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Original Paper

Differential Effects of 3,5-Diiodo-L-Thyronine and 3,5,3′-Triiodo-L-Thyronine On Mitochondrial Respiratory Pathways in Liver from Hypothyroid Rats

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Key Words
Mitochondria • Energy metabolism • Diiodothyronine • Thyroid hormones

Abstract
Background/Aims: Both 3,5-diiodo-L-thyronine (3,5-T2) and 3,5,3′-triiodo-L-tyronine (T3) affect energy metabolism having mitochondria as a major target. However, the underlying mechanisms are poorly understood. Here, using a model of chemically induced hypothyroidism in male Wistar rats, we investigated the effect of administration of either 3,5-T2 or T3 on liver oxidative capacity through their influence on mitochondrial processes including: proton-leak across the mitochondrial inner membrane; complex I-, complex II- and glycerol-3-phosphate-linked respiratory pathways; respiratory complex abundance and activities as well as individual complex aggregation into supercomplexes. ***Methods:*** Hypothyroidism was induced by propylthiouracil and iopanoic acid; 3,5-T2 and T3 were intraperitoneally administered at 25 and 15 µg/100 g BW for 1 week, respectively. Resulting alterations in mitochondrial function were studied by combining respirometry, Blue Native-PAGE followed by in-gel activity, and Western blot analyses. ***Results:*** Administration of 3,5-T2 and T3 to hypothyroid (hypo) rats enhanced mitochondrial respiration rate with only T3 effectively stimulating proton-leak (450% vs. Hypo). T3 significantly enhanced complex I (+145% vs. Hypo), complex II (+66% vs. Hypo), and glycerol-3 phosphate dehydrogenase (G3PDH)-linked oxygen consumptions (about 6- fold those obtained in Hypo), while 3,5-T2 administration selectively restored Euthyroid values of complex II- and increased G3PDH- linked respiratory pathways (+165% vs. Hypo). The mitochondrial abundance of all respiratory complexes and of G3PDH was increased by T3 administration whereas 3,5-T2 only increased complex V

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and G3PDH abundance. 3,5-T2 enhanced complex I and complex II in gel activities with less intensity than did T3, and T3 also enhanced the activity of all other respiratory complexes tested. In addition, only T3 enhanced individual respiratory component complex assembly into supercomplexes. ***Conclusions:*** The reported data highlight novel molecular mechanisms underlying the effect elicited by iodothyronine administration to hypothyroid rats on mitochondrial processes related to alteration in oxidative capacity in the liver. The differential effects elicited by the two iodothyronines indicate that 3,5-T2, by influencing the kinetic properties of specific mitochondrial respiratory pathways, would promote a rapid response of the organelle, while T3, by enhancing the abundance of respiratory chain component and favoring the organization of respiratory chain complex in supercomplexes, would induce a slower and prolonged response of the organelle.

Introduction

In mitochondria the oxidation of the NADH- and FADH2-reducing equivalents by mitochondrial respiratory complexes is coupled to pumping of protons into the intermembrane space; the resulting proton motive force is then used to form ATP [1]. Electron transport through the respiratory chain, however, is not perfectly coupled to ATP synthesis, since a portion of the protons, actively pumped by the respiratory chain into the intermembrane space, can cross the phospholipid bilayer and return into the matrix, thus dissipating the energy produced by the proton motive force as heat (a process termed proton-leak) [2].

It has recently been observed that interactions between individual respiratory complexes result in the formation of structures termed supercomplexes [3-5]. Such supramolecular organizations have been hypothesized to provide i) kinetic advantage (channeling), ii) control of reactive oxygen species (ROS) generation by the electron transport chain and iii) stabilization of individual complexes [3-5].

One major endocrine regulator of mitochondrial functionality is 3, 5,3'-triiodo-L-tyronine (T3). T3 greatly influences the tissue's mitochondrial content by promoting mitochondriogenesis, thus enhancing the cell's oxidative capacity [6] and, at the same time, controlling mitophagy by removing damaged mitochondria but also by sustaining oxidative phosphorylation [7]. At the mitochondrial level, T3 i) affects the reactions involved in substrates oxidations, ii) enhances the reactions involved in the synthesis and the export of ATP and ii) causes oxidative phosphorylation to be inefficient, by promoting mitochondrial proton-leak [8-10], and, as a result, iv) participates in the regulation of basal metabolic rate and to body heat production.

Besides T3, the thyroid hormone (TH) derivative 3, 5-diiodo-L-thyronine (3,5-T2), which is present in biological fluids, shows significant effects on energy metabolism [11, 12]. When administered to hypothyroid rats, 3,5-T2 increases the animals' metabolic rate [13, 14], cold tolerance [15], and their ability to use lipids as metabolic substrates [16-20]. In addition, when chronically administered to high fat diet fed rodents, 3,5-T2 contrasts fat accumulation and various dysmetabolic diseases associated with ectopic fat accumulation in lean tissues, such as liver steatosis and insulin resistance [21-24].

Ample evidence suggests that mitochondria are the major targets of 3,5-T2. Indeed, the ability of 3,5-T2 to stimulate mitochondrial function [25-27] underlies this hormone's effect on the increase of the animal's metabolic rate [13-17, 28] and on the prevention of ectopic accumulation of lipids induced by a high-fat diet [21, 23, 24, 28]. However, the cellular and molecular mechanisms through which 3,5-T2 elicits mitochondrial energetic adaptations in metabolically active tissues such as the liver await deeper investigation. Indeed, no study has so far examined the effects of iodothyronines on individual electron transport chain (ETC) complexes, their organization in supercomplexes and their relationship with mitochondrial oxidative capacity in hypothyroid liver.

Here, we individuate the mitochondrial processes influenced by a daily administration of either 3, 5-T2 or T3 for one week to chemically induced hypothyroid rats, in terms of respiratory pathway activities, efficiency of ATP synthesis as well as organization of respiratory complexes into supercomplexes. The obtained data provide new perspectives which aid in our understanding on the involvement of mitochondria in iodothyronine-mediated control of energy metabolism.

Materials and Methods

Animal treatment

Male Wistar rats weighing 275-300 g were obtained from Envigo RMS Srl (Udine Italy). Rats were housed in a temperature-controlled room at 28°C under a 12:12-h light-dark cycle, and food and water were available ad libitum. In the present study 4 groups of animals were used, with each group including 6 animals. Sample size was calculated based on a G* Power Test that was performed using software obtained from the University of Dusseldorf: http://www.gpower.hhu.de/. The power was 0.90, the effect size (f) was 1.27 and the α was 0.05. Group "Eu" consisted of Euthyroid rats, sham injected. Group "Hypo" consisted of hypothyroid rats. Hypothyroidism was induced by a daily intraperitoneal (i.p.) administration of propylthiouracil (1 mg/100 g bw) and a weekly i.p. administration of iopanoic acid (6 mg/100 g bw). This chemical approach induces severe hypothyroidism and inhibition of the three known types of deiodinase enzymes. This allows to attribute the observed effect to the administered iodothyronines rather than to any of their deiodinated products [14]. Hypothyroidism was complete after 4 weeks of treatment. Group "Hypo+T2" consisted of rats treated similarly to the "Hypo" group but in addition receiving a daily i.p administration of 3, 5-T2 (25 μg/100 g bw) during the last week of treatment. 3, 5-T2 was purchased from Sigma-Aldrich Corp. (St. Louis, MO) Group "Hypo+T3" consisted of rats treated similarly to the "Hypo" group but receiving a daily i.p administration of T3 (15 μg/100 g bw) during the last week of treatment. T3 was purchased from Sigma-Aldrich Corp. (St. Louis, MO).

The chosen doses of the iodothyronines have been shown to be effective in inducing enhancement of metabolic rate and mitochondrial respiration rate, without inducing significant variations in the animals' body weights [17]. The efficacy of the treatment was assessed by measuring serum levels of total T3 (TT3) and total T4 (TT4) that were significantly reduced by hypothyroidism [the obtained values were: TT4 (nM) 60±5 and 7±2 for Eu and Hypo, respectively; TT3 (nM) 0.84 ±0.02 and 0.19±0.05, for Eu and Hypo, respectively]. The administration of 3, 5-T2 to hypothyroid rats did not influence total TT4 and TT3 levels (6 ± 3 nM and 0.18 ± 0.01 nM for TT4 and TT3, respectively). On the other hand, the administration of T3 to hypothyroid rats enhanced TT3 levels to values higher than Euthyroid ones (6 ± 2 nM and 1.4 ± 0.08 nM for TT4 and TT3, respectively). Since TSH levels have not been detected we have no indication on the response of the hypophysis -thyroid axis to 3,5-T2 and T3 administration. At the end of each treatment, rats were anesthetized by i.p. injection of chloral hydrate (40 mg/100 body weight) and killed by decapitation. Livers were excised, weighed, and immediately processed for mitochondrial isolation or frozen in liquid nitrogen for later use. The present study was carried out in strict accordance with recommendations in the European Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize animal pain and suffering. All animal protocols were approved by the Committee on the Ethics of Animal Experiments of the University of Napoli Federico II (Italy) and the Italian Minister of Health.

Mitochondria isolation

Liver fragments were immersed in ice-cold isolation buffer, consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, 5 mM EGTA, and 0.1 % fatty acid-free bovine serum albumin pH 7.4, and subsequently homogenized in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 500 x g for 10 min at 4°C, the resulting supernatant was centrifuged at 3000 x g for 10 min at 4°C to obtain a mitochondrial pellet. Mitochondrial pellets were washed twice and resuspended in a minimal volume of isolation medium and kept on ice and either immediately used for measurement of respiratory rate or stored at -80°C for later processing. To detect proton-leak kinetics, fatty acid- free bovine serum albumin was omitted from the isolation buffer.

Mitochondrial respiration rate and proton-leak kinetics

Mitochondrial respiration rate was detected polarographically by using a Clark type electrode (Rank Brothers, Cambridge, UK). Mitochondria were incubated in a respiration buffer (0.5 ml of 80 mM KCl, 50 mM HEPES (pH 7.0), 1 mM EGTA, 5 mM K2HPO4, 0.1% BSA (wt/vol) at 37°C, for about 2-3 minutes, then respiration was initiated by adding succinate (5 mM) (in the presence of 4 μM rotenone) or glutamate (5 mM) + malate (2.5 mM) as substrates. After approximately 4 min, ADP (300 μM) was added to the incubation medium to induce State 3 respiration (i.e. respiration in which phosphorylation of ADP is at the maximal rate). After exhaustion of ADP, State 4 of respiration was reached, with mitochondria respiring principally to balance proton-leak. The proton-leak across the mitochondrial membrane was estimated by evaluating the change in oxygen consumption in succinate supported- oligomycin inhibited- respiration rate as a function of membrane potential ($\Delta\psi$) [*29*]. $\Delta\psi$ was measured using a triphenylmethylphosphonium (TPMP+) sensitive electrode [30]. A TPMP-binding correction 0.4 was applied. Oxygen consumption was determined simultaneously to $\Delta\psi$. For these measurements 0.5 mg of mitochondrial protein was incubated in 1 ml of respiration buffer deprived of BSA and supplemented with oligomycin (1 μg/ml) and nigericin (80 ng/ml). Mitochondria were energized using 5 mM succinate, and respiration was then titrated with an increasing amount of malonate (up to 2 mM).

Mitochondrial respiratory pathway activities

Mitochondrial respiratory pathway activities were detected in frozen then thawed mitochondria, treated with digitonin. This detergent allows to remove mitochondria external membranes without influencing the assembly of individual respiratory complexes in supramolecular organizations. Mitochondria (2 mg of proteins) were diluted in mitochondria isolation buffer supplemented with digitonin (4 g/g protein) and left in ice for 15 minutes. Mitochondria were centrifuged at 10.000 x g for 10 min at 4°C and the pellets were resuspended in a minimal volume. Part of the pellet was used for protein quantification.

To detect NADH-complex I- linked respiratory pathways, NADH driven oxygen consumption, which is inhibited by rotenone, has been measured. To this aim digitonin-treated mitochondria were incubated in 500 ml respiration buffer supplemented with cytochrome c (20 μM). Respiration rate was started by addition of NADH (1mM) and subsequent addition of rotenone (40 μM) to the respiration buffer after approximately 4 minutes. To evaluate complex II-linked respiratory pathways, respiratory buffer was supplemented with rotenone (40 μM) and cytochrome c (20 mM), and oxygen consumption was measured in the presence of succinate (5 mM) as substrate. To detect glycerol-3-phosphate dehydrogenase (G3PDH)-linked respiratory pathways, respiratory buffer was supplemented with rotenone 40 μM and cytochrome c 20 mM, oxygen consumption was then measured in the presence of glycerol-3-phosphate (G3P) (10 mM) as substrate. Complex IV activity was detected by incubating digitonin-treated mitochondria in respiration buffer supplemented with cytochrome c (20 mM), rotenone (40 μM) and antimycin (0.02 mM); oxygen consumption was started by addition of N,N,N',N' tetramethyl -p-phenylene-diamine TMPD (0.3 mM) and sodium ascorbate (4 mM).

Separation of respiratory complexes and supercomplexes by Blue-Native Page (BN-PAGE) and histochemical staining for in-gel activity

Solubilization of mitochondrial membranes by detergents, BN-PAGE, staining, and densitometric quantification of oxidative phosphorylation complexes was performed essentially as described by Scagger et al [31]. and Lombardi et al [32]. with minor modifications. Briefly, the mitochondria-containing sediment was suspended in a low-salt buffer (50 mM NaCl, 50 mM imidazole, pH 7.0) and solubilized with 10% (w/v) dodecyl-maltoside (for solubilization of individual respiratory chain complexes) or digitonin (4 g/g protein, for solubilization of respiratory chain supercomplexes). Immediately after the electrophoretic run (carried out on 4–13% gradient polyacrylamide gels), enzymatic colorimetric reactions were performed essentially as reported by others [33]. Complex I activity was determined by incubating the gel slices with 2 mM Tris–HCl, pH 7.4, 0.1 mg/ml NADH, and 2.5 mg/ml nitro blue tetrazolium (NTB) at room temperature. Complex II activity was evaluated after incubating the gel slices at room temperature in a 100 mM Tris/glycine buffer at pH 7.4 containing 1 mg/mL NTB and 1 mM sodium succinate. Complex IV activity was estimated by incubating BN-PAGE gels with 5 mg 3, 3′-diaminobenzidine tetrahydrochloride (DAB) dissolved in 9 mL phosphate buffer (0.05 M, pH 7.4), 1 mL catalase (20 μg/mL), 10 mg cytochrome c, and 750 mg sucrose. The original color of the complex I, II, or IV-reacting bands was preserved by fixing the gels in 50% methanol and

10% acetic acid. In parallel, another electrophoretic run was performed to stain the gels with Coomassie Blue G and obtain the total band pattern of respiratory complexes or supercomplexes. After gel scanning, the areas of the bands were expressed as absolute values (arbitrary units).

Western blot

To obtain total tissue lysates, livers were homogenized in RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with a broad-range antiprotease cocktail (SIGMA Aldrich). Homogenates were left on ice for 1 h and then centrifuged at 17.000 x g for 30 min, at 4°C; the resulting supernatants were collected.

To obtain mitochondrial lysates, mitochondria were isolated as described above with the isolation medium being supplemented with the antiprotease cocktail (SIGMA Aldrich). Isolated mitochondria were diluted in RIPA buffer left on ice for 1h and then centrifuged at 17.000 x g for 30 min at 4°C. The obtained supernatants containing lysate without debris were collected.

Primary antibodies used throughout the study were the following: oxophos ab110413, a cocktail of antibody used to detect CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1 and CV-ATP VA subunits, ab 188585 used to detect mitochondrial glycerol-3-phosphate dehydrogenase, ab 110322 used to detect adenine nucleotide transferase (ANT) protein levels.

Equal loading was verified by Ponceau S staining. Protein concentrations were determined by using the Bio Rad's DC method (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

Data are reported as mean ± SEM and have been analyzed by one-way ANOVA followed by a Student-Newman-Keuls post-hoc test. Analyses have been performed with Graphpad Prism 5 software. Differences have been considered statistically significant when $P<0.05$.

Results

Effects of iodothyronines administration to hypothyroid rats on food intake and body weight gain

Hypothyroidism significantly reduced body weight gain (-27% and -52% vs. Eu, respectively) (Table 1). Although over the whole treatment period (i.e. 4 weeks of treatment with PTU and IOP with or without either T3 or 3, 5-T2 administration during the last week of treatment) there were no significant variations in body weight-gain (Table 1), T3 administration during the last week reduced body weight whereas 3, 5-T2 administration increased it significantly. Food intake was reduced significantly by hypothyroidism, and a tendency toward an increased food intake was observed following administration of either T3 or 3, 5-T2 to Hypo rats (Table 1).

Effects of iodothyronines administration to hypothyroid rats on liver mitochondrial respiratory parameters and proton-leak kinetics

When using succinate (+rotenone) as substrate, hypothyroidism significantly reduced both State 3 and State 4 respiratory rates (-25% and -30% vs. Eu, respectively) (Table 2). The administration of either 3, 5-T2 or T3 to hypothyroid rats induced an increase in the above parameters, the effects of T3 being higher than those of 3, 5-T2 (Table 2). No differences

Table 1. Effects of iodothyronines administration to hypothyroid rats on body weight gain and food intake. Values reported represent mean ± SEM of 6 different experiments. * P <0.05 vs. Eu, § P<0.05 vs. Hypo, ° P <0.05 vs. Hypo+T2

	Eu	Hypo	Hypo+T2	Hypo+T3
Body weight gain (g)	100 ± 3	48 ± 4 *	53 ± 4 *	46 ± 3 *
Body weight gain during last week of treatment, g	18 ± 2	1 ± 0.2*	9 ± 2 *§	-5 ± 3 *°
Total food intake g/rat	498 ± 12	360 ± 10*	395 ± 18*	400 ± 15 *
Food intake during last week of treatment, g/rat	110 ± 7	68 ± 3*	82 ± 3*	80 ± 3*

in RCR values were observed between the analyzed groups (Table 2). When using glutamate (+malate) as substrate, hypothyroidism significantly reduced both State 3 and State 4 respiratory rates, which were only effectively stimulated by T3 administration (Table 2).

Hypothyroidism also significantly reduced proton-leak kinetics, since liver mitochondria from hypothyroid rats respired less with respect to those from Euthyroid rats to maintain the same ΔΨ value (Fig. 1A). The administration of 3, 5-T2 to hypothyroid rats did not affect proton-leak kinetics (i.e. the curves obtained with mitochondria from hypothyroid- and 3, 5-T2-treated groups were superimposable across most of their range). Interestingly, the plot relative to mitochondria from 3, 5-T2-treated rats extends further towards higher respiration rates and ΔΨ (Fig. 1A), thus indicating enhanced activity of the reactions involved in substrate oxidation. T3 administration to hypothyroid rats significantly affected proton-leak kinetics (Fig. 1A), since to maintain the same ΔΨ value, mitochondria from T3-treated hypothyroid rats respired more with respect to those from hypothyroid and Euthyroid rats. When the differences in proton conductance between the four animal groups were quantified by comparing the averaged values of respiration obtained at the highest ΔΨ of proton-leak kinetics common to all the groups (185 mV, in our case, dotted line in Fig. 1A), hypothyroidism reduced proton-leak by 61% vs. Eu and the administration of T3 increased it by about 450% and 120% vs. Hypo and Eu, respectively.

As assessed by Western Blot analyses, ANT levels were significantly reduced by hypothyroidism (-35 % vs. Eu). T3 administration to hypothyroid rats significantly enhanced ANT levels (+ 90% vs. Hypo) while restoring Euthyroid values observed in Eu rats (Fig. 1 B), thus indicating that ANT may be involved in the T3-induced proton-leak process.

Effect of iodothyronines administration to hypothyroid rats on liver mitochondria respiratory pathways

We next investigated the effects of iodothyronines on the mitochondrial complex-linked respiratory pathways (Fig. 2). Complex I-linked respiratory pathways (involving complexes I, III, IV), were

Table 2. Effects of iodothyronines administration to hypothyroid rats on liver mitochondrial respiratory rates. Respiratory rates (State 4 and State 3) were expressed as nAtoms oxygen/min/mg of mitochondrial proteins. Respiratory control ratio (RCR) is the ratio between State 3 and State 4 respiration rate. Values reported represent mean ± SEM of 6 different experiments. * P <0.05 vs. Eu, § P <0.05 vs. Hypo, ° P <0.05 vs. Hypo+T2

	Eu	Hypo	Hypo+T2	Hypo+T3
Succinate + rotenone				
State 4	36 ± 3	27 ± 2 *	38 ± 2 §	45 ± 2 *§°
State 3	270 ± 15	190 ± 12 *	258 ± 9 §	300 ± 12 §
RCR	7.5 ± 0.8	7.1 ± 0.8	7.0 ± 0.5	7.0 ± 0.5
Glutammate + malate				
State 4	13.1 ± 0,8	10.1 ± 0.8 *	11.0 ±0.9 *	15.0 ± 0.7 *§°
State 3	75.7 ± 3.6	54.7 ± 3.3 *	62.7 ± 6.8	84.8 ± 6.4 §°
RCR	5.8 ± 0.2	5.6 ± 0.5	5.7 ± 0.5	5.7 ± 0.5

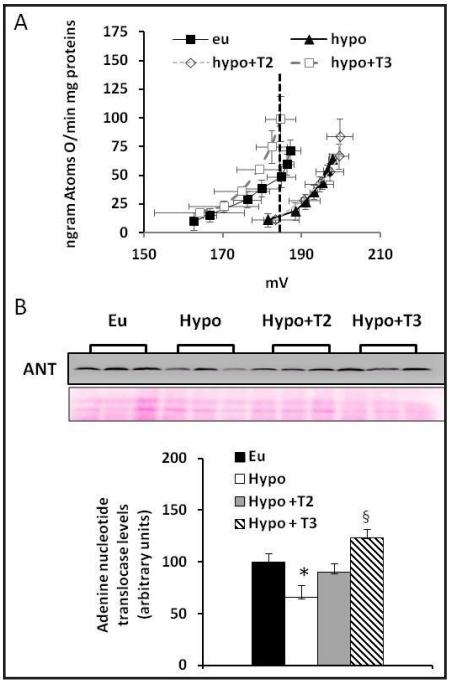

Fig. 1. (A) Effects of iodothyronines administration to hypothyroid rats on liver mitochondrial proton-leak. Data points are means ± SEM of 4 independent experiments. (B) Representative Western blots of Adenine nucleotide translocase levels detected in isolated mitochondria (15 µg of protein/rat/lane). The bar charts show blot signal quantification relative to Eu animals, set as 100, and data represent means ± SEM of 5-6 independent experiments. * P <0.05 vs. Eu, § P <0.05 vs. Hypo.

detected in digitonin-treated mitochondria as NADH driven, rotenone-sensible oxygen consumption. As reported in Fig. 2A, complex I-linked respiratory pathways were significantly reduced by hypothyroidism (-35% vs. Eu), were not affected by 3, 5-T2, and were significantly stimulated by T3 (+145% vs. hypo and + 60% vs. Eu). Complex II-linked respiratory pathways (involving complex II, III and IV), were detected in digitonin-treated mitochondria as succinate (+ rotenone)-driven oxygen consumption.

As reported in Fig. 2B, complex II-linked respiratory pathways were significantly reduced by hypothyroidism (-15% vs. Eu) and were significantly increased by both 3, 5-T2 and T3, with 3,5-T2 restoring Euthyroid values and T3 enhancing levels above Euthyroid levels (T3 induced increases were +66% vs. Hypo and +35 vs. Eu).

Complex IV activity was detected in digitonin-treated mitochondria as TMPD + ascorbate (+ rotenone + antimycin)- driven oxygen consumption. As reported in Fig. 2C, complex IV activity was significantly reduced by hypothyroidism (-38% vs. Eu) and not affected by 3,5-T2. T3 administration to hypothyroid rats significantly stimulated complex IV activity (+42% vs. hypo), reaching values not significantly different from those detected in mitochondria from Euthyroid rats. Finally, G3PDH-linked respiratory pathways (involving G3PDH, complexes

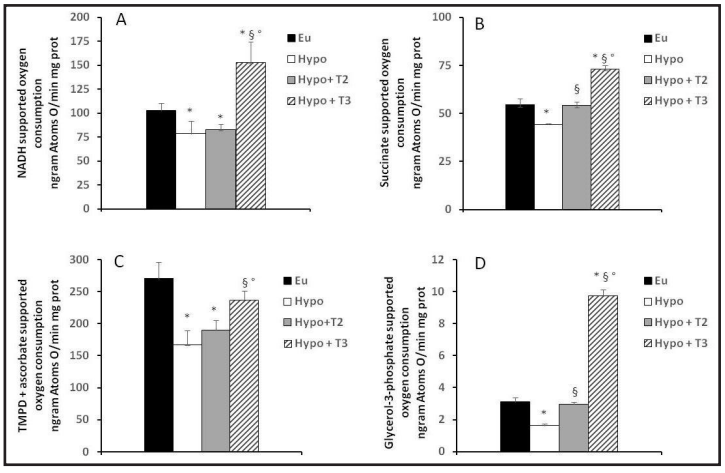

Fig. 2. Effects of iodothyronine administration to hypothyroid rats on liver mitochondrial complex I- (A), complex II- (B), complex IV- (C), and G3PDH-linked (D) respiratory pathways. Data represent means ± SEM of 6 independent experiments. * <0.05 vs. Eu, § P <0.05 vs. Hypo, ° P<0.05 vs. Hypo+T2.

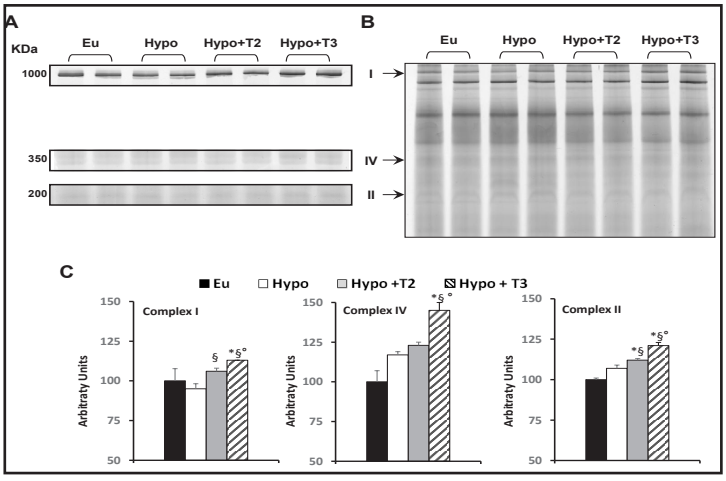

Fig. 3. BN-PAGE-based analysis of individual respiratory complexes from dodecylmaltoside-solubilized crude mitochondria from liver of Euthyroid (Eu), Hypothyroid (Hypo), 3,5-T2-treated hypothyroid (Hypo+T2) and T3-treated hypothyroid (Hypo+T3) rats. (A) Representative images of histochemical staining of complex I (I), complex IV (IV), and complex II (II) in-gel activity. (B) Representative image of a Coomassie blue stained BN-PAGE gel. Bands characteristic of individual OXPHOS complexes are recognizable. Molecular weights of standard proteins and the relative position of the respiratory complexes are indicated. (C) Densitometric quantification of colored bands corresponding to in gel-activity of complex I, complex IV, and complex II. Protein extracts were prepared for each animal, and each individual was assessed separately. Protein load was 15 μg/lane. Data were normalized to the value obtained for Eu animals, set as 100, and presented separately for each treatment (means ± SEM; n=4). * P <0.05 vs. Eu, § P <0.05 vs. Hypo, ° P < 0.05 vs. Hypo+T2.

III and IV) were detected in digitonin-treated mitochondria as glycerol-3-phosphate (G3P) (+rotenone) driven oxygen consumption. As reported in Fig. 2D, G3P-supported oxygen consumption was significantly reduced by hypothyroidism (-60% vs. Eu), and subsequent iodothyronines administration significantly increased its value. 3,5-T2 administration to hypothyroid rats increased G3PD-supported oxygen consumption (+165% vs. hypo), and restored Euthyroid values; on the other hand, the effect of T3 was much higher, since values observed in T3 treated animals were about 6- and 3- fold those obtained in Hypo and Eu, respectively.

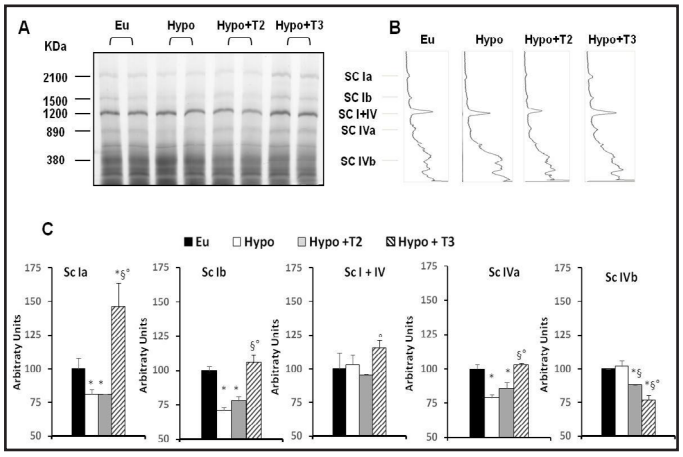

Fig. 4. Blue native PAGE based analysis of digitonin-solubilized crude mitochondria from liver of Euthyroid (Eu), Hypothyroid (Hypo), 3,5-T2-treated hypothyroid (Hypo+T2) and T3-treated hypothyroid (Hypo+T3) rats. (A) Representative image of a Coomassie blue stained BN-PAGE gel. Bands characteristic of OXPHOS supercomplexes are recognizable in all the experimental groups. (B) Representative density traces for OXPHOS supercomplex bands. (C) Densitometric quantification of the areas of the blue-colored bands of OXPHOS supercomplexes containing Complex I and Complex IV activities (namely, SC Ia, SC Ib, SC I+IV, SC IIa, SC IVb). Data were normalized to the value obtained for Eu animals, set as 100, presented separately for each treatment (means ± SEM; n=4) and expressed as arbitrary units. * P <0.05 vs. Eu, § P <0.05 vs. Hypo, ° P<0.05 vs. Hypo+T2.

Effect of iodothyronines administration to hypothyroid rats on the in-gel activities of liver mitochondria respiratory complexes

To further investigate the effects of iodothyronine administration on the phenotype and biochemical properties of hepatic mitochondria, the respective respiratory chain complexes from Eu, Hypo, Hypo+T2, and Hypo+T3 rats were extracted either with dodecylmaltoside or the mild detergent digitonin and resolved by BN-PAGE (Fig. 3 and Fig. 4). For each individual respiratory complex (I, II and IV) the in-gel activity was analyzed (Fig. 3). Digitonin-solubilized respiratory supercomplexes were assayed for their electrophoretic profiles as well as complex I and IV activities (Fig. 4). Regarding the dodecylmaltoside-solubilized individual respiratory complexes, densitometric analysis revealed that hypothyroidism did not result in significant changes in the in-gel activity of all assayed enzymes (Fig. 3). 3,5-T2-administration significantly enhanced the activities of complex I (+12% vs. Hypo) and complex II (+5% vs. Hypo; +12% vs. Eu, see Fig. 3). T3-administration produced a significant stimulation of the in-gel activities of all assayed complexes, displaying the highest effect on complex IV activity (+24% vs. Hypo; +45% vs. Eu) (Fig. 3). Fig. 4 shows the BN-PAGE-based electrophoretic profiles of digitonin-solubilized respiratory supercomplexes from liver of Eu, Hypo, Hypo+T2, and Hypo +T3 rats. The separated bands were then assayed for complex I and complex IV in-gel activities. Five bands showed complex I or/and complex IV activities. For a clearer description of the results obtained, based on the in-gel activity they present, the five bands were named SC Ia, Sc Ib, SC I+IV, SC IVa, SC IVb, starting from band of the highest molecular weight, (Fig. 4). Three bands within the molecular mass range of 1200–2100 kDA showed complex I activity (SC Ia, Sc Ib, SC I+IV). Complex IV in-gel activity was revealed in three bands within the molecular mass range of 380-1200 kDA (SC I+IV, SCIVa, SC IVb). The lightest band (1200 kDa) containing complex I activity corresponded to the heaviest band showing complex IV activity, (namely, SC I+IV in Figs. 4A and 4B), thus representing a supercomplex containing both the respiratory complexes.

Densitometric analysis of the electrophoretic profiles of the obtained supercomplexes revealed that hypothyroidism produced a significant reduction (vs. Eu) of the SC Ia, SC Ib and SC IVa bands (Figs. 4B and 4C). 3, 5-T2-administration induced no significant changes in the levels of all the considered supercomplexes (vs. Hypo), except for SC IVb of which the level was slightly but significantly reduced (-5% vs. Hypo; -4% vs. Eu) (Figs. 4B and 4C). T3-administration induced a significant increase in the levels of the heaviest supercomplexes showing complex I activity, namely SC Ia and SCIb (+80% an +49% vs. Hypo, respectively), barely altering those of SC I-IV. T3 administration did not significantly change activity of the earlier-mentioned supercomplexes of complex IV, SC I-IV, enhanced that of SC IVa (+37% vs. Hypo) and significantly reduced that of the lightest supercomplex (i.e SC IVb) (-24% vs. Hypo) (Fig. 4B and 4C).

Effect of iodothyronine administration to hypothyroid rats on respiratory chain complex-abundance in liver mitochondria

As reported in Fig. 5 and 6, Western blot analysis indicated that the levels of complex I and G3PDH were significantly reduced by hypothyroidism (-43% and -50% vs. Eu, respectively). 3, 5-T2 significantly increased mitochondrial levels of complex V (+30% vs. Hypo) as well as those of G3PDH (more than 2-folds vs. Hypo and + 38% vs. Eu) (Fig.6).

T3 significantly increased the mitochondrial content of all five respiratory complexes and the increases were as follows: complex I +94% vs. Hypo and +67 % vs. Eu, complex II +40% vs. both Hypo and Eu rats, complex III +140 % vs. Hypo and +97% vs. Eu, complex IV + 100% vs. Hypo and +62% vs. Eu, complex V about +55% vs. both Hypo and Eu (Fig. 5). In addition, T3 had a marked effect on G3PDH levels (+ 250% and +560%, vs. Hypo and Eu, respectively) (Fig. 6).

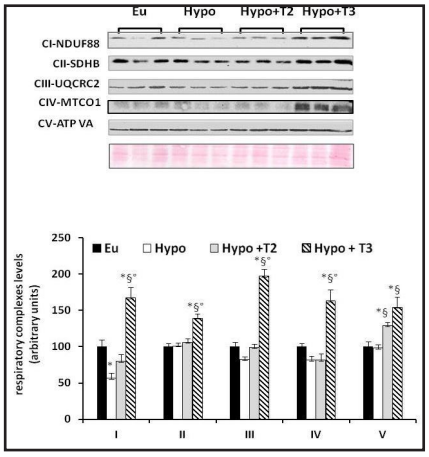

Fig. 5. Effects of iodothyronines administration to hypothyroid rats on liver mitochondrial respiratory complexes abundance. Representative Western blots of CI-NDUF88, CII-SDHB, CIII-UQCRC2, CIV-MTCO1 and CV-ATP VA subunits detected in isolated mitochondria (15 µg of protein/rat/lane). Data were normalized to the value obtained for Eu animals, set as 100 and represent means ± SEM of 6 independent experiments. * P <0.05 vs. Eu, § P <0.05 vs. Hypo, ° P<0.05 vs. Hypo+T2.

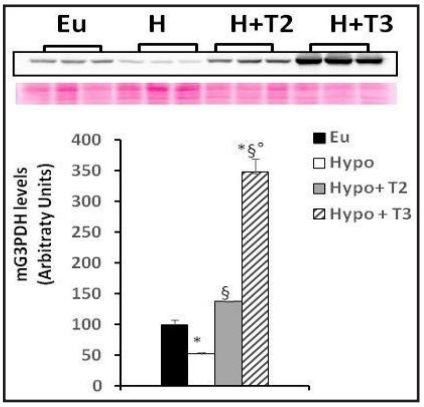

Fig. 6. Effects of iodothyronines administration to hypothyroid rats on liver mitochondria glycerol- 3-phosphate dehydrogenase (G3PDH) abundance. Representative Western blots of G3PDH detected in isolated mitochondria (15 µg of protein/rat/lane). Data were normalized to the value obtained for Eu animals, set as 100 and represent means ± SEM of 6 independent experiments. * P <0.05 vs. Eu, § P <0.05 vs. Hypo, ° P<0.05 vs. Hypo+T2.

Discussion

Data reported in the present paper give further insight into how administration of T3 or 3,5-T2 to hypothyroid rats differentially influences the mitochondrial processes responsible for change in liver oxidative capacity.

Hypothyroidism significantly reduced both coupled and uncoupled respiration, when using succinate+rotenone as substrate, thus confirming data reported in the literature [34-37]. Inhibition of respiratory chain activity and reduced ANT levels

lead to a lower capacity of hypothyroid mitochondria to synthesize and export ATP. At the same time, the concomitant inhibition of proton-leak processes lead to a reduced dissipation of energy as heat. The reduction in ANT levels reported here (which have been shown to be correlated with mitochondrial proton-leak/conductance in non-phosphorylating mitochondria [38, 39]), could likely contribute to the lower proton-leak observed in liver mitochondria from hypothyroid rats. Alterations in mitochondrial inner membrane phospholipid composition, which take place in hypothyroid mitochondria [40] could also be responsible for the reduced proton-leak.

Administration of 3,5-T2 to hypothyroid rats for one week enhanced both coupled and uncoupled respiration in succinate+rotenone-energized mitochondria.

The effect of 3,5-T2 on uncoupled respiration does not involve proton leak but is likely attributed to an enhanced activity of reactions involved substrate oxidation as revealed by:

i) the evidence that the plot relative to proton-leak kinetics of mitochondria from 3, 5-T2 treated rats, besides largely overlapping that of mitochondria from hypothyroid rats (i.e. absence of proton conductance variation), extends further towards higher respiration rates and proton motive force, which is diagnostic of higher activity of overall reactions involved the oxidation of substrate, being succinate in this specific case;

ii) the enhanced activity of complex II-linked mitochondrial respiratory pathways;

iii) the enhanced "in gel activity" of complex II.

Since 3,5-T2 was not able to influence the abundance of respiratory complexes the points mentioned under ii) and iii), are likely the result of variations of the kinetic properties of the respiratory chain. One aspect of the mechanism underlying the stimulatory effect of 3, 5-T2 on coupled respiration may be the rise in the activity of the above-described reactions involved in the oxidation of substrates, associated with the enhancement of ATP synthase levels.

Although 3,5-T2 does not activate proton-leak, this iodothyronine influences other processes involved in mitochondrial heat production by selectively activating FADH2 linked respiratory pathways. Indeed, the amount of ATP generated by FADH oxidation (produced by G3P via G3PDH or by succinate via complex II-succinate dehydrogenase) is lower than that observed when the NADH-reducing equivalents enter the respiratory chain in complex I [41]. Hence, the evidence that 3,5-T2 has a selective effect in increasing respiration in mitochondria energized with substrates producing FADH2 (such as succinate and G3P), and that did not affect NADH oxidation (rotenone- inhibitable), suggests a specific cellular mechanism that could affect a thermogenic process at the mitochondrial level.

T3 shares with 3,5-T2 the ability to activate complex II- and G3PDH-linked respiratory pathways and increased complex V and G3PDH levels, but the effects of T3 are more pronounced with respect to those elicited by 3,5-T2. T3 but not 3,5-T2 was able to significantly activate proton-leak; the T3-associated increase in the mitochondrial ANT [39, 40] and changes in the inner membrane phospholipid composition in liver mitochondria [42] could contribute to the T3- induced proton-leak [40]. Moreover, the strong T3-induced activation of G3PDH-linked respiratory pathways, considering their implications in mitochondrial heat production, might be considered as thermogenic action that adds to the T3-induced proton-leak. Despite more factors contribute to whole animal body weight gain, the high thermogenic action of T3 could contribute to the reduced metabolic efficiency observed in T3 treated animals, which indeed is the only animal group losing weight during the last week of treatment

Even though the stimulatory effect of 3,5-T2 is principally directed to the FADH2-linked respiratory pathways, T3 enhances the activities of all the respiratory pathways analyzed as well as the in-gel activity of the individual complexes. These T3-induced effects could be explained by the higher abundance of the mitochondrial respiratory complexes as well as by their supramolecular assembly. Data reported here show, for the first time, the ability of T3 to influence the assembly of individual respiratory component complexes in supercomplexes when administered to hypothyroid rats. In view of the proposed role for supercomplexes, based on the data described here, their importance in mitochondrial

physiology associated to different thyroid states can be hypothesized. On the one hand, the formation of supercomplexes, observed in mitochondria from hyperthyroid rats, and the resulting improvement of electron transport rate, would enhance the tissue's oxidative capacity, necessary to sustain the increased metabolic rate. On the other hand, since hyperthyroidism is associated with mitochondrial oxidative stress [36], assembly of supercomplexes may be advantageous to limit or balance ROS generation, of which overproduction can have disastrous consequences for the cell. This is particularly important in view of evidence presented in the literature that fatty acid composition of the major mitochondrial phospholipid classes is affected by thyroid hormones which, by decreasing their degree of saturation, enhance the probability of formation of lipid hydroperoxides and damages induced to the membranes [42].

Conclusion

Data presented here reveal novel molecular mechanisms underlying the effect elicited by iodothyronine administration to hypothyroid rats on hepatic mitochondrial processes that lead to increased fuel oxidation for energy extraction, mitochondrial respiratory rate, heat production and release, hence contributing to support whole-animal increased energy expenditure.

Interestingly, the presented data indicate that 3,5-T2 and T3 share only some of their effects at the mitochondrial level and that the molecular mechanisms underlying them substantially vary. They further support the idea that 3,5-T2, by influencing the kinetic properties of specific mitochondrial respiratory pathways, would promote a rapid response of the organelle to variations in energy demand. Instead, T3, by enhancing the abundance of respiratory chain component, and by favoring the organization of respiratory chain complex in supercomplexes, would induce a slower, prolonged and more sustained response of the organelle.

It should be considered that, since this study is restricted to liver, it formally cannot be excluded that molecular mechanisms underlying the mitochondrial response to hypothyroidism and to iodothyronine administration may differ among various iodothyronine-target tissues. Thus, it needs to be underlined that further studies regarding tissue-specificity of the above observed metabolic effects are warranted.

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Disclosure Statement

The authors declare no conflict of interests.

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