **a** Total RNA was isolated from liver of euthyroid, hypothyroid (Hypo) and T_2-treated hypothyroid rats (Hypo+T_2), β-subunit gene expression was assessed by Real-Time qPCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control for normalization. The data are expressed as percentage of β-subunit mRNA abundance of euthyroid rats (100%) and are means ± SD of four different experiments. *Values with different letters* differ significantly $p < 0.05$. **b** Liver-mitochondrial proteins (15 μg), isolated from different rat groups, were separated by electrophoresis and transferred onto nitrocellulose membranes. The latter were incubated with antibodies against α/β subunits of ATP synthase or porin. A representative experiment is shown in this panel. The histogram represents the means ± SD of the values obtained from densitometric analysis of five different experiments after normalization with porin. The data are expressed as percentage of the protein level of euthyroid rats (100%). *Values sharing a different letter* differ significantly

Chainy 2001; Yilmaz et al. 2003; Senatore et al. 2010). Therefore, to investigate whether the decrease in the content of CL observed in hypothyroid mitochondria could be due to ROS-induced CL peroxidation, the amount of peroxidised CL was directly measured in mitochondria by HPLC. A 23% increase in the level of peroxidized CL was observed in hypothyroid as compared to euthyroid mitochondria. T_2 administration to hypothyroid rats reduced the amount of mitochondrial peroxidized CL to values observed in mitochondria from euthyroid rats (Fig. 3).

Chemical changes in mitochondrial membrane lipid composition were further investigated by analyzing the fatty acid composition of mitochondrial membranes. Table 3 shows that in hypothyroid rats, the level of linoleic acid ($C_{18:2}$, n-6) increased in parallel with a decrease in the percentage of arachidonic acid ($C_{20:4}$, n-6). Regarding n-3 polyunsaturated fatty acids, eicosapentaenoic acid level ($C_{20:5}$) was higher and docosahexaenoic acid ($C_{22:6}$) level lower in hypothyroid mitochondria. This decrease was less pronounced following T_2 administration to hypothyroid rats. No significant change in the sum of saturated and unsaturated fatty acids and in their unsaturation index (U.I.) was observed among the three groups of rats.

Discussion

Thyroid hormones act in two different ways on the energy metabolism of higher organisms. The long-term effects induce within days the expression of specific genes of energy metabolism via interaction of T_3 with thyroid hormone receptors (Goglia et al. 1999). The short-term effects refer to stimulation within minutes to a few hours of mitochondrial activities. T_2 effects on different metabolic pathways have been shown to be more rapid than those of T_3 and often independent of protein synthesis (Wrutniak-Cabello et al. 2001; Lombardi et al. 2007). It has been shown that short-term stimulation of lipogenesis by T_2 occurs in cultured hepatocytes from hypothyroid rats (Giudetti et al. 2005). Unlike other enzyme activities of the oxidative phosphorylation pathway, studies of rapid effects of T_2 on F_oF_1-ATP synthase are lacking (Izquierdo and Cuezva 1993). Very recently, we demonstrated that chronic T_2 administration to hypothyroid rats upregulates rat-liver mitochondrial F_oF_1-ATP synthase activity by a genomic mechanism involving the GA-binding protein/nuclear respiratory factor-2 (Mangiullo et al. 2010).

Short-term control events, however, may precede the onset of the long-term induction. In the present study we show that T_2 administration to hypothyroid rats induces, besides a stimulation of respiratory parameters, a rapid (within 1 h) increase in the F_oF_1-ATP synthase activity. In agreement with previous findings (Paradies and Ruggiero 1988; Nogueira et al. 2002; Mangiullo et al. 2010) we found in hypothyroid mitochondria a reduced succinate-induced respiration rate both in State 3 and State 4 (Table 1).

Table 2 The effect of thyroid state on phospholipid composition and cholesterol content in rat-liver mitochondria

Lipid fraction	Euthyroid	Hypothyroid	Hypothyroid+T_2
Cardiolipin	14.3±1.2[a]	9.2±0.8[b]	12.5±1.4[a]
Phosphatidylethanolamine	33.3±1.9[a]	28.8±1.2[b]	31.8±2.2[a]
Phosphatidylinositol	9.3±1.1	11.2±1.6	8.9±1.2
Phosphatidylserine	4.0±0.6	5.6±0.8	4.2±0.8
Phosphatidylcholine	40.0±2.2[a]	44.9±2.6[b]	43.0±2.9[b]
Phosphatidylethanolamine/Phosphatidylcholine	0.83±0.06[a]	0.64±0.08[b]	0.74±0.06[b]
Total phospholipids	166.3±12.8	140.0±9.2	156.9±8.9
Cholesterol	10.2±1.2	10.1±1.4	10.1±1.2
Ratio cholesterol/total phospholipids	0.06±0.01	0.07±0.01	0.06±0.01

Phospholipids were extracted from mitochondria of euthyroid and treated rats. Total phospholipids are expressed as nmol phospholipid phosphorus/mg protein and cholesterol as nmol/mg protein. Single phospholipids were separated by HPLC, quantified by determining inorganic phosphate and are expressed as % of the total phospholipids. Data are the means ± S.D. of six independent experiments. Values sharing different letters differ significantly

Treatment of hypothyroid rats with T_2 reverses the impairments observed in their mitochondria. T_2 administration to hypothyroid rats noticeably increased both coupled (state 3), uncoupled (state 4) and FCCP-stimulated respiration, while no change in the ADP/O ratio was observed. These findings add further support to those of Lombardi et al. (2009) who found that in mitochondria from rat-skeletal muscle T_2 rapidly enhanced succinate-supported respiration as well as the rate of fatty acid oxidation. Our results highlight the ability of T_2, when injected into hypothyroid rats, to induce a rapid stimulation of the ATP synthase activity (Fig. 2a). To determine whether the increased ATP synthesis observed upon T_2 treatment could be ascribed to a direct effect of T_2 on ATP synthase, we measured oligomycin-sensitive ATPase activity in ESMP. The latter, which expose the catalytic sites to the suspension medium, allow to study direct effects of T_2 on ATP synthase, differentiating them from effects on ANT or the adenine nucleotide content (Hafner et al. 1990). Data of Fig. 2b show that the F_oF_1-ATPase activity is influenced by T_2 treatment in a way similar to the ATP synthase activity, thus suggesting a direct effect of T_2 on complex V. The

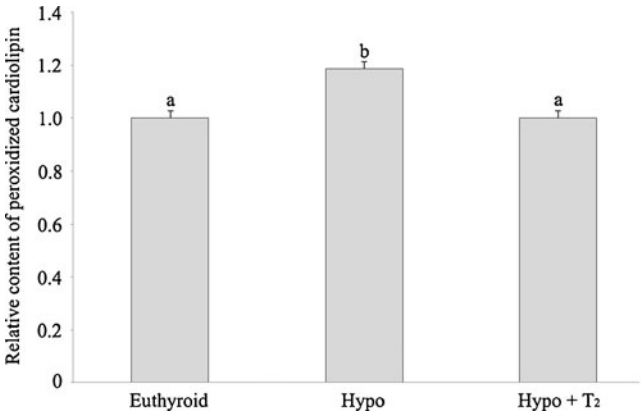

Fig. 3 Relative content of peroxidized cardiolipin in mitochondria from euthyroid, hypothyroid and T_2-treated hypothyroid rats. Mitochondrial content of peroxidized cardiolipin, calculated by peak area and quantified by determining inorganic phosphate was assayed by HPLC at 234 nm. Peak area of the euthyroid control is assumed as the unit. The values are the means ± SD of 5 independent experiments, $p<0.05$ vs control

Table 3 Fatty Acid composition (mol%) of mitochondrial membrane phospholipids

Fatty Acid	Euthyroid	Hypothyroid	Hypothyroid+T_2
$C_{14:0}$	0.4±0.1	0.5±0.1	0.6±0.1
$C_{16:0}$	19.9±0.7	18.8±1.2	19.7±0.9
$C_{16:1(n-7)}$	1.0±0.2	0.5±0.1	0.8±0.1
$C_{18:0}$	20.5±0.7	18.4±1.0	18.6±1.4
$C_{18:1(n-9)}$	7.6±1.1	8.3±0.9	9.1±1.1
$C_{18:2(n-6)}$	21.9±1.9 [a]	25.7±2.0[b]	22.8±2.1 [a]
$C_{18:3\ (n-3)}$	0.3±0.1[a]	1.0±0.1 [b]	1.1±0.1 [b]
$C_{20:4\ (n-6)}$	21.8±0.8 [a]	18.6±0.9 [b]	19.9±1.2 [a]
$C_{20:5\ (n-3)}$	0.9±0.1 [a]	1.4±0.3 [b]	1.1±0.2 [b]
$C_{22:5\ (n-3)}$	0.6±0.1	1.0±0.2	1.2±0.2
$C_{22:6\ (n-3)}$	6.2±0.5 [a]	2.9±0.8 [b]	4.1±0.6
∑ saturated	40.8±0.5	37.69±0.4	38.9±0.7
∑ unsatured	60.3±0.6	59.35±0.6	60.1±0.6
∑ sat./∑ unsat.	0.7±0.04	0.63±0.03	0.7±0.03
$C_{20:4}/C_{18:2}$	1.0	0.7	0.9
UI	185.2±13.2	166.9±2.1	174.5±5.3

Fatty acids were extracted from mitochondrial membrane phospholipids. After derivatization with methanolic boron trifluoride, fatty acid methyl esters (FAME) were separated by gas–liquid chromatography and identified by using known standards. FAME are expressed as mol %. The data are the means ± S.D. of five independent experiments. ∑ saturated and ∑ unsaturated = sum of saturated and unsaturated fatty acids, respectively. U.I. = unsaturation index, is defined as ∑ mol% of each fatty acid x number of double bonds of the same fatty acid. Values with different letters differ significantly, $P<0.05$

possibility that the observed increase in F_oF_1-ATP synthase activity could be ascribed to an increased expression of Complex V can reasonably be excluded by our results, since both the abundance of the mRNA of the catalytic β-subunit and the immunodetected α-β F_1 protein content were unaffected by T_2 treatment (see Fig. 2). Similarly, Lombardi et al. (2007) reported that acute administration of T_2 to hypothyroid rats was able to stimulate bioenergetic parameters in mitochondria from rat-skeletal muscle even in the presence of actinomycin D and/or cycloheximide, inhibitors of RNA and protein synthesis, respectively. Our results suggest that T_2 can affect the F_oF_1-ATP synthase activity by modulating the mitochondrial membrane lipid composition, in particular the CL level. CL is emerging as an important factor in the regulation of mitochondrial bioenergetics. It has been reported that CL, a phospholipid which is concentrated on the matrix side of the mitochondrial inner membrane where it is synthesized, is required for full activity of the F_oF_1-ATP synthase (Schlame et al. 2000). The mitochondrial phosphorylation machinery is the result of integrated functions of F_oF_1-ATP synthase complex, phosphate carrier and ANT. The activities of both these carriers, which are reduced in mitochondria from hypothyroid rats (Dummler et al. 1996; Siculella et al. 2006), are greatly influenced by the mitochondrial CL level (Paradies et al. 1991; Hoffmann et al. 1994; Claypool et al. 2008). The content of CL in the mitochondrial inner membrane may change either as a consequence of an alteration of the enzymatic steps involved in its biosynthetic process (Schlame et al. 2000) or as a consequence of oxidative damage by ROS (Chicco and Sparagna 2007; Petrosillo et al. 2007). Thyroid hormone has been shown to directly modulate the mitochondrial CL content by influencing the activity of CL biosynthesis enzymes (for review see Chicco and Sparagna 2007). Here, we show for the first time that the significant loss in CL content, observed in liver mitochondria of PTU-treated rats by other authors (Paradies et al. 1991) and confirmed in the present study, is also associated with a simultaneous increase in the amount of peroxidised CL occurring in mitochondria from hypothyroid rats. A reduction in CL content, associated with an increase in the level of oxidized CL, has been detected in mitochondria isolated from heart and brain tissues of aged animals (Paradies et al. 2010). CL is highly vulnerable to oxidative damage. Indeed, CL molecules are particularly rich in unsaturated fatty acids and they are located in mitochondria near the ROS production sites (Chicco and Sparagna 2007; Petrosillo et al. 2007), which are represented by the respiratory chain complexes I and III. A large body of evidence indicates that H_2O_2 production and oxidative stress significantly increase in liver mitochondria of PTU-treated rats (Das and Chainy 2001; Yilmaz et al. 2003; Venditti et al. 2003; Taş et al. 2006). The results in Fig. 3 show that T_2 administration to hypothyroid rats abolished the increase in peroxidised CL detected in the mitochondria from hypothyroid rats, restoring them to values observed in mitochondria from euthyroid rats.

Taken together, our results indicate that T_2 administration to hypothyroid rats increased in liver mitochondria the F_oF_1-ATP synthase activity in both the ATP synthesis and hydrolysis direction by a short-term mechanism. This effect is already evident within 1 h after hormone administration. Mitochondria from hypothyroid rats, as compared to mitochondria from euthyroid ones, showed a lower amount of the level of CL associated with a simultaneous increase in the level of oxidized CL. These findings provide evidence that the increase in F_oF_1-ATP synthase activity observed in T_2-treated hypothyroid rats can be ascribed, at least in part, to a T_2-induced increase in the amount of mitochondrial CL, occurring in parallel with a decreased level of the CL oxidized form.

The short-term effect of T_2 on different metabolic pathways represents a well established phenomenon. Even if the physiological role of T_2 is not yet completely defined it has been suggested that the rapidity by which T_2 acts in stimulating the mitochondrial respiration rate may play an important role in physiological conditions in which rapid energy utilization is required such as cold exposure or overfeeding (Lombardi et al. 1998; Goglia 2005). Our results strengthen this suggestion and contribute to better understand of how T_2 regulates metabolic rates.

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