The TRβ-selective agonist, GC-1, stimulates mitochondrial oxidative processes to a lesser extent than triiodothyronine

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Abstract

Specific tissue responses to thyroid hormone are mediated by the hormone binding to two subtypes of nuclear receptors, TRα and TRβ. We investigated the relationship between TRβ activation and liver oxidative metabolism in hypothyroid rats treated with equimolar doses of triiodothyronine (T_3) and GC-1, a TRβ agonist. T_3 treatment produces increases in O_2 consumption and H_2O_2 production higher than those elicited by GC-1. The greater effects of T_3 on oxidative processes are linked to the higher hormonal stimulation of the content of respiratory chain components including autoxidizable electron carriers as demonstrated by the measurement of activities of respiratory complexes and H_2O_2 generation in the presence of respiratory inhibitors. It is conceivable that these differential effects are dependent on the inability of GC-1 to stimulate TRα receptors that are likely involved in the expression of some components of the respiratory chain. The greater increases in reactive oxygen species production and susceptibility to oxidants exhibited by mitochondria from T_3-treated rats are consistent with their higher lipid and protein oxidative damage and lower resistance to Ca^{2+} load. The T_3 and GC-1 effects on the expression levels of nuclear respiratory factor-1 and -2 and peroxisome proliferator-activated receptor-γ coactivator-1α suggest the involvement of respiratory factors in the agonist-linked changes in mitochondrial respiratory capacities and H_2O_2 production.

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Introduction

Triiodothyronine (T_3) is involved in a variety of physiological processes including energy expenditure regulation. It, indeed, increases basal metabolic rate as a result of the stimulation of numerous metabolic pathways (Silva 1995). Because of its metabolic effects, an excess of thyroid hormone might be beneficial in some disorders such as obesity (Krotkiewski 2002) and dyslipidemia (Hansson *et al.* 1983). However, thyroid hormone also elicits unwanted side effects, including cardiac dysfunction (Klein & Ojamaa 2001), bone mass loss (Wagasugi *et al.* 1994), and oxidative stress development in target tissues (Venditti & Di Meo 2006).

Most of the T_3 effects are due to the hormone binding to two subtypes of nuclear receptors (TRs), TRα and TRβ, which are encoded by two different genes and can selectively mediate some specific thyroid hormone responses (Forrest & Vennström 2000). Studies on mutant mice have shown that TRα mediates T_3 effects on heart rate and modulates body temperature, whereas TRβ mediates the cholesterol-lowering and TSH-suppression effects of T_3 (Forrest & Vennström 2000). Recently, the availability of the TRβ-selective agonist, 3,5-dimethhyl-4-(4′-hydroxy-3′-isopropylbenzyl)phenoxy acetic acid (GC-1), has allowed to confirm the above results. Indeed, GC-1 has been shown to lower both serum cholesterol and triglycerides, in measure equal to or greater than T_3, without significant stimulation of the heart rate (Trost *et al.* 2000), and elicit body weight (BW) loss, resulting from a modest increase in metabolic rate (Grover *et al.* 2004). The potential use of GC-1 for obesity and hypercholesterolemia treatment has been highlighted by the finding that it induces other desirable effects of thyroid hormone. Indeed, GC-1 treatment prevents fat mass accumulation, does not elicit deleterious effects on skeletal muscle (Villicev *et al.* 2007), and does not result in bone loss typical of T_3-induced thyrotoxicosis (Freitas *et al.* 2003). More recently, it has been reported that GC-1 treatment of hypothyroid rats produces increases in tissue oxidative damage smaller than those elicited by T_3 treatment (Venditti *et al.* 2009*a*), thus showing that GC-1 limits another detrimental effect of T_3.

To date, information on mechanisms underlying the relationship between TRβ activation and tissue oxidative metabolism is lacking. Because aerobic metabolism in eukaryotic cells is largely dependent on mitochondrial

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oxidative phosphorylation, and mitochondria also represent the main source of reactive oxygen species (ROS), the above-mentioned mechanisms can be understood by studying the effects of TRβ activation on mitochondrial population characteristics. Thus, in the present study, we compared the effects of T_3 and GC-1 administration to hypothyroid rats on respiratory capacities and ROS generation in rat liver mitochondria. The metabolic responses were related to changes in the expression levels of nuclear respiratory factor-1 and -2 (NRF-1 and -2) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1 or PPARGC1A), which play a role in the mitochondrial biogenesis (Scarpulla 2002), and they are regulated by thyroid hormone (Weitzel *et al.* 2001, Venditti *et al.* 2009*b*).

Materials and Methods

Materials

All chemicals used (Sigma Chimica) were of the highest available grades. GC-1 was synthesized as described by Chiellini *et al.* (1998). Response to oxidative stress was determined using reagents and instrumentation of the commercially available Amerlite system (Ortho-Clinical Diagnostics, Milano, Italy).

Animals

The experiments were carried out on 70-day-old male Wistar rats, supplied by Nossan (Correzzana, Italy) at day 45 of age. From day 49, animals were randomly assigned to one of four groups: euthyroid control group (C), hypothyroid rats (H), hypothyroid rats treated with T_3 (H + T_3), and hypothyroid rats treated with GC-1 (H + GC-1). In H rats, both thyroid and deiodinase activities were chronically inhibited by i.p. administration of 6 propyl-2-thiouracil (PTU) (1 mg/100 g BW, once per day for 3 weeks) and iopanoic acid (IOP; 6 mg/100 g BW, at days 10, 13, 16, 19, and 21 after the first PTU injection) respectively. The other rats, which underwent the same treatment of H rats, were also i.p. administered with equimolar doses of T_3 or GC-1 (15·36 nmol/100 g BW, once a day for 10 days before killing). All rats were kept under the same environmental conditions and were provided with water *ad libitum* and commercial rat chow diet (Nossan).

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

The animals, anesthetized with Ethrane (Abbot), were killed by decapitation, and livers were rapidly excised and were 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). Liver fragments were cut and used for western blotting. Subsequently, liver was freed from connective tissue, weighed, finely minced, and washed with HM. Tissue fragments were gently homogenized (20% w/v) in HM using a glass Potter-Elvehjem homogenizer set at a standard velocity (500 r.p.m.) for 1 min. Aliquots of homogenates were used for cytochrome oxidase determination 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 were centrifuged at the same sedimentation velocity. Mitochondrial preparations were washed in this manner twice before final suspension in WB. The protein concentrations of the mitochondrial fractions were measured by the biuret method (Gornall *et al.* 1949).

Analytical procedures

For determination of liver cytochrome *c* oxidase (COX) activity, homogenate suspensions were diluted with modified Chappel–Perry medium so that the preparations contained 100 mg of tissue per ml. COX activity was determined polarographically at 30 °C using a Gilson glass respirometer equipped with a Clark oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, OH, USA) by the procedure of Barré *et al.* (1987).

Mitochondrial oxygen consumption was monitored at 30 °C by a Gilson respirometer in 1·6 ml of incubation medium (145 mM KCl, 30 mM HEPES, 5 mM KH_2PO_4, 3 mM $MgCl_2$, and 0·1 mM EGTA, pH 7·4) with 0·25 mg of 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 activities of the mitochondrial respiratory chain complexes were also measured. The activities of the first three complexes of the electron transport system were measured by a Beckman (Fullerton, CA, USA) spectrophotometer (model DU 640) using the method of Ragan *et al.* (1987). Complex IV (COX) activity was determined polarographically, as described previously (Barrè *et al.* 1987), on mitochondrial suspensions diluted with modified Chappel–Perry medium so that the preparations contained 0·2 mg of mitochondrial proteins per ml.

The extent of the peroxidation processes was determined by measuring the level of lipid hydroperoxides (HPs) according to Heath & Tappel (1976).

Protein oxidation was assayed by the reaction of 2,4-dinitrophenylhydrazine with protein carbonyls according to procedure of Schild *et al.* (1997) for mitochondria.

Table 1 Effect of treatment with triiodothyronine (T_3) or GC-1 of hypothyroid rats on oxygen consumption by rat liver mitochondria. Values are means ± S.E.M. of eight experiments. One rat was used for each experiment. Oxygen consumption rates are expressed in nmol O/min per mg protein

	Group			
	C	H	H+T_3	H+GC-1
Substrate				
Succinate				
State 4	42·6±1·1	38·2±1·0	105·5±3·9[a,b]	50·3±0·4[a,b,c]
State 3	212·4±5·4	187·7±9·0[a]	515·5±4·0[a,b]	239·4±9·5[a,b,c]
RCR	4·7±0·5	4·3±0·2	4·8±0·4	4·6±0·2
Pyruvate/malate				
State 4	15·5±1·0	13·1±0·4	29·9±1·0[a,b]	15·2±0·2[c]
State 3	35·4±0·9	27·5±0·6[a]	60·8±0·7[a,b]	40·7±0·9[a,b,c]
RCR	2·4±0·2	2·1±0·2	2·0±0·2	2·6±0·2

C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H-GC-1, hypothyroid GC-1-treated rats. The level of significance was chosen as $P<0·05$.
[a]Significant versus C rats.
[b]Significant versus H rats.
[c]Significant versus H+T_3 rats.

Ubiquinols ($CoQH_2$) from 2 mg/ml mitochondrial suspensions were oxidized to ubiquinones (CoQs) with 0·5 ml of 2% $FeCl_3$ and 2·0 ml of ethanol. The total content of CoQs ($CoQH_2$+CoQ) was then determined as described by Lang *et al.* (1986). Vitamin E (Vit 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 H_2O_2 release was measured at 30 °C following the increase in fluorescence (excitation at 320 nm and emission at 400 nm) due to oxidation of *p*-hydroxyphenylacetate (PHPA) by H_2O_2 in the presence of HRP (Hyslop & Sklar 1984) in a computer-controlled Jasko 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 Rot, 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 KH_2PO_4, 3 mM $MgCl_2$ and 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: Rot, which blocks the transfer of electrons from complex I to CoQ (Palmer *et al.* 1968), and antimycin A (AA), which interrupts electron transfer within the CoQ-cytochrome b site of complex III (Turrens *et al.* 1985). Inhibitor concentrations (5 µM Rot and 10 µM AA) which do not interfere with the detection PHPA–HRP system were used (Venditti *et al.* 2003).

Capacity to remove H_2O_2 was determined by comparing the ability of mitochondrial samples and desferrioxamine solutions to reduce the fluorescence increase linked to the PHPA oxidation, induced by the H_2O_2 produced in a system containing glucose and glucose oxidase (Venditti *et al.* 2001). Thus, the capacity of mitochondrial samples to remove H_2O_2 was expressed as equivalent desferrioxamine concentration.

Response to oxidative challenge was determined as described previously (Venditti *et al.* 1999*a*). Shortly, several dilutions of the mitochondrial suspensions in the range of protein concentrations from 20 to 0·005 mg/ml were prepared in 15 mM Tris (pH 8·5). The assays were performed in microtiter plates. Enhanced chemiluminescence reactions were

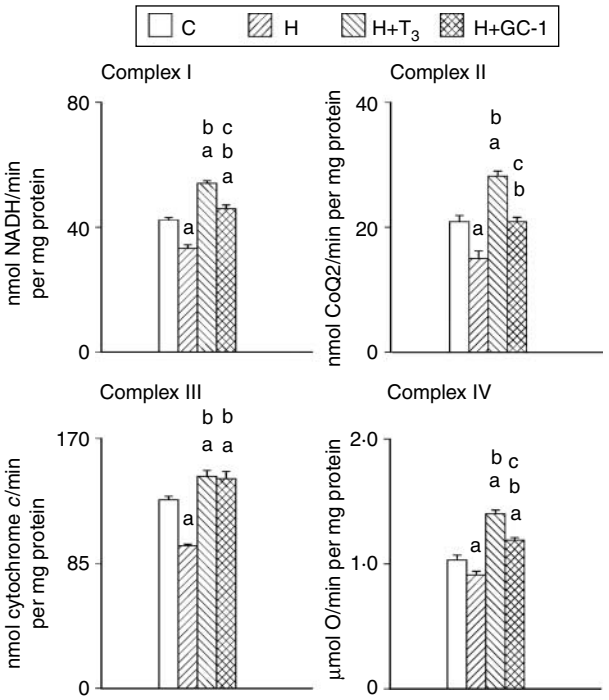


Figure 1 Activities of mitochondrial respiratory complexes from euthyroid (C), hypothyroid (H), hypothyroid T_3-treated (H+T_3), and hypothyroid GC-1-treated (H+GC-1) rats. Values are means ± S.E.M. of eight experiments. One rat was used for each experiment. [a]Significant versus C rats; [b]significant versus H rats; [c]significant versus H+T_3 rats. The level of significance was chosen as $P<0·05$.

Table 2 Effect of treatment with triiodothyronine (T_3) or GC-1 of hypothyroid rats on H_2O_2 release by succinate and pyruvate/malate-supplemented mitochondria from rat liver. Values are means±S.E.M. of eight experiments. One rat was used for each experiment. H_2O_2 release rates are expressed in pmol/min per mg protein

	Group			
	C	H	H+T_3	H+GC-1
Substrate and additions				
Succinate	102·0±0·4	92·9±1·3[a]	123·6±1·3[a,b]	105·0±1·0[b,c]
Succinate+ADP	62·5±0·7	58·7±0·7[a]	77·3±0·8[a,b]	63·6±0·8[b,c]
Pyruvate/malate	238·7±0·9	183·9±2·4[a]	260·1±1·5[a,b]	242·9±0·5[b,c]
Pyruvate/malate+ADP	171·4±0·8	102·4±1·3[a]	190·2±1·4[a,b]	174·3±1·2[b,c]

C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H-GC-1, hypothyroid GC-1-treated rats. The level of significance was chosen as $P<0·05$.
[a]Significant versus C rats.
[b]Significant versus H rats.
[c]Significant versus H+T_3 rats.

initiated by addition of 250 µl of the reaction mixture to 25 µl of the samples. The reaction mixture was obtained by mixing 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 were 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 Rot, 0·3 mg mitochondrial protein/ml reaction mixture, 100 µM Ca^{2+}, and 50 mM EGTA or 100 µM cyclosporin A (CSA) where indicated.

Mitochondrial membrane potential ($\Delta\Psi$) was estimated through fluorescence changes of safranin (8 µM) recorded on the Jasko fluorometer (excitation wavelength 495 nm and 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 Rot, 0·3 mg mitochondrial protein/ml reaction mixture, and 100 µM Ca^{2+}. $\Delta\Psi$ 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 Rot, 0·38 EDTA, 8 µM safranin, 38·5 ng/ml valinomycin, and KCl at concentrations from 0 to 0·96 mM.

For determination of protein expression, liver fragments were gently homogenized (1:10, w/v) in 500 mM NaCl, 0·5% Nonidet P-40, 6 mM EDTA, 6 mM EGTA, 1 mM dithiothreitol, 40 mM Tris–HCl, pH 8·0, in the presence of antiprotease mixture including 40 µg/ml phenylmethylsulphonyl fluoride, 5 µg/ml leupeptin, 5 g/ml aprotinin, 7 g/ml pepstatin. Homogenates were centrifuged at 1000 ***g*** for 10 min at 4 °C, and samples were prepared by diluting 10 µl of the resulting supernatants, containing 1·5 mg/ml protein, with 5 µl of 3% SDS, 30% glycerol, 15% β-mercaptoethanol, 0·1% bromophenol blue, 0·187 M Tris base, pH 6·8, and they were

Table 3 Effect of inhibitors on H_2O_2 release by liver mitochondrial fractions from hypothyroid, hypothyroid triiodothyronine (T_3)-treated, and hypothyroid GC-1-treated rats. Values are means±S.E.M. of eight experiments. One rat was used for each experiment. H_2O_2 release rates are expressed in pmol/min per mg protein

	Group			
	C	H	H+T_3	H+GC-1
Substrate and additions				
Succinate (Succ)	152·3±0·8	137·0±1·0[a]	187·0±2·0[a,b]	170·9±0·5[a,b,c]
Succ+Rot	102·6±1·0	92·7±1·4[a,d]	123·2±1·4[a,b,d]	104·5±1·5[b,c]
Succ+Rot+AA	832·9±7·2	771·6±30·3[a,d]	964·7±19·6[a,b,d]	907·6±2·9[a,b,c,d]
Pyruvate/malate (Pyr/mal)	238·9±0·8	184·2±1·7[a]	260·6±1·9[a,b]	242·0±1·5[b,c]
Pyr/mal+AA	939·0±6·7	920·2±6·4[a,d]	1082·8±3·8[a,b,d]	978·2±3·4[a,b,c,d]
Pyruvate/malate	238·9±1·0	183·8±1·0[a]	259·6±1·7[a,b]	242·1±0·9[b,c]
Pyr/mal+Rot	249·5±6·0	198·0±5·4[a,d]	274·3±1·5[a,b,d]	242·9±1·4[b,c]

C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H-GC-1, hypothyroid GC-1-treated rats. The level of significance was chosen as $P<0·05$.
[a]Significant versus C rats.
[b]Significant versus H rats.
[c]Significant versus H+T_3 rats.
[d]Significant effect of the last inhibitor added versus mitochondria under same conditions without that inhibitor.

boiled for 5 min before loading on 6% stacking and 12% running SDS-PAGE gels. Gel was run in the mini protean equipment (Bio-Rad) for about 1 h at constant voltage (25 V). Separated hepatic proteins were transferred to nitrocellulose membranes by electroblotting. Membranes were incubated with a 1:1000 dilution of antibodies to PGC-1, NRF-1, and NRF-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 154 mM NaCl, 10 mM Tris–HCl, pH 8·0, 2·5% nonfat dry milk, 10% Tween 20. Rabbit polyclonal antibodies raised against amino acids 1–300 mapping near the N-terminus of PGC-1, 204–503 mapping at the C-terminus of NRF-1, and 1–180 of NRF-2α were used. Antibody binding was detected by carrying out secondary antibody incubations using peroxidase-conjugated anti first IgG antibodies (Santa Cruz Biotechnology) diluted 1:4000. Secondary antibody was detected using the ECL system according to the manufacturer's recommendation (Santa Cruz Biotechnology). The blots were stripped by treating them for 10 min with 0·2 M NaOH followed by 5-min wash with H_2O and two 5-min washes with 154 mM NaCl, 10 mM Tris–HCl, pH 8·0, 0·1% Tween 20. The blots were again blocked for 30 min with 154 mM NaCl, 10 mM Tris–HCl, pH 8·0, 2·5% nonfat dry milk, and 10% Tween 20; washed as described above; and incubated for 2 h with a 1:2000 dilution of anti-actin antibody (Santa Cruz Biotechnology) in blocking solution. Remaining procedures, as described for other antibodies, were followed. The actin was used for loading standardization. To compare protein expression levels among groups, a standard euthyroid sample was run on each gel, and all group values were then compared with the euthyroid sample that was assigned a value of 1.

Statistical analysis

The data, obtained in eight different experiments and expressed as means ± S.E.M., were analyzed with a one-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. Probability values (P) $<0{\cdot}05$ were considered significant. The results of the experiments are presented as sample curves.

Results

O_2 consumption

The results dealing with effects of T_3 and GC-1 treatments on rates of mitochondrial oxygen consumption are reported in Table 1. In the presence of succinate, such rates were lower in hypothyroid group than in euthyroid group during state 3, but not during state 4. Conversely, the rates were increased by T_3 and GC-1 treatments during both respiration states, with the highest rates reached in H + T_3 group. The values of respiratory control ratio (RCR) were not significantly modified by treatments. In the presence of pyruvate/malate, oxygen consumption rates, which were again lower in hypothyroid group than in euthyroid group during state 4, were increased by T_3 treatment in both respiratory states resulting higher values than control values. Conversely, GC-1 treatment increased only pyruvate/malate-supported state 3 respiration. RCR values were not modified by treatments.

Respiratory complex activities

As shown in Fig. 1, the activity of complex I was decreased by PTU + IOP and increased by T_3, and to a lesser extent by GC-1 so that in H + GC-1 group, it was not significantly

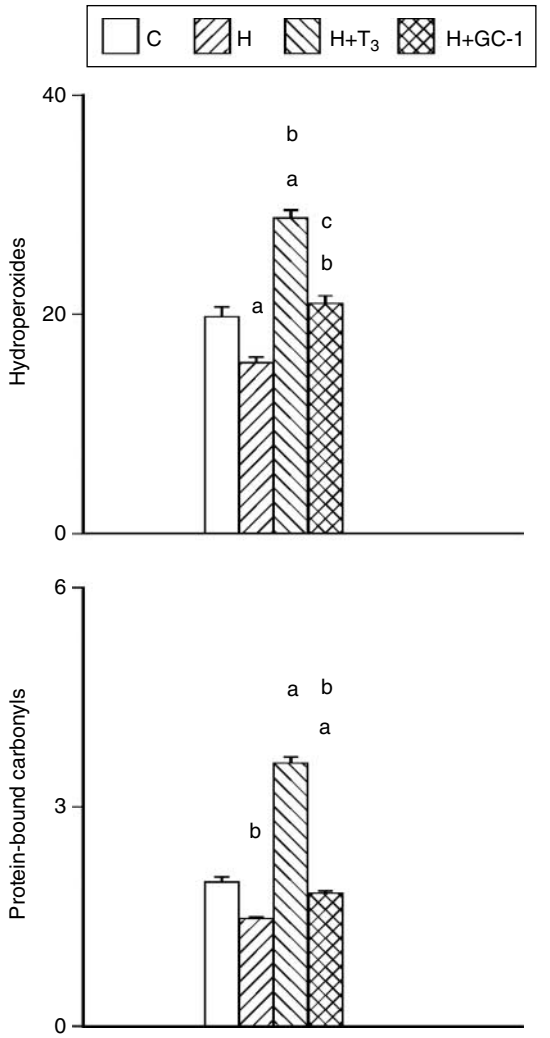


Figure 2 Oxidative damage of rat liver mitochondria from euthyroid (C), hypothyroid (H), hypothyroid T_3-treated (H + T_3), and hypothyroid GC-1-treated (H + GC-1) rats. Hydroperoxides (HPs) (above) are expressed in pmol NADPH/min per mg protein. Protein-bound carbonyls (CO) (below) are expressed in nmol/mg protein. Values are means ± S.E.M. of eight experiments. One rat was used for each experiment. [a]Significant versus C rats; [b]significant versus H rats; [c]significant versus H + T_3 rats. The level of significance was chosen as $P<0{\cdot}05$.

Table 4 Effect of triiodothyronine (T_3) or GC-1 treatment of hypothyroid rats on mitochondrial antioxidants. Values are means±S.E.M. of eight experiments. One rat was used for each experiment

	Group			
	C	H	H+T_3	H+GC-1
Parameter				
Vit E	0·34±0·01	0·26±0·01[a]	0·46±0·01[a,b]	0·39±0·01[a,b,c]
CoQ9	1·57±0·04	1·35±0·06[a]	2·04±0·08[a,b]	1·89±0·05[a,b,c]
CoQ10	0·21±0·01	0·14±0·01[a]	0·24±0·01[b]	0·20±0·02[b]
GSH	21·5±1·5	26·2±0·6	22·1±2·3	21·2±0·5

Vitamin E (Vit E), coenzyme Q9 (CoQ9), coenzyme Q10 (CoQ10), and reduced glutathione (GSH) levels are expressed in nmol/mg protein. C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H-GC-1, hypothyroid GC-1-treated rats. The level of significance was chosen as $P<0·05$.
[a]Significant versus C rats.
[b]Significant versus H rats.
[c]Significant versus H+T_3 rats.

different from controls. Similar results were found for complex II. Conversely, the activity of complex III was similarly increased by agonists so that in both H+T_3 and H+GC-1 groups, it was higher than in controls. This result was also found for the activity of complex IV even though it was increased to a lesser extent by GC-1 treatment.

Homogenate COX activity and mitochondrial protein content

COX activities were lower in hypothyroid homogenates than in euthyroid homogenates (65·6±1·3 and 73·7±0·7 respectively). Treatment with agonists of hypothyroid rats was associated with increases in COX activities, which were lower in GC-1-treated animals than in T_3-treated animals (90·3±1·5 and 97·3±1·8 respectively).

The ratio between the cytochrome oxidase activities in homogenates and mitochondria supplied a rough estimate of hepatic contents of mitochondrial proteins. They were 71·5±4·9, 72·1±2·5, 69·5±2·0, and 74·6±2·4 for C, H, H+T_3, and H+GC-1 groups respectively. No significant difference was found among such values.

Mitochondrial H_2O_2 release and capacity to remove H_2O_2

The results concerning the rates of H_2O_2 mitochondrial release are reported in Table 2. They show that, during both respiratory states and in the presence of both succinate and pyruvate/malate, such rates were decreased by PTU+IOP and increased by agonist treatment. The effect of GC-1 was lower so that rates of H_2O_2 release were not significantly different in C and H+GC-1 groups.

It is known that H_2O_2 produced within mitochondria is partially removed by H_2O_2 metabolizing enzymes and hemoproteins (Venditti *et al.* 2001), so that the determination of the rates of H_2O_2 release does not allow us to deduce anything about the rate of its production. However, we measured the capacities of mitochondria to remove H_2O_2 they produce, and found that they were 3·27±0·06, 2·37±0·10, 4·27±0·12, and 3·48±0·10 for C, H, H+T_3, and H+GC-1 groups respectively. Because, as the rates of H_2O_2 release, the capacities of H_2O_2 removal reached the highest and lowest values in T_3-treated and hypothyroid rats respectively, whereas they were not significantly different in euthyroid and GC-1-treated rats, the differences among the groups in the H_2O_2 release rates reflected those in the peroxide generation.

Effects of inhibitors on H_2O_2 release

As shown in Table 3, in the absence of Rot, the rates of mitochondrial succinate-supported H_2O_2 release were decreased by PTU+IOP and increased by agonist treatment above the control value. The highest rate was reached following T_3 treatment. Rot addition decreased the rates of H_2O_2 release in all groups, stopping what was occurring at

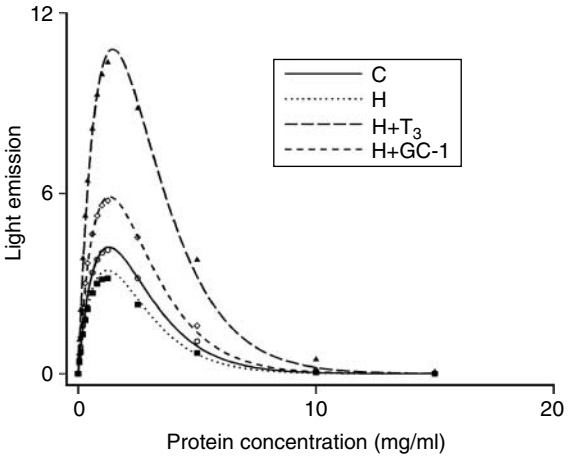


Figure 3 Response to oxidative challenge *in vitro* of mitochondrial preparations from rat liver. The susceptibility to stress was evaluated by determining the variations with concentrations of light emission from a luminescent reaction. Emission values are given as percentage of an arbitrary standard (44 ng/ml peroxidase). The curves are computed from experimental data using the equation: $E=a\times C/\exp(b\times C)$. Preparations from euthyroid (C), hypothyroid (H), hypothyroid T_3-treated (H+T_3), and hypothyroid GC-1-treated (H+GC-1) rats. One representative experiment of eight similar experiments is shown.

complex I, due to the reverse electron flow from coenzyme Q (Ernster & Lee 1967). However, the lowest and highest rates were again found in H and H+T_3 groups, whereas the rates in C and H+GC-1 groups were not significantly different. The further addition of antimycin increased H_2O_2 release rates in all groups and made the significance of the differences among groups similar to that found in the presence of the succinate alone. Addition of antimycin or Rot to pyruvate/malate-supported mitochondria increased H_2O_2 release rates in all groups. However, whereas the significance of differences between groups was not modified, in the presence of Rot, the rates of H_2O_2 release were higher in GC-1-treated rats than in control rats in the presence of antimycin.

Oxidative damage

HP and protein-bound carbonyls levels were used as indices of oxidative damage to lipids and proteins respectively, in rat liver mitochondria. As shown in Fig. 2, lipid and protein damage was decreased by PTU+IOP and increased by agonist treatment. The GC-1-elicited increase was lower so that the oxidative damage was not significantly different in control and H+GC-1 groups.

Antioxidants

The results dealing with effects of T_3 or GC-1 administration to hypothyroid rats on mitochondrial antioxidants are reported in Table 4. They show that Vit E content was increased by T_3 treatment and to a lesser extent by GC-1 treatment. Similar changes of CoQ9 and CoQ10 content were induced by treatments, which did not modify mitochondrial GSH content.

Response to oxidative challenge

The luminescence response to changes of concentration of the homogenates (Fig. 3) was described by the equation ($E = a \times C / \exp(b \times C)$). The parameters *a* and *b*, which determine the light emission maximum ($E_{max} = a/e \times b$), are dependent on the concentration of substances that are able to induce (iron or cuprum ligands) and inhibit (antioxidants) respectively the H_2O_2-induced luminescent reaction. Examination of the curves in Fig. 3 and data reported in Table 5 show that the emission maximum was not significantly different in hypothyroid and euthyroid rats and was increased by T_3 and to a lesser extent by GC-1 treatment. The values of the parameters *a* and *b* indicate that the increase induced by T_3 treatment in emission peak is due to lower *b* values and higher *a* values, while the increase induced by GC-1 was only to impute to higher *a* value.

Mitochondrial swelling

As shown by the absorbance changes in Fig. 4, the extent of the swelling was higher in Ca^{2+}-loaded mitochondria by T_3-treated rats. Mitochondrial swelling was drastically reduced by CSA or EGTA (unreported results), pointing to the role played by permeability transition pore (PTP). Ca^{2+}-induced swelling was preceded by a rapid decrease in membrane potential ($\Delta\Psi$), which was higher in preparations from T_3-treated rats. No difference in Ca^{2+}-induced swelling and fall in membrane potential of mitochondrial preparations among the other groups.

Protein expression

Western blot experiments were conducted using identical amounts of total protein extract from livers of hypothyroid and treated rats that were loaded onto an SDS-PAGE, and were blotted according to standard protocols. The high specificity of the antibodies and molecular weight markers allowed us to easily identify the PGC-1, NRF-1, and NRF-2 proteins.

The results reported in Fig. 5 show that PGC-1, NRF-1, and NRF-2 levels were decreased, increased, and unmodified respectively, following PTU+IOP treatment. Furthermore, all levels were increased by agonist treatment. However, PGC-1 level was increased by T_3 and in greater measure by GC-1 treatment without reaching the value of euthyroid controls, whereas NRF-1 and -2 levels were increased by GC-1 and in greater measure by T_3 treatment exceeding the euthyroid value with both agonists.

Table 5 Effect of treatment with triiodothyronine (T_3) or GC-1 of hypothyroid rats on parameters characterizing the response to oxidative stress of rat liver mitochondria. Values are means ± S.E.M. of eight experiments. One rat was used for each experiment. For explanation of symbols see text

	Group			
	C	H	H+T_3	H+GC-1
Parameters				
a	8·7±0·21	7·6±0·2	19·9±1·1[a,b]	12·3±1·0[a,b,c]
b	0·82±0·04	0·87±0·04	0·70±0·03[b]	0·80±0·05
E_{max}	4·1±0·2	3·2±0·2	10·4±0·8[a,b]	5·6±0·4[a,b,c]

C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H-GC-1, hypothyroid GC-1-treated rats. The level of significance was chosen as $P<0·05$.
[a]Significant versus C rats.
[b]Significant versus H rats.
[c]Significant versus H+T_3 rats.

Discussion

It is well known that the electron transfer through the respiratory chain can produce either energy necessary for cell viability, by tetravalent reduction of O_2 to H_2O, or oxidative damage and sometime cell death, by univalent reduction of O_2 to superoxide radical.

Changes in tissue O_2 consumption and ROS production involve changes in tissue content of mitochondrial proteins and/or in rate of electron flow through inner membrane of each mitochondrion due to modified amount of respiratory chain proteins.

Previously, we reported that treatment of hypothyroid rats with GC-1 produces increases in liver respiration smaller than treatment with equimolar doses of T_3 (Venditti *et al.* 2009*a*). The present results, concerning the liver content of mitochondrial proteins, show that the increases in tissue respiration, induced by both treatments, were not due to mitochondrial proliferation, thus suggesting differential agonist effects on mitochondrial population characteristics. In fact, mitochondrial O_2 consumption and activities of the multi-subunit complexes, composing mitochondrial electron transport system, demonstrate that the differential T_3 and GC-1 modulation of liver respiration is due to different agonist effects on rates of electron flow through the mitochondrial inner membrane. The T_3 effect is in agreement with the early observations that hyperthyroidism increases respiratory chain protein amount and inner surface area (Jacovcic *et al.* 1978) of mitochondria without changing their number (Goglia *et al.* 1989) and total protein mass (Venditti *et al.* 2006). It is conceivable that the lower effect of GC-1 on O_2 consumption is due to smaller increase in mitochondrial content of respiratory chain components. This idea is supported by our observation that the changes in state 3 O_2 consumption, induced by T_3 and GC-1, are in agreement with the respective increases in the activities of complexes I, II, and IV. On the other hand, the complex III activity was similarly increased by T_3 and GC-1 showing that the components of the mitochondrial respiratory chain do not respond as a unit to GC-1 stimulation.

It is not clear what are the factors responsible for different increases in liver content of respiratory chain components elicited by T_3 and GC-1. Owing to the similar ratio between liver and plasma concentrations that GC-1 and T_3 exhibit (Chiellini *et al.* 1998), differences in tissue uptake of the agonists should seem unlikely. However, other effects including different half life, altered nuclear import, impaired inactivation by deiodinases could contribute to liver agonist concentrations. Differences in TRβ receptors affinity do not seem to be involved because agonists also exhibit similar binding affinity for TRβ receptors (Chiellini *et al.* 1998). Conversely, the observation that most of the thyroid hormone actions are mediated by both receptor isoforms (Brent 2000) suggests that the expression of some components of respiratory chain is also regulated through the agonist binding to TRα receptors, even though TRα is the minority isoform (20%) in the liver (Schwartz *et al.* 1992). This is an intriguing hypothesis, but it has to be supported by additional experiments demonstrating an effect of selective TRα agonist on respiratory chain components.

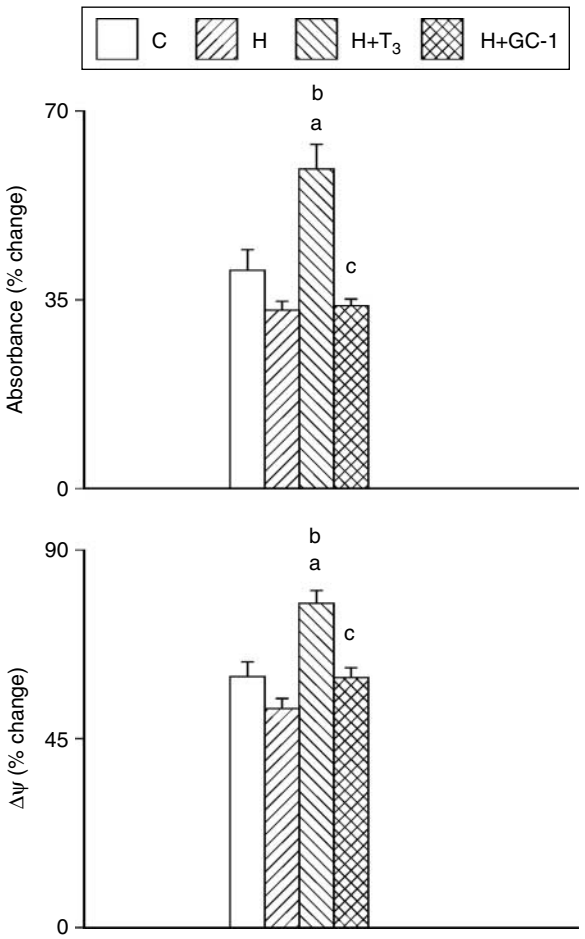
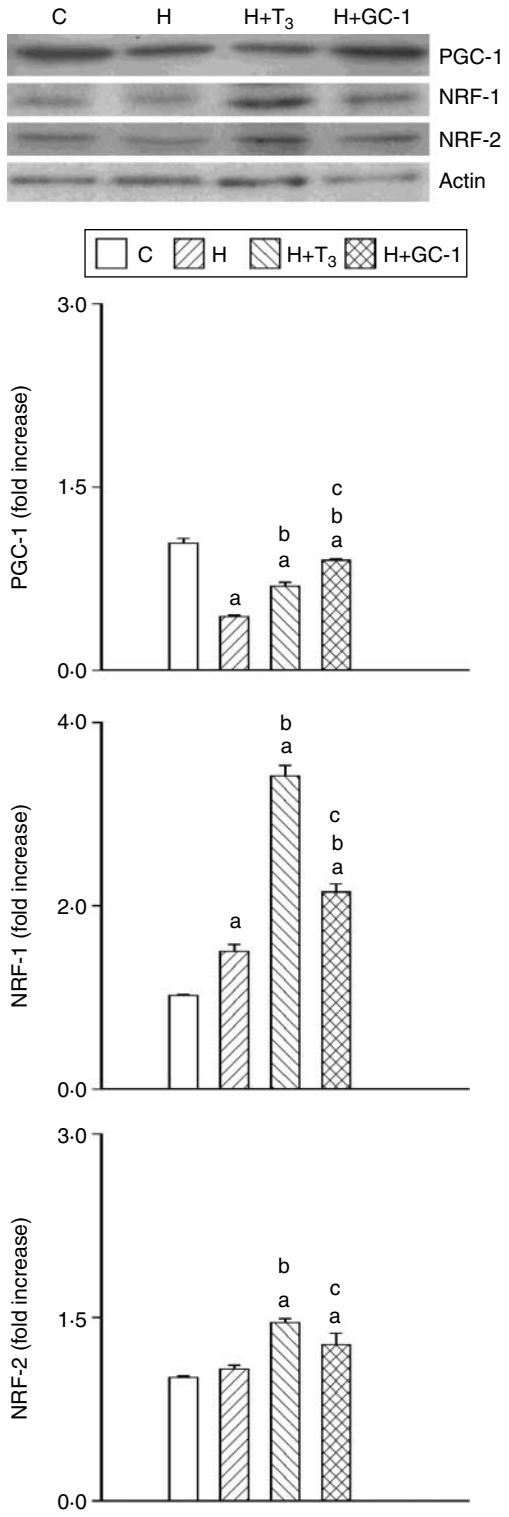


Figure 4 Ca^{2+}-induced swelling (above) and membrane potential dissipation (below) of mitochondrial preparations from euthyroid (C), hypothyroid (H), hypothyroid T_3-treated (H+T_3), and hypothyroid GC-1-treated (H+GC-1) rats. Swelling of mitochondrial preparations (0·3 mg/ml) was monitored as decrease of the absorbance at 540 nm in a standard medium containing 100 μM Ca^{2+} and was expressed as percent of the initial value before Ca^{2+} addition. Membrane potential (ΔΨ) of mitochondrial preparations (0·3 mg/ml) was estimated through fluorescence changes of safranin (8 μM) (excitation wavelength 495 nm and emission wavelength 586 nm) in a standard medium containing 100 μM Ca^{2+}. ΔΨ was calculated using a suitable calibration curve. The decrease of ΔΨ for each preparation was expressed as percent of the initial value before Ca^{2+} addition. The initial values of absorbance of mitochondrial preparations were 1·00±0·07, 0·96±0·06, and 0·98±0·05 for H, H+T_3, and H+GC-1 rats respectively. Initial values of ΔΨ were 191·2±18·9 mV, 182·9 ±12·7 mV, 185·6±12·3 mV, for H, H+T_3, and H+GC-1 rats respectively. Values are means±S.E.M. of eight experiments. One rat was used for each experiment. [a]Significant versus C preparations; [b]significant versus H preparations; [c]significant versus H+T_3 preparations. The level of significance was chosen as $P<0·05$.



Further information on the effects of the agonists on respiratory chain component concentration was obtained by the combined measurements of mitochondrial H_2O_2 release and removal. Indeed, the finding that the increases in ROS production were smaller in H+GC-1 than in H+T_3 preparations suggests that T_3 and GC-1 treatments of hypothyroid rats induce differential increases in mitochondrial concentration of autoxidizable electron carriers. Such an idea was confirmed by the changes in H_2O_2 release rates induced by inhibitors of the respiratory chain, which render the concentration of the autoxidizable carriers the only factor affecting ROS production rate.

Mitochondria are at the same time the main source and main target of ROS, which lead to oxidative damage of their components. Such a damage has to increase in conditions in which ROS production increases. Thus, our results show an enhancement in the oxidatively damaged mitochondrial lipids and proteins after T_3 and GC-1 treatments of hypothyroid rats. Furthermore, oxidative damage of H+GC-1 mitochondria did not exceed that of euthyroid mitochondria consistently with their similar H_2O_2 production.

Determination of the content of some free radical scavengers was not able to show a possible relationship between mitochondrial antioxidant protection and oxidative damage in the agonist-treated rats. In fact, we found that both treatments differently modified liposoluble and hydrosoluble antioxidant concentration. Mitochondrial Vit E and coenzyme Q levels increased in T_3-and GC-1-treated rats according to what previously found in hepatic tissue (Venditti *et al.* 2009*a*), whereas GSH levels were not modified by treatments although the cytosolic levels decreased (Venditti *et al.* 2009*a*). This result is consistent with the observation that, during oxidative stress-induced tissue GSH depletion, liver mitochondria conserve GSH level (Venditti *et al.* 1999*b*) by transport systems located on the inner membrane (Zhong *et al.* 2008).

Besides antioxidant content, other factors, such as the levels of polyunsaturated fatty acids and cytochromes in membrane could affect mitochondrial response to oxidants. Therefore, we tested susceptibility to oxidative stress of mitochondrial preparations challenging them with hydrogen peroxide or Ca^{2+} load *in vitro*.

Figure 5 Liver PGC-1, NRF-1, and NRF-2 protein expressions evaluated via western blotting. Liver total proteins from euthyroid (C), hypothyroid (H), hypothyroid and T_3-treated (H+HT_3), and hypothyroid and GC-1-treated (H+GC-1) rats were isolated and analyzed. Representative blots of PGC-1, NRF-1, NRF-2 and actin protein expressions are shown (above). Analysis was performed as described in Materials and Methods. Bar graphs (below) correspond to the respective densitometric quantification. Actin was used for loading standardization. Ratios of band intensities to the β-actin band intensities were compared with a standard euthyroid sample that was assigned a value of 1. Values are means±S.E.M. of three independent experiments. One rat was used for each experiment. [a]Significant versus C preparations; [b]significant versus H preparations; [c]significant versus H+T_3 preparations. The level of significance was chosen as $P<0{\cdot}05$.

In the former case, we found that the mitochondrial susceptibility to oxidants was increased by both agonist treatments. The analysis of the curves of light emission showed that the lower susceptibility exhibited by H + GC-1 preparations was attributable to lower content of iron ligands, able to interact with H_2O_2 and produce $^{\cdot}OH$ radicals, and higher content of substances, able to scavenge such ROS.

In the latter case, we found that only Ca^{2+}-loaded mitochondria from T_3-treated rats underwent enhanced swelling, which was prevented by EGTA or cyclosporine A, and therefore, could be attributed to Ca^{2+}-dependent modulation of the gated PTP. The lack of changes in GC-1 preparations can be due to lower production of gating inducers, such as ROS (Crompton *et al.* 1987), which seem to be involved in the oxidation of membrane protein thiols unmasked by matrix Ca^{2+} (Castilho *et al.* 1998). If so, the small increase in peroxide-induced light emission and lack of increase in Ca^{2+}-dependent swelling, shown in mitochondria from GC-1-treated rats, should be a consequence of scant GC-1-induced enhancement in susceptibility of such mitochondria to oxidative challenge.

Taken together, our results seem to indicate that the increases in O_2 consumption, ROS production and susceptibility to oxidants shown in liver mitochondria from H + T_3 and H + GC-1 rats are largely dependent on treatment-linked changes in the mitochondrial content of respiratory chain components.

The expression of respiratory apparatus is controlled by nuclear regulatory proteins including NRF-1, NRF-2, and PGC-1. NRF-1 and NRF-2 are transcriptional factors which should play a role in the transcriptional control of many genes involved in mitochondrial function and biogenesis, whereas PGC-1 is a transcriptional coactivator which should act as a coactivator of NRF-1- and NRF-2-dependent transcription. (Scarpulla 2002).

It has been shown that T_3 triggers processes, such as mitochondrial biogenesis, adaptive thermogenesis, and hepatic gluconeogenesis (Yen 2001), which resemble those regulated by PGC-1, which, in turn, interacts with several nuclear hormone receptors including TRβ (Puigserver *et al.* 1998). It has been reported that short- (Weitzel *et al.* 2003) and long-term (Irrcher *et al.* 2003, Venditti *et al.* 2009*b*) T_3 treatments increase PGC-1 protein levels in rat liver. Scarce information is available on T_3 effect on NRF-1 and -2 expressions. However, recent report has shown that the liver levels of both activators are increased by T_3 treatment of hypothyroid rats (Venditti *et al.* 2009*b*).

Therefore, we measured PGC-1, NRF-1, and NRF-2 protein expression levels, and we found that PTU + IOP treatment decreased PGC-1 levels and increased those of NRF-1 and -2. Conversely, agonist treatment led to increased levels of all proteins, even though PGC-1 levels remained lower than the euthyroid ones. The higher increases induced by T_3 in NRF-1 and -2 levels were consistent with the observation that liver COX activities were higher in T_3-treated rats than in GC-1-treated rats. Because thyroid hormone increases expression of mitochondrial and nuclear encoded subunits of COX in rat liver (Sheehan *et al.* 2004), it is conceivable that NRF-1 and -2 protein expressions is linked to COX protein concentration. On the other hand, our finding that T_3 treatment was not able to restore PGC-1 levels to control value and increased such levels less than GC-1 was not expected on the basis of previous observation that 5 days of T_3 treatment induced increases in PGC-1 protein expression in rat skeletal muscle and liver (Irrcher *et al.* 2003). At the present time, we are not able to suggest a straightforward explanation for these discrepant results. One possibility is that they are due to different protocols used to induce hyperthyroid state in the rats since Irrcher *et al.* (2003) administered daily doses of 40 μg of T_3 per 100 g BW to euthyroid rats. Both the higher dose of T_3 and its administration to euthyroid animals might alter the response to the hormone. It is conceivable that in euthyroid rats, factors, including T_4 and T_3 derivatives, are present at concentrations which can modify the response to T_3. This idea is supported by our previous work (Venditti *et al.* 2009*b*), dealing with hypothyroid rats subjected to different treatments (T_3 or T_4 administration and cold exposure), in which a strong correlation was found between COX activity and level of PGC-1 protein expression. Apart from these considerations, previous results (Irrcher *et al.* 2003), suggesting that PGC-1 activation via posttranslational modifications is more important than protein amount in determining COX content in rat liver, make conceivable that relatively low PGC-1 levels can coexist with the high COX activities found in T_3-treated rats.

In conclusion, our data supply indicates that NRFs are responsible for the different increases, induced by T_3 and GC-1 in respiratory chain components, thus determining not only the respiratory capacities, but also the mitochondrial ROS production and oxidative damage in hepatic tissue.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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