Tri-iodothyronine treatment differently affects liver metabolic response and oxidative stress in sedentary and trained rats

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Abstract

We investigated whether swim training modifies the effects of tri-iodothyronine (T_3) treatment on the metabolic response and oxidative damage of rat liver. Respiratory capacities, oxidative damage, levels of antioxidants, and susceptibility to oxidative challenge of liver homogenates were determined. Mitochondrial respiratory capacities, rates of H_2O_2 release, and oxidative damage were also evaluated. Training modified most of the measured parameters in both thyroid states, although the extent of changes was higher in hyperthyroid preparations. T_3 treatment enhanced homogenate respiratory capacity, which was further enhanced by training despite the decrease in mitochondrial respiratory capacity. Hormonal treatment also induced liver oxidative damage and glutathione depletion, and increased tissue susceptibility to oxidative challenge. These effects were lower in trained animals. The extensive oxidative

damage found in liver homogenates from hyperthyroid sedentary rats was due to low tissue antioxidant protection and high mitochondrial H_2O_2 production rate, which were increased and decreased respectively by animal training. The training effect on H_2O_2 production was associated with lower oxidative damage and susceptibility to Ca^{2+} -induced swelling of mitochondria. Measurements with respiratory inhibitors indicated that the differences in H_2O_2 release in hyperthyroid groups were due to differences in mitochondrial content of autoxidizable electron carrier located at Complex III. We conclude that moderate training is able to reduce hyperthyroid state-linked cellular and subcellular oxidative damage in liver increasing its antioxidant defenses and decreasing the mitochondrial generation of reactive oxygen species.

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Introduction

It is long known that in vertebrates that experimentally induced hyperthyroidism leads to accelerated basal metabolic rate and oxygen consumption in several tissues (Schwartz & Oppenheimer 1978). It is also well established that the increased respiratory capacity displayed from rat tissues following prolonged thyroid hormone treatment is associated with oxidative stress, as documented by the high levels of oxidative damage indicators (Videla 2000, Venditti & Di Meo 2006). Accumulating evidence has demonstrated that in the liver such effects are primarily due to increased tissue content of mitochondrial respiratory chain components (Brand & Murphy 1987), including autoxidizable electron carriers (Venditti *et al.* 2003). This increase enhances tetravalent and univalent oxygen reduction, leading to higher oxygen consumption and reactive oxygen species (ROS) production respectively (Fernández & Videla 1993, Venditti *et al.* 2003).

Studies on the effects of tri-iodothyronine (T_3) treatment on the antioxidant defense system of rat liver have shown that the changes in both the activities of antioxidant enzymes and the levels of low molecular weight scavengers are unbalanced and often opposite, although the whole antioxidant capacity

significantly decreases in hyperthyroid state (Videla 2000, Venditti & Di Meo 2006). This decrease is associated with a higher susceptibility of hepatic tissue to oxidative challenge both *in vitro* (Venditti *et al.* 1997, 1999a) and *in vivo* (Videla *et al.* 1995, Troncoso *et al.* 1997). Interestingly, T_3 effects appear to be strongly dependent on treatment duration. Indeed, ischemia-reperfusion injury, which is exacerbated by a 2-day T_3 treatment (Troncoso *et al.* 1997), is reduced by a single T_3 dose (Fernández *et al.* 2007).

Hyperthyroid state-linked liver oxidative damage can be attenuated by strengthening antioxidant status, as demonstrated by the observation that the administration of vitamin E (Venditti *et al.* 1999a) and caffeic acid phenylethyl ester (Mohamadin *et al.* 2007) protects against lipid peroxidation in hyperthyroid liver.

Some studies indicate that antioxidant administration and moderate physical training can have similar protective effects in conditions leading to increased free radical production. For example, the appearance of signs of increased free radical generation induced by acute swimming was prevented in rat liver by both vitamin E administration (Brady *et al.* 1979) and training (Venditti & Di Meo 1996). The training effect on liver was associated with an increase in tissue whole antioxidant

defenses (Venditti & Di Meo 1996). However, it is not possible to exclude that, like in rat skeletal muscle (Venditti *et al.* 1999b, Servais *et al.* 2003), a reduced free radical activity contributes to delayed peroxidative reactions in the hepatic tissue.

To shed some light on this matter, we evaluated the effects of physical training on respiratory capacity and oxidative damage of liver homogenates from euthyroid and hyperthyroid rats. Moreover, because mitochondria are a possible target of ROS, respiratory capacity and oxidative damage of mitochondrial preparations were also evaluated. Markers of lipid and protein damage were related with scavenger levels and susceptibility to oxidants of liver homogenates and with mitochondrial H_2O_2 production rates.

Materials and Methods

Materials

All chemicals used (Sigma Chimica) were of the highest grades available. The response to oxidative stress was determined using reagents and instrumentation of the commercially available Amerlite System (Ortho-Clinical Diagnostics, Milano, Italy).

Serum levels of free T_3 (FT₃) and thyroxine (FT₄) were determined using commercial RIA kits (DiaSorin, Salluggia, Italy).

Animals

Male Wistar rats (50 days old), supplied by Nossan (Correzzana, Italy) at day 45 of age, were randomly divided into sedentary and trained animals. Swimming, used as the exercise for the trained rats, was administered 5 days per week for 10 weeks. Our program provided initially brief swimming periods and their gradual lengthening to both facilitate the learning process and reduce the stress reaction. Therefore, in the first week, the rats were made to swim for 15 min daily to familiarize them with the water immersion. In the second week, the training session lasted 50 min and from the third to the tenth week the rats swam for 60 min a day. Swimming was performed in a plastic container that was 100 cm high, filled with water maintained at 35 °C to a depth of 55 cm. Untrained animals were kept in a small chamber holding about 3 cm water maintained at 35 °C.

Ten days before the end of the training program half of sedentary and trained rats were made hyperthyroid by the treatment with daily i.p. injections of T_3 (10 µg/100 g body weight). Thus, there were four groups of rats: euthyroid sedentary (ES), euthyroid trained (ET), hyperthyroid sedentary (HS), and hyperthyroid trained (HT).

All the rats were subjected to the same conditions (one per cage, constant artificial circadian cycle of 12-h light:12-h darkness, and $50 \pm 10\%$ relative humidity), and fed the same diet, a commercial rat chow purchased from Nossan, and water on an *ad libitum* basis.

The treatment of animals in these experiments was in accordance with the guidelines set forth by the University's Animal Care Review Committee.

Liver homogenate preparation

Twenty-four hours following the end of the training program, at about 120 days of age, all animals were killed by decapitation while under ether anesthesia. Arterial blood samples were collected and later analyzed to determine the plasma levels of FT₃ and FT₄. The livers were rapidly excised and placed into ice-cold homogenization medium (HM; 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.1% fatty acid-free albumin, 10 mM Tris, pH 7.4). Tissues were freed from connective tissue and were weighed, finely minced, and washed with HM. Finally, tissue fragments were gently homogenized (20% w/v) in the same solution using a glass Potter–Elvehjem homogenizer set at a standard velocity (500 r.p.m.) for 1 min. Aliquots of homogenates were used for analytical procedures and preparation of mitochondrial fractions.

Preparation of mitochondria

The homogenates, diluted 1:1 with HM, were freed of debris and nuclei by centrifugation at 500 g for 10 min at 4 °C. The resulting supernatants were centrifuged at 10 000 g for 10 min. The mitochondrial pellets were resuspended in washing buffer (WB; 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 20 mM Tris, pH 7.4) and centrifuged at the same sedimentation velocity. Mitochondrial preparations were washed in this manner twice before final suspension in WB. Mitochondrial protein was measured by the biuret method (Gornall *et al.* 1949).

Analytical procedures

Cytochrome oxidase (COX) activity was determined by the procedure of Barré *et al.* (1987) using liver homogenates and mitochondrial suspensions diluted with modified Chappel–Perry medium so that the preparations contained per ml either 100 mg tissue or 0.2 mg mitochondrial proteins.

Oxygen consumption of homogenates and mitochondria was monitored at 30 °C by a Hansatech respirometer in 1.6 ml incubation medium (145 mM KCl, 30 mM HEPES, 5 mM KH_2PO_4 , 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4) with 50 µl homogenate or 0.25 mg mitochondria per ml and succinate (10 mM), plus 5 µM rotenone (Rot), or pyruvate/malate (10/2.5 mM) as substrates, in the absence (State 4) and in the presence (State 3) of 500 µM ADP.

The extent of the peroxidative processes in liver homogenates and mitochondrial preparations was determined by measuring the level of lipid hydroperoxides (HPs) according to Heath & Tappel (1976). Determination of protein oxidative damage was performed measuring protein-bound carbonyl levels by the procedure of Reznick & Packer (1994) for homogenates and by the modified procedure of Schild *et al.* (1997) for mitochondria.

Glutathione peroxidase (GPX) activity was assayed at 37 °C according to Flohé & Günzler (1984) with H₂O₂ as substrate. Glutathione reductase (GR) activity was measured at 30 °C according to Carlberg & Mannervik (1985).

Ubiquinols (CoQH₂) from 0.5 ml of 10% homogenate were oxidized to ubiquinones (CoQs) with 0.5 ml of 2% FeCl₃ and 2.0 ml ethanol. The content of CoQs was then determined according to Lang *et al.* (1986). Vitamin E content was determined using the HPLC procedure of Lang *et al.* (1986). Reduced glutathione (GSH) concentration was measured as described by Griffith (1980).

The rate of mitochondrial H₂O₂ release was measured at 30 °C following the increase in fluorescence (excitation at 320 nm, emission at 400 nm) due to oxidation of *p*-hydroxyphenylacetate (PHPA) by H₂O₂ in the presence of horseradish peroxidase (HRP; Hyslop & Sklar 1984) in a computer-controlled Jasco fluorometer equipped with a thermostatically controlled cell-holder. The reaction mixture consisted of 0.1 mg/ml mitochondrial proteins, 6 U/ml HRP, 200 µg/ml PHPA, and 10 mM succinate, plus 5 µM rotenone, or 10 mM pyruvate/2.5 mM malate added at the end to start the reaction in a medium containing 145 mM KCl, 30 mM HEPES, 5 mM KH₂PO₄, 3 mM MgCl₂, 0.1 mM EGTA, pH 7.4. Measurements with the different substrates in the presence of 500 µM ADP were also performed. Furthermore, the effects of two respiratory inhibitors were investigated: rotenone, which blocks the transfer of electrons from Complex I to ubiquinone (Palmer *et al.* 1968), and antimycin A (AA), which interrupts electron transfer within the ubiquinone–cytochrome *b* site of Complex III (Turrens *et al.* 1985). Inhibitor concentrations (5 µM Rot, 10 µM AA) that do not interfere with the detection PHPA-HRP system were used (Venditti *et al.* 2003).

Capacity to remove H₂O₂ was determined by comparing the ability of mitochondrial samples to reduce H₂O₂-linked fluorescent emission with that of desferrioxamine solutions (Venditti *et al.* 2001). Thus, the capacity of mitochondrial samples to remove H₂O₂ was expressed as equivalent desferrioxamine concentration.

Response to oxidative challenge was determined as previously described (Venditti *et al.* 1999c). Briefly, the samples of 10% (w/v) homogenates were obtained by diluting the 20% homogenates with equal volumes of 0.2% Lubrol in 15 mM Tris, pH 8.5. Several dilutions of samples up to a tissue concentration of 0.002% were prepared in 15 mM Tris (pH 8.5). The assays were performed in microtiter plates. Enhanced chemiluminescence reactions were initiated by addition of 250 µl of the reaction mixture to 25 µl of the samples. The reaction mixture was obtained by mixing the solutions containing substrate in excess and signal generating reagents respectively in buffer at pH 8.6 (Vitros Signal Reagent). The plates were incubated at 37 °C for 30 s under continuous shaking and then transferred to a luminescence analyzer (Amerlite Analyzer). The emission values were fitted to dose–response curves using the statistical facilities of the Fig.P graphic program (Biosoft, Cambridge, UK).

Mitochondrial swelling was spectrophotometrically measured by determining the apparent absorbance at 540 nm in a medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH 7.2, 2 mM succinate, 4 µM rotenone, 0.3 mg mitochondrial protein per ml of reaction mixture, 100 µM Ca²⁺, and 50 mM EGTA or 100 µM cyclosporin A (CSA) where indicated.

Mitochondrial membrane potential ($\Delta\Psi$) was estimated through the fluorescence changes of safranin (8 µM) recorded on the Jasco fluorometer (excitation wavelength 495 nm, emission wavelength 586 nm) in a medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH 7.2, 2 mM succinate, 6 µM rotenone, 0.3 mg mitochondrial protein per ml of reaction mixture, 100 µM Ca²⁺. $\Delta\Psi$ was calculated according to Åkerman & Wikström (1976) using a calibration curve obtained incubating mitochondria in a medium containing 200 mM sucrose, 10 mM HEPES, pH 7.2, 6 µM rotenone, 0.38 EDTA, 8 µM safranin, 38.5 ng/ml valinomycin, and KCl at concentrations from 0 to 0.96 mM.

Statistical analysis

The data obtained in eight different experiments are expressed as means $\pm$ S.E.M. Data were analyzed with a two-way ANOVA method. When a significant *F* ratio was found, the Student–Newman–Keuls multiple range test was used to determine the statistical significance between means. The effects of respiratory inhibitors in the same group were statistically analyzed with the Student's *t*-test. Probability values (*P*) < 0.05 were considered significant.

Results

The animal thyroid state was documented by their heart mass/body mass ratio and plasma levels of FT₃ and FT₄. Heart mass/body mass ratios were 2.25 $\pm$ 0.04, 2.95 $\pm$ 0.05, 3.15 $\pm$ 0.07, and 3.30 $\pm$ 0.12 in ES, ET, HS, and HT rats respectively. They were significantly (*P* < 0.05) increased by training in the euthyroid rats and by T₃ treatment in the sedentary and trained rats. As it is shown in Fig. 1, plasma levels of FT₃ increased, whereas FT₄ levels decreased in hyperthyroid rats in comparison with respective euthyroid controls. Conversely, there were not differences in FT₃ and FT₄ levels between sedentary and trained animals.

COX activity

In liver homogenates, COX activity was increased by T₃ administration in both sedentary and trained rats and was not modified by training in euthyroid and hyperthyroid animals. In liver mitochondria, COX activity was increased by T₃ treatment in sedentary and trained group and was significantly decreased by training in both thyroid states (Table 1).

The ratio between COX activities in homogenates and mitochondria provided a rough estimate of tissue content of

Figure 1 Serum thyroid hormone levels in euthyroid sedentary (ES), euthyroid trained (ET), hyperthyroid sedentary (HS), and hyperthyroid trained (HT) rats. Values are means $\pm$ S.E.M. of eight different experiments. FT₃ and FT₄ are serum level of free tri-iodothyronine and thyroxine respectively. ^aSignificant versus euthyroid controls. The level of significance was chosen as $P<0.05$.

mitochondrial proteins. Such a content was higher in hyperthyroid rats than in respective euthyroid controls and was increased by training in both thyroid states.

O₂ consumption

The rates of O₂ consumption by liver homogenates are reported in Fig. 2 (upper panel). The rates of succinate-supported O₂ consumption were higher in hyperthyroid rats than in euthyroid controls during both State 4 and State 3. Respiration rates were increased by training only in hyperthyroid rats during State 4. Respiratory control ratio (RCR) values, which were 4.3 ± 0.2 , 4.0 ± 0.3 , 4.8 ± 0.2 , and 4.6 ± 0.2 for ES, ET, HS, and HT rats respectively, were not significantly ($P>0.05$) affected by T₃ and training. The rates of pyruvate/malate-supported O₂ consumption were higher in hyperthyroid than in euthyroid controls during State 4 and State 3. Furthermore, they were increased by training in

Table 1 Cytochrome oxidase activities and mitochondrial protein content in rat liver

Group	Cytochrome oxidase		Mitochondrial protein
	Homogenate	Mitochondria	
ES	69.3 $\pm$ 1.3	0.94 $\pm$ 0.01	72.7 $\pm$ 3.3
ET	67.5 $\pm$ 0.5	0.84 $\pm$ 0.01 [†]	80.4 $\pm$ 1.9 [†]
HS	113.0 $\pm$ 1.6*	1.19 $\pm$ 0.02*	95.0 $\pm$ 3.0*
HT	108.5 $\pm$ 2.3*	0.99 $\pm$ 0.02* [†]	109.5 $\pm$ 1.7* [†]

Values are means $\pm$ S.E.M. For each value eight rats were used. Cytochrome oxidase activity (COX) is expressed in $\mu\text{mol O/min per milligram mitochondrial protein or g tissue}$. Mitochondrial protein is expressed as mg protein per g tissue. ES, euthyroid sedentary rats; ET, euthyroid trained rats; HS, hyperthyroid sedentary rats; HT, hyperthyroid trained rats. *Significant versus euthyroid controls. [†]Significant versus sedentary animals. Treatment $\times$ training interaction significant for mitochondrial COX. The level of significance was chosen as $P<0.05$.

Figure 2 Rates of O₂ consumption by rat liver homogenates (upper panel) and mitochondria (bottom panel) from euthyroid sedentary rats (ES); euthyroid trained rats (ET); hyperthyroid sedentary rats (HS); and hyperthyroid trained (HT) rats. Values are means $\pm$ S.E.M. of eight different experiments. Rates of O₂ consumption were measured in the absence (State 4) and in the presence (State 3) of ADP with Complex II (succinate) and Complex I (pyruvate/malate) linked substrates. ^aSignificant versus euthyroid controls. ^bSignificant versus sedentary animals. Treatment $\times$ training interaction significant for State 4 homogenate respiration supported by pyruvate/malate, State 4 mitochondria respiration supported by succinate, and State 3 mitochondria respiration supported by succinate or pyruvate/malate. The level of significance was chosen as $P<0.05$.

euthyroid rats during State 4. RCR values, which were 2.8 ± 0.1 , 2.1 ± 0.1 , 2.2 ± 0.1 , and 2.6 ± 0.1 for ES, ET, HS, and HT rats respectively, were significantly ($P<0.05$) increased by T₃ in trained rats and decreased in sedentary ones, whereas they were decreased by training in the euthyroid rats and increased in the hyperthyroid ones.

The rates of O₂ consumption by liver mitochondria are reported in Fig. 2 (bottom panel). The rates of succinate-supported O₂ consumption were higher in hyperthyroid rats than in euthyroid controls during both State 4 and State 3. Respiration rates were reduced by training in euthyroid rats during State 4 and in both thyroid states during State 3. RCR values, which were 4.7 ± 0.1 , 5.1 ± 0.3 , 3.8 ± 0.1 , and 3.3 ± 0.1 for ES, ET, HS, and HT rats respectively, were significantly ($P<0.05$) reduced by T₃ in sedentary and trained rats.

The rates of pyruvate/malate-supported O₂ consumption were increased by T₃ in sedentary and trained rats during State 4 and in sedentary rats during State 3. Furthermore, the rates were decreased by training in both thyroid states during State 4 and State 3. RCR values, which were 1.7 ± 0.1 , 2.3 ± 0.1 , 1.7 ± 0.1 , and 1.8 ± 0.1 for ES, ET, HS,

Figure 3 Oxidative damage in liver preparations from euthyroid sedentary (ES), euthyroid trained (ET), hyperthyroid sedentary (HS), and hyperthyroid trained (HT) rats. Values are means $\pm$ S.E.M. of eight different experiments. Hydroperoxides (HPs) are expressed in nmol NADP min/g tissue and pmol NADP min/mg mitochondrial protein. Protein-bound carbonyls (CO) are expressed in nmol/mg protein. ^aSignificant versus euthyroid controls. ^bSignificant versus sedentary animals. Treatment $\times$ training interaction significant for mitochondrial HP and carbonyl levels. The level of significance was chosen as $P < 0.05$.

and HT rats respectively, were significantly ($P < 0.05$) decreased by T_3 in trained rats and increased by training in euthyroid ones.

Oxidative damage

In liver homogenates and mitochondria, the levels of hydroperoxides and protein-bound carbonyls were significantly higher in hyperthyroid rats than in respective

euthyroid controls. Furthermore, they were reduced by training in both thyroid states (Fig. 3).

Antioxidants

The activities of antioxidant enzymes and the levels of low molecular weight antioxidants in the liver homogenates are reported in Table 2. T_3 treatment increased GPX activities in sedentary rats and decreased GR activities in both sedentary and trained animals. Training increased GPX activity in euthyroid but not in hyperthyroid rats and did not affect GR activities in both thyroid states. Changes in vitamin E were found only in HT liver, which exhibited levels lower than their euthyroid and untrained controls. CoQ9 and CoQ10 levels were not modified by T_3 administration or training. GSH levels were decreased by T_3 treatment in trained and untrained rats, and were increased by training in both thyroid states.

Response to oxidative challenge

The luminescence response to changes of concentration of the homogenates (Fig. 4) was described by the equation ($E = a \cdot C / \exp(b \cdot C)$). The parameters a and b , which determine the light emission maximum ($E_{\max} = a/e \cdot b$), are dependent on the concentration of substances able to induce (iron or cuprum ligands) and inhibit (antioxidants) respectively the H_2O_2 -induced luminescent reaction. Examination of the curves in Fig. 4 and data reported in Table 3 show that the emission maximum was increased by T_3 treatment in sedentary and trained rats, and was decreased by training in both thyroid states. The values of the parameters a and b indicate that the increase in emission peak induced by T_3 treatment is due to lower b values and higher a values, while the decrease induced by training is mainly imputed to higher b values.

Mitochondrial H_2O_2 release and capacity to remove H_2O_2

During both State 4 and State 3 respiration, the rates of succinate and pyruvate/malate supported H_2O_2 mitochondrial

Table 2 Antioxidants levels in rat liver homogenates. Values are means $\pm$ S.E.M. For each value eight rats were used. Glutathione peroxidase activity (GPX) is expressed in μ mol NADPH min/g tissue. Glutathione reductase (GR) is expressed in μ mol NADPH min/g tissue. Vitamin E (Vit E), coenzyme Q9 (CoQ9), and coenzyme Q10 (CoQ10) content is expressed in nmol/g tissue. Reduced glutathione (GSH) is expressed in μ mol/g tissue

Parameter	Group			
	ES	ET	HS	HT
GPX	75.7 $\pm$ 1.4	85.0 $\pm$ 0.8 [†]	85.5 $\pm$ 2.8*	87.6 $\pm$ 2.1
GR	9.0 $\pm$ 0.5	9.8 $\pm$ 0.3	7.7 $\pm$ 0.3*	8.3 $\pm$ 0.1*
Vit E	49.94 $\pm$ 1.63	45.44 $\pm$ 0.69	47.31 $\pm$ 0.85	35.70 $\pm$ 1.50* [†]
CoQ9	76.98 $\pm$ 1.63	82.27 $\pm$ 3.50	82.77 $\pm$ 1.26	76.72 $\pm$ 1.02
CoQ10	7.44 $\pm$ 0.22	8.59 $\pm$ 0.31	7.93 $\pm$ 0.37	7.76 $\pm$ 0.62
GSH	4.79 $\pm$ 0.09	5.49 $\pm$ 0.39 [†]	2.61 $\pm$ 0.07*	3.48 $\pm$ 0.09* [†]

ES, euthyroid sedentary rats; ET, euthyroid trained rats; HS, hyperthyroid sedentary rats; HT, hyperthyroid trained rats. *Significant versus euthyroid controls. [†]Significant versus sedentary animals. Treatment $\times$ training interaction significant for vitamin E and CoQ9 content. The level of significance was chosen as $P < 0.05$.

Figure 4 Response to *in vitro* oxidative challenge of liver homogenates from euthyroid sedentary (ES), euthyroid trained (ET), hyperthyroid sedentary (HS), and hyperthyroid trained (HT) rats. Tissue susceptibility to stress was evaluated by determining the variations, with concentration of homogenates, of light emission from a luminescent reaction. Emission values are given percentage of an arbitrary standard (44 ng/ml peroxidase). The curves were computed from experimental data by equation $E = a.C/\exp(b.C)$.

release were increased by T₃ treatment in sedentary and trained rats, and were decreased by training in both thyroid states (Fig. 5).

The capacities to remove H₂O₂ were increased by T₃ treatment ($P < 0.05$) in trained and untrained rats and were decreased by training in both thyroid states ($P < 0.05$). The values, expressed as equivalent concentration of desferroxamine (nmol/mg protein), were 3.18 ± 0.09 , 2.77 ± 0.05 , 4.13 ± 0.10 , and 3.45 ± 0.07 for ES, ET, HS, and HT rats respectively.

Effect of inhibitors on H₂O₂ release

As shown in Table 4, T₃ treatment increased the rate of mitochondrial succinate-supported H₂O₂ release, in the absence of rotenone, in both sedentary and trained rats, whereas training reduced it in both thyroid states. As expected, rotenone addition decreased the rates of H₂O₂ release in all groups, by stopping the release occurring at

Complex I, due to the reverse electron flow from coenzyme Q (Ernster & Lee 1967). However, the rates were again increased by T₃ and decreased by training. Analogous results were obtained after further addition of antimycin, which increased H₂O₂ release rates in all groups. Addition of antimycin or rotenone to pyruvate/malate supported mitochondria increased H₂O₂ release rates in all groups. However, whereas in the presence of antimycin, the significance of differences between groups was not modified, in the presence of rotenone the rates of H₂O₂ release were not different in sedentary and trained hyperthyroid rats.

Mitochondrial swelling

As shown by the absorbance changes in Fig. 6, the extent of swelling was increased by T₃ treatment in Ca²⁺-loaded mitochondria from sedentary rats, but not in mitochondria from trained rats. Mitochondrial swelling was drastically reduced by CSA or EGTA (unreported results), pointing to the role played by the permeability transition pore. Ca²⁺-induced swelling was preceded by a rapid decrease in membrane potential, which was greater in preparations from sedentary hyperthyroid rats than in those from trained rats. No difference in Ca²⁺-induced mitochondrial swelling and $\Delta\Psi$ decrease were found in euthyroid preparations (Fig. 6).

Discussion

A first consideration, elicited from the results reported in this paper, concerns the effects on the liver oxidative metabolism capacity of T₃ prolonged administration to sedentary and trained rats. The aerobic metabolic capacity of tissues can be evaluated measuring the *in vitro* COX activity, which is positively correlated to the maximal oxygen consumption (Simon & Robin 1971). It has been reported that training does not modify COX activity and, therefore, the oxidative capacity of liver (Terblanche *et al.* 2001), which, conversely, increases in experimental (Venditti *et al.* 1999a, 2006) and functional (Venditti *et al.* 2004, 2006) hyperthyroidism. The present results confirm the above reports and indicate

Table 3 Parameters characterizing the response to oxidative stress of rat liver homogenates. Values are means $\pm$ S.E.M. For each value eight rats were used. For explanation of symbols see text. The relation between light emission and homogenate concentration is described by the equation: $E = a.C/\exp(b.C)$. $E_{\max} = a/e.b$

Parameter	Group			
	ES	ET	HS	HT
<i>a</i>	72.8 $\pm$ 5.4	70.3 $\pm$ 4.2	98.7 $\pm$ 3.7*	90.0 $\pm$ 6.5*
<i>b</i>	1.34 $\pm$ 0.03	1.81 $\pm$ 0.12 [†]	0.75 $\pm$ 0.08*	1.03 $\pm$ 0.10*. [†]
<i>E</i> _{max}	20.0 $\pm$ 1.1	14.3 $\pm$ 1.5 [†]	48.4 $\pm$ 2.4*	32.12 $\pm$ 2.1*. [†]

ES, euthyroid sedentary rats; ET, euthyroid trained rats; HS, hyperthyroid sedentary rats; HT, hyperthyroid trained rats. *Significant versus euthyroid controls. [†]Significant versus sedentary animals. Treatment $\times$ training interaction significant for *E*_{max}. The level of significance was chosen as $P < 0.05$.

Figure 5 Rates of H_2O_2 release by liver mitochondria from euthyroid sedentary (ES), euthyroid trained (ET), hyperthyroid sedentary (HS), and hyperthyroid trained (HT) rats. Values are means $\pm$ S.E.M. of eight different experiments. Rates of H_2O_2 release were measured in the absence (State 4) and in the presence (State 3) of ADP with Complex II (succinate) and Complex I (pyruvate/malate) linked substrates. ^aSignificant versus euthyroid controls. ^bSignificant versus sedentary animals. Treatment $\times$ training interaction significant for H_2O_2 release sustained by succinate during State 4 and State 3, and H_2O_2 release sustained by pyruvate/malate during State 3. The level of significance was chosen as $P < 0.05$.

that T_3 induces similar increases in liver oxidative capacity in sedentary and trained rats.

These increases were due, in both animal groups, to enhanced mitochondrial aerobic capacity and tissue content of mitochondrial proteins. However, the changes in aerobic capacity were greater in HS group, whereas those in protein content were greater in HT group. Our results also suggest that training does not increase liver aerobic capacity, because the proliferation of mitochondrial population it seems to induce is accompanied by a decrease in the aerobic capacity of the organelles.

The mitochondrial proliferation found in hyperthyroid liver can be attributed to the capacity of T_3 to increase the expression of peroxisomal proliferator-activated receptor gamma co-activator (PGC)-1, a transcriptional coactivator that plays a pivotal role in mitochondrial biogenesis (Weitzel

et al. 2001). On the other hand, catecholamines may be responsible for mitochondrial proliferation in the liver from trained rats. In fact, recent studies have shown i) an increased PGC-1 expression in muscle from swimming trained rats (Goto *et al.* 2000), ii) an increased plasma catecholamine level during single swimming sessions (Park *et al.* 2005) and an increased PGC-1 expression in murine muscle in response to exercise linked to β -adrenergic receptor activation (Miura *et al.* 2007).

Interestingly, the prolonged T_3 administration and training elicited changes in the oxidative capacity of homogenates and mitochondria were reflected in the changes in succinate and pyruvate/malate sustained State 3 respiration of these preparations. This suggests that both treatments modify liver mitochondrial population without alter the ratios among the components of respiratory chain.

Some changes in State 4 respiration rate induced by training in mitochondria and homogenates remain to be understood. The increase in basal respiration rates represents a compensatory response to increased leak of protons back in the mitochondrial matrix. Thus, the increases induced by T_3 are consistent with an enhanced basal proton conductance dependent on oxidative modifications of inner mitochondrial membrane lipids (Brand 2000). In this light, the respiration rates should have to decrease in mitochondrial preparations from trained rats, but this does not happen in succinate-supplemented mitochondria from HT rats. Moreover, although the changes found in homogenates can be explained by the increased tissue content in mitochondrial proteins, the pyruvate/malate-supported respiration rate measured in homogenates from ET rats appears to be out of proportion to the mitochondrial protein content.

There are a few studies concerning the chronic swimming effects on liver oxidative damage in euthyroid rats, which reported discrepant results (Venditti & Di Meo 1996, 1997, Aydin *et al.* 2007), likely due to different training protocols. Conversely, there is full agreement about thyroid hormone

Table 4 Effects of inhibitors specific for different segments of the respiratory chain on rates of H_2O_2 release by rat liver mitochondria. Values are means $\pm$ S.E.M. For each value eight rats were used. Mitochondrial H_2O_2 release rate is expressed in $\text{pmol min}^{-1} \text{mg}^{-1} \text{protein}$

Substrate	Group			
	ES	ET	HS	HT
Succinate (Succ)	115.3 $\pm$ 1.0	101.2 $\pm$ 0.8 [†]	179.0 $\pm$ 2.3*	132.6 $\pm$ 0.6* [†]
Succ + Rot	101.4 $\pm$ 1.1 [†]	92.0 $\pm$ 2.9 ^{†,‡}	146.5 $\pm$ 2.4* [†]	111.4 $\pm$ 0.8* ^{†,‡}
Succ + Rot + AA	803.8 $\pm$ 9.8 [†]	718.3 $\pm$ 1.8 ^{†,‡}	919 $\pm$ 14.8* ^{†,‡}	830.7 $\pm$ 3.4* ^{†,‡}
Pyruvate/Malate (Pyr/Mal)	194.7 $\pm$ 1.5	181.1 $\pm$ 1.1 [†]	310.4 $\pm$ 2.7*	300.2 $\pm$ 4.3* [†]
Pyr/Mal + AA	992.9 $\pm$ 4.2 [†]	924.4 $\pm$ 2.2 ^{†,‡}	1036.8 $\pm$ 11.4* [†]	1020.6 $\pm$ 17.3* ^{†,‡}
Pyr/Mal	194.6 $\pm$ 2.5	182.0 $\pm$ 1.2 [†]	310.9 $\pm$ 2.6*	300.5 $\pm$ 3.4* [†]
Pyr/Mal + Rot	227.6 $\pm$ 2.6 [†]	214.8 $\pm$ 1.2 ^{†,‡}	332.8 $\pm$ 2.4* [†]	329.5 $\pm$ 2.5* [†]

ES, euthyroid sedentary rats; ET, euthyroid trained rats; HS, hyperthyroid sedentary rats; HT, hyperthyroid trained rats. *Significant versus euthyroid controls. [†]Significant versus sedentary animals; [‡]significant effect of the last inhibitor added versus mitochondria under same conditions without that inhibitor. Treatment $\times$ training interaction significant for H_2O_2 release in the presence of succinate, succinate plus rotenone, pyruvate/malate plus antimycin or rotenone. The level of significance was chosen as $P < 0.05$.

Figure 6 Ca^{2+} -induced swelling (upper panel) and membrane potential dissipation (lower panel) of liver mitochondria from euthyroid sedentary (ES), euthyroid trained (ET), hyperthyroid sedentary (HS), and hyperthyroid trained (HT) rats. Swelling, monitored as decrease in the absorbance at 540 nm, is expressed as percentage of the initial value before Ca^{2+} addition. Membrane potential ($\Delta\psi$), estimated through fluorescence changes of safranine, was calculated using a suitable calibration curve. Its decrease is expressed as percentage of the initial value before Ca^{2+} addition. Values are means $\pm$ S.E.M. of eight different experiments. The initial absorbance values were 0.85 ± 0.04 , 0.90 ± 0.07 , 0.86 ± 0.05 , and 0.83 ± 0.05 for ES, ET, HS, and HT preparations respectively. Initial values of $\Delta\psi$ were 149.1 ± 11.5 , 152.7 ± 10.4 , 140.6 ± 9.7 , and 147.0 ± 8.9 for ES, ET, HS, and HT preparations respectively. ^aSignificant versus euthyroid controls. ^bSignificant versus sedentary animals. Treatment $\times$ training interaction significant for swelling and membrane potential. The level of significance was chosen as $P < 0.05$.

promotion of liver oxidative damage (Videla 2000, Venditti & Di Meo 2006). The present results show that moderate training reduces lipid and protein oxidation in euthyroid animals and attenuates its increase associated with T_3 administration, suggesting that training exerts a strong protective effect against T_3 -induced oxidative stress.

Oxidative stress results from a disturbance of the normal cell balance between production of ROS and the capacity to neutralize their action. Thus, in order to understand the mechanisms underlying the differential effects of T_3 on liver

oxidative damage in sedentary and trained rats, we examined changes in liver capacity to generate and neutralize free radicals.

In previous reports, we showed that the total antioxidant capacity of rat liver is increased by training (Venditti & Di Meo 1996, 1997) and decreased by T_3 treatment (Venditti *et al.* 1997, 1999a). The determination of total antioxidant capacity on tissue preparations presents several limits. On the other hand, due to the complexity of the antioxidant defense systems, investigations on tissue antioxidant status based on the determination of single antioxidant compounds or enzymes often does not offer a coherent pattern. This also happens for the data here reported, concerning some components of the antioxidant defense system, which do not allow to establish whether the effectiveness of such system is differently modified by T_3 treatment in sedentary and trained rats. Indeed, the levels of some antioxidants are not modified by training in hyperthyroid rats, whereas those of vitamin E and GSH undergo opposite changes, likely because the different degree of GSH depletion is not the cause, but the consequence of the different levels of oxidative stress in the two rat groups. However, some information was obtained by examining the liver sensitivity to oxidative processes by measuring the levels of light emission resulting from *in vitro* exposure to H_2O_2 of liver homogenates. As the emission maximum indicates the susceptibility of the preparations to oxidative challenge (Venditti *et al.* 1999c), our results reveal that such a susceptibility is reduced by training, but in greater measure in T_3 -treated animals. Also, the results indicate that the lower susceptibility to oxidants found in trained groups is mainly due to a higher antioxidant capacity. Thus, there is indirect evidence that a greater effectiveness of the antioxidant defense system contributes to the lower degree of oxidative damage induced by T_3 in the liver from trained rats.

More clear information has been obtained on ROS generation contribution to the different extent of oxidative damage found in sedentary and trained rats. ROS are produced in several cellular sites, but mitochondria have long been recognized as the major source of free radicals (Liu *et al.* 2002). The enhancement in mitochondrial protein content of hyperthyroid livers should lead to increased ROS flow from mitochondria to cytoplasm. However, it is apparent that training protects the hepatic tissue against oxidative damage by inducing adaptive modifications that prevent the elevated mitochondrial H_2O_2 efflux. Indeed, training limits the T_3 -induced increase in H_2O_2 mitochondrial release rates. The effect concerns mainly the H_2O_2 release during State 4 respiration sustained by Complex II-linked substrate, so that in the trained group the rate of H_2O_2 release per g of tissue is 87% of that found in sedentary group. A training-induced reduction of succinate-supported ROS release occurs also in euthyroid rats. However, such reduction is small and is able only to compensate the increase in mitochondrial protein content.

The observation that the mitochondrial capacity to remove H_2O_2 is higher in hyperthyroid than in euthyroid groups and in sedentary than in trained groups indicates that the differences in

H₂O₂ release reflect differences in the production of the H₂O₂ precursor, the superoxide radical (O₂^{•-}). Such a production, in turn, depends on concentration and reduction degree of the autoxidizable electron carriers located at Complex I and Complex III of the respiratory chain. Information on the relative contribution of autoxidizable carriers to the O₂^{•-} generation in sedentary and trained rats can be obtained by analyzing the effects of respiratory inhibitors and substrates on H₂O₂ release. This analysis indicates that the increase in H₂O₂ release shown in hyperthyroid rats is due to an increased concentration of both autoxidizable electron carriers. This idea is supported by previous reports showing that hyperthyroidism increases the content of several components of respiratory chain in liver mitochondria (Jacovcic *et al.* 1978, Horrum *et al.* 1985, 1986). However, our results are not able to show the presence within the respiratory chain of a main site responsible for the enhanced H₂O₂ production. On the other hand, it is possible, as previously suggested (Venditti *et al.* 2003), that mitochondria undergo a generalized thyroid hormone-linked change in electron carrier content.

Conversely, it is apparent that the prolonged T₃ administration to trained rats induces a smaller increase in the mitochondrial content of the ROS generator located at Complex III. Indeed, in the presence of rotenone, succinate-supported mitochondrial H₂O₂ release is lower in trained group than in the sedentary one, whereas the release supported by pyruvate/malate is not significantly different in the two groups. Further support comes from the results obtained in the presence of antimycin A, which makes ROS production dependent only on the concentration of autoxidizable carriers. Such results show that H₂O₂ release is lower in the trained group when it depends on the Complex III generator, i.e. in the presence of succinate, whereas there are not differences between the groups when it depends on the Complex I and III generators, i.e., in the presence of pyruvate/malate. Finally, when pyruvate/malate-supplemented mitochondria are treated with rotenone, which makes H₂O₂ release dependent only on the concentration of the Complex I generator, such release is not significantly different in the two hyperthyroid groups. We also observed that, in the presence of rotenone, pyruvate/malate-sustained H₂O₂ release was lower in trained than in sedentary euthyroid rats. However, to date we are not able to offer a convincing explanation for this different effect of training on autoxidizable carrier of Complex I in euthyroid and hyperthyroid rats.

The T₃ and training-dependent changes in ROS generation have important implications for the function and even the viability of mitochondria, because these organelles are the main target of the ROS they produce. It is known that hyperthyroidism leads to an increase in total liver Ca²⁺ concentration (Hummerich & Soboll 1989). In the presence of this ion, oxidative alterations of protein thiols of the mitochondrial inner membrane promote a membrane permeabilization referred to as mitochondrial permeability transition (Zoratti & Szabo 1995), which leads to mitochondrial swelling. Our results show that mitochondrial

susceptibility to Ca²⁺-induced swelling is significantly lower in trained than in sedentary hyperthyroid rats. This observation is consistent with the failure of T₃ to induce similar levels of oxidative stress and ROS production in mitochondria from sedentary and trained animals.

In conclusion, this work supplies strong indication that training limits the oxidative stress brought out in the hepatic tissue by prolonged thyroid hormone treatment, reducing mitochondrial ROS production and preserving the antioxidant system effectiveness. Because it has been recently shown that functional recovery from ischemia reperfusion of hyperthyroid hearts is increased by training (Venditti *et al.* 2008), it is apparent that, together with antioxidant integration, properly graded physical activity can protect hyperthyroid tissues against the oxidative damage-linked dysfunctions.

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