The Journal of Experimental Biology 212, 986-993
Published by The Company of Biologists 2009
doi:10.1242/jeb.021808

T_3 and the thyroid hormone β-receptor agonist GC-1 differentially affect metabolic capacity and oxidative damage in rat tissues

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Accepted 6 January 2009

SUMMARY

We compared the changes in tissue aerobic metabolism and oxidative damage elicited by hypothyroid rat treatment with T_3 and its analog GC-1. Aerobic capacities, evaluated by cytochrome oxidase activities, were increased more by T_3 than by GC-1. Furthermore, the response of the tissues to T_3 was similar, whereas the response to GC-1 was high in liver, low in muscle and scarce in heart. Both treatments induced increases in ADP-stimulated O_2 consumption, which were consistent with those in aerobic capacities. However, unlike T_3, GC-1 differentially affected pyruvate/malate- and succinate-supported respiration, suggesting that respiratory chain components do not respond as a unit to GC-1 stimulation. According to the positive relationship between electron carrier levels and rates of mitochondrial generation of oxidative species, the most extensive damage to lipids and proteins was found in T_3-treated rats. Examination of antioxidant enzyme activities and scavenger levels did not clarify whether oxidative damage extent also depended on different antioxidant system effectiveness. Conversely, the analysis of parameters determining tissue susceptibility to oxidants showed that pro-oxidant capacity was lower in GC-1- than in T_3-treated rats, while antioxidant capacity was similar in treatment groups. Interestingly, both agonists decreased serum cholesterol levels, but only GC-1 restored euthyroid values of heart rate and indices of tissue oxidative damage, indicating that GC-1 is able to lower cholesterolemia, bypassing detrimental effects of T_3.

Key words: thyroid hormone, thyroid hormone agonist, oxidative metabolism, oxidative damage.

INTRODUCTION

It is well known that in mammals the thyroid hormone triiodothyronine (T_3) serves as a general regulator of body metabolism. Indeed, it stimulates basal metabolic rate and the metabolism of carbohydrates, lipids and proteins (Hoch, 1974), and increases cardiac contractile performance (Buccino et al., 1967) and heart rate (Di Meo et al., 1994) in response to the demand for greater tissue perfusion.

Thyroid hormone is used in the treatment of hypothyroidism and some thyroid cancers. Because of its metabolic effects, therapeutic thyroid hormone administration might also be extended to other disorders such as obesity (Krotkiewsky, 2002) and dyslipidaemia (Hansson et al., 1983). However, the therapeutic utilization of T_3 might be accompanied by harmful side effects, particularly cardiac dysfunction (Klein and Ojamaa, 2001). Thus, there is interest in developing analogues of T_3 that elicit desirable but not unwanted effects.

It is theoretically possible to discriminate among the T_3 effects since they are due to the interaction of the hormone with two subtypes of nuclear receptor, TRα and TRβ, which selectively mediate tissue-specific thyroid hormone responses (Forrest and Vennström, 2000). TRα mediates T_3 effects on heart rate and modulates body temperature, whereas TRβ mediates the cholesterol-lowering and thyroid-stimulating hormone (TSH)-suppressant effects of T_3 (Forrest and Vennström, 2000). Therefore, the development of T_3 analogues that bind preferentially to TRβ has been considered a possible strategy to obtain beneficial T_3 effects whilst avoiding the harmful ones. Recently, the availability of 3,5-dimethyl-4-(4′-hydroxy-3′-isopropylbenzyl)phenoxy acetic acid (GC-1), a TRβ-selective agonist, has allowed discrimination amongst the different effects of thyroid hormone. Indeed, GC-1 lowers serum cholesterol and triglycerides in measure equal to or greater than T_3, without significant stimulation of heart rate (Trost et al., 2000). The observed preferred accumulation of GC-1 in the liver *vs* the heart probably also contributed to the marked lipid-lowering effect in the absence of an effect on heart rate (Trost et al., 2000). More recently, in euthyroid monkeys a significant GC-1-induced cholesterol lowering and body mass loss has been observed, resulting from a modest increase in metabolic rate (Grover et al., 2004). A moderate increase in O_2 consumption and a large decrease in plasma cholesterol was also found in cholesterol-fed euthyroid rats (Grover et al., 2004). The above results suggest the possibility that selective agonists, such as GC-1, can be used for the treatment of obesity and hypercholesterolaemia even though further studies are necessary to establish the cellular mechanisms of the metabolic rate regulation. It is well known that the T_3-induced increase in basal metabolic rate is dependent on accelerated O_2 consumption in target tissues, secondary to an increased content of mitochondrial respiratory chain components (Nishiki et al., 1978; Horrum et al., 1985). This metabolic response is accompanied, as a side effect, by an enhanced generation of reactive oxygen species (ROS) leading to oxidative stress development in rodent tissues as well as in hyperthyroid patients, as documented by enhanced levels of indicators of lipid and protein oxidation (reviewed by Venditti

and Di Meo, 2006; Fernández et al., 2006). Because the ability of a substance to induce conditions of oxidative stress represents a contraindication for its therapeutic utilization, the necessity to investigate the relationship between TRβ activation and both aerobic metabolism and oxidative damage of the tissues is apparent. To shed light on this subject, we compared the effects of T_3 and GC-1 treatment of hypothyroid rats on the aerobic metabolism and oxidative damage of liver, heart and skeletal muscle. Accordingly, we determined the cytochrome oxidase (COX) activity, and oxygen consumption in the presence of complex I- and complex II-linked substrates (pyruvate/malate and succinate, respectively). We also determined hydroperoxide and protein-bound carbonyl levels as markers of oxidative damage to lipids and proteins, respectively. Finally, to evaluate the possible impact of the antioxidant defence system on the extent of tissue oxidative damage in T_3- and GC-1-treated rats, we determined: (i) activities of antioxidant enzymes, such as glutathione peroxidase (GPX) and glutathione reductase (GR); (ii) levels of free radical scavengers, such as vitamin E, coenzyme Q (CoQ) and reduced glutathione (GSH); and (iii) the *in vitro tissue* susceptibility to oxidants.

MATERIALS AND METHODS

Materials

All chemicals used (Sigma Chimica, Milano, Italy) were of the highest grade available. GC-1 was synthesized in our laboratory as previously described (Chiellini et al., 1998). The response to oxidative stress was determined using the reagents and instrumentation of the commercially available Amerlite System (Ortho-Clinical Diagnostics, Milano, Italy). Serum levels of free triiodothyronine (FT_3) and thyroxine (FT_4) were determined using a commercially available RIA kit (DiaSorin, Salluggia, Italy). Serum cholesterol levels were measured using a commercial kit available from Pokler Italia (Genova, Italy).

Animals

The experiments were carried out on 70-day-old male Wistar rats (*Rattus norvegicus albius* Berkenkault 1769), supplied by Nossan (Correzzana, Italy). From day 49, animals were randomly assigned to one of four groups: control euthyroid rats (C); hypothyroid rats (H); and hypothyroid rats treated with T_3 (H+T_3) or GC-1 (H+GC-1). In H rats, both thyroid and deiodinase activities were chronically inhibited by intraperitoneal administration of propylthiouracil (PTU; 1 mg 100 g^{-1} body mass, once per day for 3 weeks), together with administration of iopanoic acid (IOP) given 10, 13, 16 and 19 days after the first PTU injection. Some rats that were subject to the same treatment also received daily intraperitoneal injections of 15.36 nmol of T_3 per 100g body mass for 10 days. The above experimental protocols induce hypothyroidism and hyperthyroidism, respectively, in the laboratory rat (Moreno et al., 1997). Moreover, the T_3 dose used to elicit the hyperthyroid state increases resting metabolic rate (Venditti et al., 2004) and induces tissue oxidative stress (Venditti et al., 1997) in rat. To compare the T_3 and GC-1 effects on metabolic and oxidative processes, the other rats were treated with 10 daily equimolar doses of GC-1 (15.36 nmol 100 g^{-1} body mass). We chose hypothyroid rats for the treatment with the agonists to obtain animals whose serum contains only T_3 or GC-1. All rats were kept under the same environmental conditions and were provided with water *ad libitum* and a 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.

Tissue preparation

After a 12 h overnight fast, the resting metabolic rate of the animals was measured using an open circuit indirect calorimetric system (Panlab Gas Analyzer LE 405, Madrid, Spain). The animals were then anaesthetized with Ethrane (Abbot, Aprilia, Italy) and subjected to electrocardiographic recording. Arterial blood samples were collected and later analysed to determine serum levels of FT_3, FT_4 and cholesterol. While still under anaesthesia, the animals were killed by decapitation and tissues (liver, heart and gastrocnemious muscle) were rapidly excised and placed into ice-cold homogenization medium (HM: 220 mmol l^{-1} mannitol, 70 mmol l^{-1} sucrose, 1 mmol l^{-1} EDTA, 0.1% fatty acid-free albumin, 10 mmol l^{-1} Tris, pH 7.4). The heart great vessels and valves were trimmed away and the ventricles and atria were cut open and rinsed free of blood. Muscle and liver were freed from connective tissue. Then the tissues were weighed, finely minced, and washed with HM. Muscular tissue fragments were incubated for 5 min with HM containing 0.1 mg ml^{-1} nagarse and washed. Finally, all tissues 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. Tissue homogenates were used for analytical procedures.

Analytical procedures

Cytochrome *c* oxidase activity was determined polarographically at 30°C using a Gilson glass respirometer equipped with a Clark oxygen electrode (Yellow Springs Instruments, OH, USA) following the procedure of Barré and colleagues (Barré et al., 1987). COX is the final respiratory enzyme, and its *in vitro* activity has been positively correlated with maximal oxygen consumption (Simon and Robin, 1981) so that such activity can be used as a measure of the aerobic metabolic capacity of tissues.

Oxygen consumption was monitored at 30°C with the above Gilson respirometer in 1.6 ml of incubation medium (145 mmol l^{-1} KCl, 30 mmol l^{-1} Hepes, 5 mmol l^{-1} KH_2PO_4, 3 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} EGTA, pH 7.4) with 50 µl of homogenate and succinate (10 mmol l^{-1}), plus 5 µmol l^{-1} rotenone, or pyruvate/malate (10/2.5 mmol l^{-1}) as substrates, in the absence (state 4) and in the presence (state 3) of 500 µmol l^{-1} ADP. Furthermore, the ratio between state 3 and state 4 respiration rates (respiratory control ratio, RCR) was calculated. Basal (state 4) respiration represents a compensatory response to the leak of protons back into the mitochondrial matrix, so that changes in its rate give information on the basal proton conductance of the inner mitochondrial membrane. Conversely, rates of ADP-stimulated (state 3) respiration supply information on the rate of oxidative phosphorylation. The respiration measurements performed in the presence of pyruvate/malate and succinate allowed us to discriminate among effects concerning different segments of the mitochondrial electron transport chain.

The extent of the peroxidative processes was determined by measuring the level of lipid hydroperoxides (HPs) (Heath and Tappel, 1976). Tissue protein oxidation was assayed by the reaction of 2,4-dinitrophenylhydrazine with protein carbonyls (Reznick and Packer, 1994).

GPX activity was assayed at 37°C (Flohé and Günzler, 1984), with H_2O_2 as the substrate. GR activity was measured at 30°C (Carlberg and Mannervik, 1985).

Ubiquinols ($CoQH_2$) from 0.5 ml of 10% homogenate 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 by HPLC (Lang et al., 1986). Vitamin E content was

determined using the HPLC procedure of Lang and colleagues (Lang et al., 1986). GSH concentration was measured in homogenates as described by Griffith (Griffith, 1980).

The response to oxidative challenge was determined as previously described (Venditti et al., 1999). In brief, samples of 10% (w/v) homogenates were obtained by diluting the 20% homogenates with equal volumes of 0.2% Lubrol in 15 mmol l^{-1} Tris, pH 8.5. Several dilutions of the samples up to a tissue concentration of 0.002% were prepared in 15 mmol l^{-1} Tris (pH 8.5). The assays were performed in microtitre 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 solutions containing substrate in excess and signal-generating reagents in buffer at pH 8.6 (Vitros Signal Reagent, Ortho Clinical Diagnostics, High Wycombe, Bucks, UK). The plates were incubated at 37°C for 30 s under continuous shaking and then transferred to a luminescence analyser (Amerlite Analyzer). The emission values were fitted to dose–response curves using the statistical facilities of the Fig.P graphic program (Biosoft, Cambridge, UK).

Data analysis

The data, expressed as means ± s.e.m., were analysed using a one-way variance method (ANOVA). 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)<0.05 were considered significant. In Fig. 2 the results of the experiments are presented as sample curves.

RESULTS

Body and serum parameters, and resting metabolic and heart rates

As shown in Table 1, body mass was lower in the T_3-treated group than in the other groups. In comparison with euthyroid controls, heart mass was lower in hypothyroid and higher in T_3-treated rats, whereas it was not different in GC-1-treated animals. Heart mass/body mass ratios, heart rates and resting metabolic rates were decreased by PTU+IOP treatment and were remarkably increased by T_3 treatment so that their values were higher than in euthyroid controls. The effect of GC-1 was more moderate so that the values of the above parameters did not differ from controls. Cholesterol level was significantly higher in hypothyroid rats and was restored to control values by agonist treatment. As expected, plasma levels of FT_3 drastically decreased in PTU+IOP rats and increased in H+T_3 but not in H+GC-1 rats, whereas FT_4 levels similarly decreased in all treatment groups.

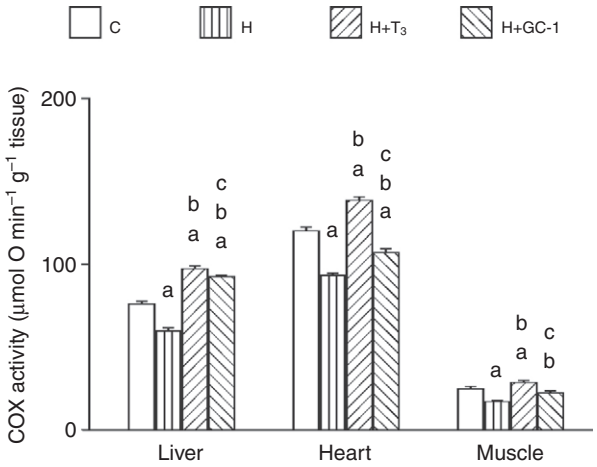

Fig. 1. Effect of T_3 or GC-1 treatment of hypothyroid rats on cytochrome oxidase (COX) activity in tissue homogenates. Values are means ± s.e.m. of eight different experiments. COX activity is expressed in µmol O min^{-1} g^{-1} tissue. C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H-GC-1, hypothyroid GC-1-treated rats. [a]Significant *vs* C rats; [b]significant *vs* H rats; [c]significant *vs* H+T_3 rats. The level of significance was chosen as P<0.05.

Cytochrome oxidase activity

As reported in Fig. 1, in all hypothyroid tissues COX activity was lower than in euthyroid ones. The absolute change in muscle COX activity was lower than that seen in liver and heart (11.6, 16.2 and 22.6 nmol O min^{-1} g^{-1} tissue, respectively), whereas the percentage change was higher (42.0%, 21.3% and 18.9%, respectively). The treatment of hypothyroid rats with T_3 increased COX activity, so that in all tissues it became significantly higher than in euthyroid controls, even though in the muscle the difference between hypothyroid and euthyroid values was small. Treatment with GC-1 was associated with tissue-dependent increases in COX activity. Thus, in comparison with euthyroid values, enzyme activities were higher in the liver, lower in the heart, and not significantly different in the muscle of GC-1-treated rats.

Oxygen consumption

Data on rates of succinate-supported oxygen consumption by tissue homogenates are reported in Table 2. In liver, such rates were lower in the hypothyroid than in the euthyroid group during both state 4 and state 3. Conversely, O_2 consumption rates were increased by

Table 1. Effect of T_3 or GC-1 treatment on body parameters, heart rate, resting metabolic rate, and serum levels of cholesterol and free iodothyronines

Parameter	C	H	H+T_3	H+GC-1
Body mass (g)	267.0±4.1	276.1±6.0	247.3±8.2[a,b]	270.4±7.3[c]
Heart mass (mg)	670±20	550±10[a]	790±50[a,b]	640±30[c]
Heart mass/body mass (mg g^{-1})	2.50±0.04	2.01±0.03[a]	3.23±0.15[a,b]	2.34±0.06[b,c]
Heart rate (beats min^{-1})	357±12	278±9[a]	534±12[a,b]	367±7[b,c]
RMR (l O_2 kg^{-1} h^{-1})	1.10±0.02	1.01±0.03[a]	1.57±0.01[a,b]	1.12±0.03[b,c]
Cholesterol (mg dl^{-1})	46.6±1.4	63.7±1.6[a]	41.8±1.7[b]	46.6±1.6[b]
FT_3 (pg dl^{-1})	301±24	25.5±5.1[a]	571.0±57.5[a,b]	26.4±4.9[a,c]
FT_4 (ng dl^{-1})	1.45±0.09	0.33±0.04[a]	0.39±0.04[a]	0.30±0.03[a]

Values are means ± s.e.m. For each value eight rats were used.

RMR, resting metabolic rate; FT_3 and FT_4, serum level of free triiodothyronine and thyroxine, respectively; C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H+GC-1, hypothyroid GC-1-treated rats.

[a]Significant *vs* C rats; [b]significant *vs* H rats; [c]significant *vs* H+T_3 rats. The level of significance was chosen as P<0.05.

Table 2. Effect of T_3 or GC-1 treatment of hypothyroid rats on succinate-sustained O_2 consumption by tissue homogenates

Tissue		C	H	H+T_3	H+GC-1
Liver	State 4	2.62±0.08	2.40±0.06[a]	4.38±0.08[a,b]	3.74±0.08[a,b,c]
	State 3	11.64±0.29	8.84±0.25[a]	24.79±0.48[a,b]	14.23±0.16[a,b,c]
	RCR	4.6±0.3	3.5±0.2[a]	5.8±0.2[a,b]	3.8±0.1[a,c]
Heart	State 4	4.87±0.17	3.72±0.07[a]	6.43±0.10[a,b]	3.91±0.05[a,c]
	State 3	11.46±0.51	10.17±0.16[a]	14.11±0.11[a,b]	10.65±0.30[c]
	RCR	2.4±0.2	2.7±0.2	2.2±0.1	2.7±0.1
Muscle	State 4	1.92±0.06	1.63±0.04[a]	2.66±0.08[a,b]	1.83±0.03[b,c]
	State 3	4.12±0.09	3.58±0.21[a]	6.55±0.10[a,b]	4.01±0.09[b,c]
	RCR	2.1±0.1	2.2±0.1	2.5±0.1[a]	2.2±0.1

Values are means ± s.e.m. For each value eight rats were used. Oxygen consumption rates are expressed in μmol O_2 min^{-1} g^{-1} tissue.
C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H+GC-1, hypothyroid GC-1-treated rats.
[a]Significant *vs* C rats; [b]significant *vs* H rats; [c]significant *vs* H+T_3 rats. The level of significance was chosen as $P<0.05$.

T_3 and GC-1 treatment during both respiration states, with the highest rates reached in the H+T_3 group. The highest values of the RCR were also found in the H+T_3 preparations, while the lowest ones were found in the H and H+GC-1 preparations. In heart, both state 4 and state 3 respiration rates were decreased by PTU+IOP treatment and increased by T_3 treatment. Following GC-1 treatment respiration rates were not significantly increased, remaining lower than control values during state 4. The RCR values were not significantly modified by the treatments. In muscle, respiration rates during state 4 and state 3 were decreased by PTU+IOP treatment. Treatment of hypothyroid rats with T_3 and GC-1 increased both state 4 and state 3 respiration in different measure so that they were higher than in controls only in H+T_3 preparations. RCR values were significantly higher in H+T_3 than in other groups.

Data on rates of pyruvate/malate-supported oxygen consumption by tissue homogenates are reported in Table 3. In liver, during both state 4 and state 3 rates were decreased by PTU+IOP treatment and increased by T_3 and GC-1 treatment. Although the greater changes were elicited by T_3, in both H+T_3 and H+GC-1 preparations respiration rates were higher than in the C group. RCR values were not affected by the treatments. In heart, state 4 and state 3 respiration rates were decreased by PTU+IOP treatment and not modified by subsequent treatment with GC-1. Conversely, respiration rates were increased by T_3 treatment, but only during state 3 were they higher in the H+T_3 than in the C group. RCR values were not affected by the treatments. In muscle, respiration rates were decreased during both state 4 and state 3 by PTU+IOP treatment. T_3 and GC-1 treatments caused different increases in such rates so that they were not different in H+GC-1 and C groups, and were higher in H+T_3 than in all the other groups. RCR values were different in the C, H and H+GC-1 groups, and were highest in the H+T_3 group.

Oxidative damage

As shown in Table 4, treatment with PTU+IOP reduced lipid peroxide and protein-bound carbonyl levels in liver and muscle, and carbonyl levels in heart. After T_3 treatment, tissue levels of both parameters significantly increased compared with hypothyroid and euthyroid values even though lower increases were found for lipid peroxide levels. GC-1 treatment was less effective than T_3 at increasing hydroperoxide and carbonyl levels, which resulted in higher than hypothyroid values in all tissues, but higher than euthyroid values only in liver.

Antioxidants

The effects of treatment with PTU+IOP, T_3 and GC-1 on tissue antioxidants are reported in Table 5. In liver, GPX activity was not modified by PTU+IOP treatment, and was significantly increased by T_3 and GC-1 compared with the euthyroid values. GR activity, which was initially lower in hypothyroid than in euthyroid rats, was higher than both the euthyroid and hypothyroid values after T_3 treatment, whereas after GC-1 treatment it was not different from either. Vitamin E content, which was decreased by PTU+IOP treatment, was restored to the euthyroid level by GC-1 and greatly increased by T_3. CoQ10 levels, which were not different in euthyroid and hypothyroid rats, were increased by the treatments. However, CoQ9 levels were lower in GC-1- than in T_3-treated rats. GSH content was increased in hypothyroid rats and was decreased by agonist treatment so that it was lower in H+T_3 and H+GC-1 rats than in euthyroid controls and reached the lowest value in T_3-treated rats.

In heart, following PTU and IOP treatment, GPX and GR activities were decreased compared with euthyroid values, which were restored by both T_3 and GC-1 treatment. Vitamin E content was lower in the hypothyroid than in the euthyroid group.

Table 3. Effect of T_3 or GC-1 treatment of hypothyroid rats on pyruvate/malate-sustained O_2 consumption by heart homogenates

Tissue		C	H	H+T_3	H+GC-1
Liver	State 4	0.88±0.03	0.66±0.04[a]	1.58±0.03[a,b]	1.21±0.05[a,b,c]
	State 3	2.76±0.22	1.98±0.05[a]	5.49±0.05[a,b]	3.75±0.08[a,b,c]
	RCR	3.1±0.1	3.0±0.2	3.5±0.3	3.1±0.2
Heart	State 4	1.36±0.05	0.92±0.02[a]	1.49±0.06[b]	1.02±0.02[a,c]
	State 3	5.85±0.22	4.38±0.05[a]	6.46±0.16[a,b]	4.81±0.20[a,c]
	RCR	4.3±0.3	4.7±0.3	4.3±0.2	4.8±0.1
Muscle	State 4	0.48±0.01	0.38±0.01[a]	0.59±0.02[a,b]	0.50±0.01[b,c]
	State 3	2.00±0.11	1.57±0.05[a]	2.94±0.07[a,b]	2.14±0.04[b,c]
	RCR	4.2±0.2	4.1±0.3	5.0±0.2[a,b]	4.3±0.2[c]

Values are means ± s.e.m. For each value eight rats were used. Oxygen consumption rates are expressed in μmol O_2 min^{-1} g^{-1} tissue.
C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H+GC-1, hypothyroid GC-1-treated rats.
[a]Significant *vs* C rats; [b]significant *vs* H rats; [c]significant *vs* H+T_3 rats. The level of significance was chosen as $P<0.05$.

Table 4. Effect of T_3 or GC-1 treatment of hypothyroid rats on oxidative damage of tissues

Tissue	Parameter	C	H	H+T3	H+GC-1
Liver	HPs	1.12±0.04	0.97±0.03[a]	2.26±0.06[a,b]	1.47±0.05[a,b,c]
	CO	2.14±0.06	1.51±0.06[a]	2.75±0.12[a,b]	1.82±0.03[a,b,c]
Heart	HPs	0.22±0.01	0.19±0.01	0.30±0.02[a,b]	0.25±0.01[b,c]
	CO	1.47±0.08	1.24±0.03[a]	2.52±0.11[a,b]	1.64±0.03[b,c]
Muscle	HPs	0.20±0.01	0.14±0.01[a]	0.24±0.02[a,b]	0.18±0.01[b,c]
	CO	1.59±0.06	1.41±0.04[a]	2.04±0.08[a,b]	1.71±0.04[b,c]

Values are means ± s.e.m. of eight different experiments. Hydroperoxides (HPs) are expressed in pmol NADP min^{-1} g^{-1} tissue. Protein-bound carbonyls (CO) are expressed in nmol mg^{-1} protein.
C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H+GC-1, hypothyroid GC-1-treated rats.
[a]Significant *vs* C rats; [b]significant *vs* H rats; [c]significant *vs* H+T_3 rats. The level of significance was chosen as $P<0.05$.

Treatment with T_3 or GC-1 was associated with different increases in vitamin content so that in comparison with control values it was higher in H+T_3 and lower in H+GC-1 rats. CoQ9 and CoQ10 levels were not different in euthyroid and hypothyroid rats and were increased by T_3 and GC-1 treatments, reaching the highest value in H+T_3 rats. GSH levels, which were decreased by treatment with PTU and IOP, were further decreased by T_3 but not by GC-1 treatment.

In muscle, GPX activity was not modified by the different treatments, whereas GR activity was higher in H+T_3 and H+GC-1 than in C and H groups. Vitamin E content was decreased by PTU+IOP treatment and was differently increased by agonist treatment. Thus, vitamin content was higher in H+T_3 and H+GC-1 than in the other groups and reached the highest value in the T_3-treated group. CoQ9 and CoQ10 levels were not significantly affected by PTU+IOP treatment and were increased by agonist treatment. CoQ9 reached the highest value in the H+T_3 group, whereas CoQ10 levels were not significantly different in T_3- and GC-1-treated rats. GSH levels were lower in the hypothyroid than in the euthyroid group. Treatment with T_3 but not GC-1 caused a further decrease in such levels.

Response to oxidative challenge

The relationship between light emission (E) and homogenate concentration (C) was described by the equation $E=aC/\exp(bC)$, where a and b are two constants (Venditti et al., 1999). A careful examination of this function shows that b is the inverse of the homogenate concentration at the emission maximum (E_{max}), which, in turn, is determined by a and b values ($E_{max}=a/eb$). As shown by the dose–response curves reported in Fig. 2, the emission levels in general and E_{max} in particular were not affected by PTU+IOP, increased by T_3, and differently affected by GC-1 treatment. These qualitative evaluations are confirmed by the results reported in Table 6, which show that GC-1 treatment increases E_{max} levels to a smaller extent than T_3 treatment in the liver and its effects are not significantly different from T_3 treatment in the muscle, whereas it does not affect E_{max} levels in the heart.

Examination of the parameters determining light emission shows that significant increases in the value of a are induced in all preparations by T_3 but not by GC-1. Significant decreases in the b values in comparison to euthyroid values were found after agonist treatment only in liver and muscle preparations.

Table 5. Effect of T_3 or GC-1 treatment of hypothyroid rats on tissue antioxidants

Tissue	Parameter	C	H	H+T_3	H+GC-1
Liver	GPX	68.7±1.5	74.5±2.9	82.5±3.9[a]	82.8±1.4[a]
	GR	10.1±0.3	9.6±0.2[a]	13.7±0.7[a,b]	9.9±0.2[c]
	Vit E	31.9±1.0	22.0±0.8[a]	38.5±2.6[a,b]	30.4±1.2[b,c]
	CoQ9	68.3±2.6	62.8±2.4	87.3±2.0[a,b]	78.1±1.9[a,b,c]
	CoQ10	6.1±0.5	6.5±0.4	8.6±0.2[a,b]	7.8±0.2[a,b]
	GSH	5.16±0.09	5.58±0.13[a]	3.78±0.09[a,b]	4.68±0.22[a,b,c]
Heart	GPX	8.6±0.3	5.8±0.4[a]	8.1±0.3[b]	8.4±0.2[b]
	GR	1.1±0.1	0.8±0.1[a]	1.3±0.1[b]	1.2±0.1[b]
	Vit E	31.5±0.4	22.4±0.8[a]	42.0±1.4[a,b]	36.2±1.2[a,b,c]
	CoQ9	79.9±6.1	83.8±3.7	127.3±1.7[a,b]	108.7±2.0[a,b,c]
	CoQ10	5.7±0.5	6.7±0.4	10.4±0.5[a,b]	8.7±0.3[a,b,c]
	GSH	1.59±0.11	1.33±0.03[a]	0.93±0.02[a,b]	1.28±0.03[a,c]
Muscle	GPX	2.6±0.1	2.3±0.2	2.8±0.2	2.5±0.1
	GR	0.67±0.04	0.61±0.03	0.81±0.05[b]	0.76±0.04[b]
	Vit E	12.7±0.3	10.0±0.7[a]	18.8±0.8[a,b]	14.3±0.7[b,c]
	CoQ9	36.4±0.8	34.8±0.6	46.9±1.6[a,b]	41.1±0.6[a,b,c]
	CoQ10	2.9±0.2	3.0±0.1	3.7±0.2[a,b]	3.6±0.2[a,b]
	GSH	1.36±0.09	1.10±0.03[a]	0.90±0.03[a,b]	1.11±0.03[a,c]

Values are means ± s.e.m. For each value eight rats were used. Glutathione peroxidase activity (GPX) is expressed in μmol NADPH min^{-1} g^{-1} tissue. Glutathione reductase (GR) is expressed in μmol NADPH min^{-1} g^{-1} tissue. Vitamin E (Vit E), coenzyme Q9 (CoQ9) and coenzyme Q10 (CoQ10) content is expressed in nmol g^{-1} tissue. Reduced glutathione (GSH) is expressed in μmol g^{-1} tissue.
C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H+GC-1, hypothyroid GC-1-treated rats.
[a]Significant *vs* C rats; [b]significant *vs* H rats; [c]significant *vs* H+T_3 rats. The level of significance was chosen as $P<0.05$.

DISCUSSION

Our results show that treatment with daily T_3 doses of 10 μg 100 g^{-1} body mass decreases the body mass of hypothyroid rats while treatment with equimolar doses of GC-1 does not modify this parameter. The body mass loss induced by T_3 agrees with previous reports using the same experimental model (Venditti et al., 1997; Venditti et al., 2004), whereas no comparable data are available in the literature concerning the GC-1 effect. On the other hand, significant differences were also found in the responses to T_3 and GC-1 of other specific thyroid hormone-responsive parameters. Thus, heart mass was increased by T_3, but not by GC-1, whereas heart mass/body mass ratio, heart rate and resting metabolic rate were increased by both agonists, but in smaller measure by GC-1, so that their values in the H+GC-1 group were not higher than those found in the euthyroid group.

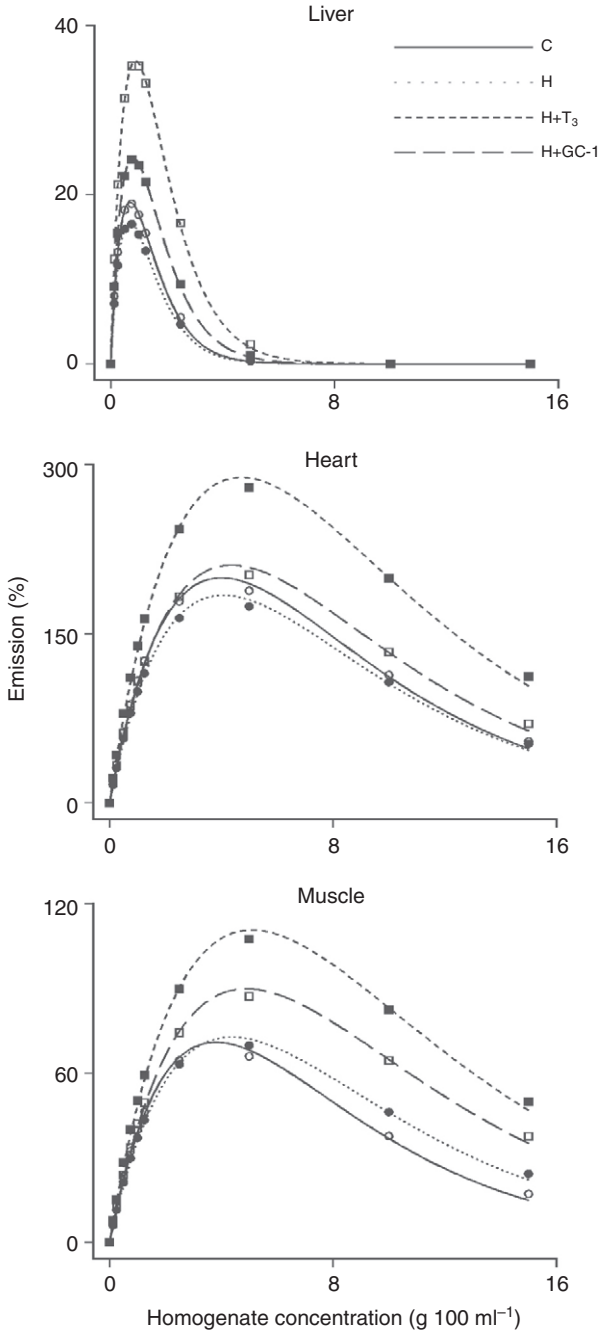

Fig. 2. Effect of treatment with T_3 or GC-1 of hypothyroid rats in response to *in vitro* oxidative challenge of tissue homogenates. Tissue susceptibility to stress was evaluated by determining the variation, with concentration of homogenate, of light emission from a luminescence reaction. Emission values are given as percentages of an arbitrary standard (44 ng ml^{-1} peroxidase). The curves were computed from experimental data by the equation $E=aC/\exp(bC)$ (see Results for details). C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H+GC-1, hypothyroid GC-1-treated rats.

In agreement with the observation that the increased capacity for heat production, induced by T_3 administration, arises from an increased capacity for oxidative metabolism of target tissues, we found increased COX activity in all examined tissues resulting from T_3 and GC-1 treatment of hypothyroid rats. However, whereas the relative increases induced by T_3 in liver, heart and muscle were similar enough (62.4%, 48.3% and 68.4%, respectively) those induced by GC-1 were remarkably different (54.8%, 14.7% and 30.4%, respectively), so that only liver COX activity underwent similar changes after T_3 and GC-1 treatment and was higher in GC-1-treated than in euthyroid control rats.

The potential mechanism underlying these and other responses to GC-1 is likely to be linked to the differences in GC-1 affinity for the T_3 nuclear receptor isoforms, as well as the different GC-1 and TRβ isoform distribution in the various tissues. Studies in cell culture have shown that T_3 and GC-1 have a similar binding affinity for the TRβ subtype, whereas T_3 affinity for the TRα subtype is approximately 10 times greater than the GC-1 affinity (Chiellini et al., 1998). It has also been shown that, whereas TRβ accounts for 80% of the TR binding in the liver (Schwartz et al., 1992), close to half of the T_3 receptors in the heart are of the TRα subtype (Schwartz et al., 1992), which is also the predominant isoform in the skeletal muscle (White et al., 2001). Moreover, measurement of GC-1 and T_3 in mouse plasma and tissues has revealed significant differences in their organ distributions (Trost et al., 2000). The tissue to plasma ratios in the liver and muscle are of the same order of magnitude for GC-1 and T_3, whereas in the heart they are 30 times higher for T_3 than for GC-1. If these results are the same in rats, they may explain why the GC-1-induced changes in aerobic capacity are lower in heart than in muscle, although the distribution of the TRβ isoform is higher in cardiac muscle. If so, the ability of GC-1 treatment to restore euthyroid values of heart rate, heart mass/body mass ratios, and aerobic capacity in hypothyroid rats might reflect indirect effects resulting from increased oxygen consumption in other tissues.

Further information on the respiratory characteristics of the tissues was obtained by measuring the *in vitro* oxygen consumption of their homogenates. As expected, we found that in all tissues basal and ADP-stimulated O_2 consumption decreased in hypothyroid and increased in T_3-treated rats. Conversely, tissue-dependent differences were shown in the respiratory responses of hypothyroid tissues to GC-1 treatment. With the exception of a report dealing with UCP1 stimulation in mouse brown adipose tissue (Ribeiro et al., 2001), the effects of GC-1 on basal and inducible mitochondrial proton conductance have not been studied. Therefore, we are not able to explain the limited changes in the state 4 respiration induced by GC-1 in liver and muscle preparations. The failure of GC-1 to induce changes in state 3 respiration in heart homogenate was consistent with its poor influence on COX activity. Thus, the GC-1-induced increases in liver and muscle respiration agreed well enough with the respective increases in COX activity. However, in both tissues state 3 respiration underwent different increases in the presence of complex I- and complex II-linked substrates. For liver, they were about 88% and 67%, respectively, whereas for muscle they were

Table 6. Effect of T_3 or GC-1 treatment of hypothyroid rats on parameters characterizing the response to oxidative stress of tissue homogenates

Tissue	Parameter	C	H	H+T_3	H+GC-1
Liver	*a*	77.4±3.6	69.0±5.2	108.8±4.8[a,b]	83.9±4.7[c]
	b	1.57±0.10	1.45±0.11	1.17±0.09[a]	1.25±0.05[a]
	E_{max}	18.1±0.9	17.4±1.2	34.2±2.7[a,b]	24.2±2.1[a,b,c]
Heart	*a*	141.3±4.3	121.7±9.1	169.5±10.4[a,b]	130.8±10.0[c]
	b	0.27±0.04	0.25±0.01	0.21±0.02	0.23±0.01
	E_{max}	192.4±20.2	186.1±14.1	283.3±24.1[a,b]	209.1±19.7[c]
Muscle	*a*	51.6±1.2	44.7±3.2	57.6±4.0[b]	49.8±3.1
	b	0.29±0.03	0.23±0.02	0.19±0.02[a]	0.20±0.01[a]
	E_{max}	65.4±5.8	71.4±6.1	109.5±7.5[a,b]	91.5±6.3[a,b]

Values are means ± s.e.m. of eight experiments. The relationship between light emission (*E*) and homogenate concentration of mitochondria (*C*) is described by the equation: $E=aC/\exp(bC)$, where *a*, *b* and *e* are constants. $E_{max}=a/eb$. For explanation see text.

C, control euthyroid rats; H, hypothyroid rats; H+T_3, hypothyroid T_3-treated rats; H+GC-1, hypothyroid GC-1-treated rats.

[a]Significant *vs* C rats; [b]significant *vs* H rats; [c]significant *vs* H+T_3 rats. The level of significance was chosen as $P<0.05$.

36% and 12%, respectively. The finding that T_3 induces a similar stimulation of state 3 respiration in the presence of pyruvate/malate and succinate (177% and 180% for liver, and 87% and 83% for muscle) suggests that the components of the respiratory chain do not respond as a unit to GC-1 stimulation unlike the case for T_3 stimulation.

It is now widely accepted that in living systems normal metabolic processes, that are essential to the cells, lead to the formation of ROS. The main biological process leading to ROS generation is electron transport within the inner mitochondrial membrane. Normally, oxygen is reduced in mitochondria to form water by concerted four-electron transfer. However, oxygen can also undergo univalent reduction by a one-electron transfer, which allows the formation of superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$), which can oxidatively damage biological molecules. Aerobic organisms are provided with a system of biochemical defences to neutralize the oxidative ROS effects, but when free radical generation exceeds the antioxidant capacity of cells oxidative stress develops. This phenomenon has been related to many pathological conditions, but it can also occur as a result of hypermetabolic state induced by normal physiological activities such as physical exercise (Barja de Quiroga, 1992). A considerable body of evidence attests that a hypermetabolic state typical of hyperthyroidism also results in oxidative injury secondary to increased ROS production and decreased antioxidant capacity in rodent tissues (Venditti and Di Meo, 2006; Fernández et al., 2006). It is also well established that thyroid hormone induces oxidative stress in humans. Clear evidence for this was provided by the finding of increased circulating levels of H_2O_2 and indices of peroxidative damage in hyperthyroid patients (Venditti and Di Meo, 2006; Fernández et al., 2006). Moreover, it was suggested that ROS play a role in myopathy and cardiomyopathy, which are the major complications of hyperthyroidism (Asayama and Kato, 1990).

Because GC-1 is able to induce changes in basal metabolic rate and aerobic metabolism of thyroid hormone target tissues, it raises the question of the extent of oxidative damage associated with metabolic changes. Thus, we investigated the relative effects of GC-1 and T_3 on tissue oxidative damage. Our results confirm a previous report indicating that T_3 treatment of hypothyroid rats induces oxidative stress in liver, heart and skeletal muscle (Venditti and Di Meo, 2006). Administration of equimolar doses of GC-1 increases tissue markers of oxidative damage to a lesser extent than T_3 treatment so that only the oxidative damage to liver lipids is significantly higher in the H+GC-1 than in the C group. Oxidative damage to lipids and proteins depends on different biochemical characteristics, such as the degree of unsaturation of hydrocarbon chains and the content of amino acids able to generate carbonyl groups, respectively. Thus, it is not surprising that in all tissues the two agonists differentially damage lipids and proteins. It is less clear how GC-1 induces oxidative damage in cardiac tissue, which is much less prone to its action. One possibility is that the oxidative damage of the cardiac muscle depends on its activity. This idea is indirectly supported by the observation that the increase in cardiac activity, induced by acute exercise, is associated with enhanced free radical generation along with augmented lipid peroxidation (Kumar et al., 1992).

To evaluate the impact of antioxidant protection changes on the different extent of tissue oxidative damage in T_3- and GC-1-treated rats, we determined the activities of antioxidant enzymes and the levels of free radical scavengers, which are major components of the antioxidant defence system. Indeed, the combined action of GPX and GR is the main determinant of tissue content of GSH, the most abundant cellular thiol. Vitamin E is the main chain-breaking antioxidant preventing free radical-initiated peroxidation (Tappel, 1972). CoQ carries out an antioxidant function in cell membranes, which can be ascribed to a synergism with vitamin E (Kagan et al., 1990) or a direct radical scavenging activity (Ernster et al., 1992). Our results show that GSH levels are higher in H+GC-1 than in H+T_3 preparations. Because the fall in GSH levels after a damaging treatment can be considered an index of oxidative stress it is not surprising that the fall is greater in H+T_3 preparations, which suffer more extensive oxidative damage. Conversely, GPX and GR activities and liposoluble antioxidant levels do not seem to be related to the different gravity of oxidative stress found in rats treated with T_3 or GC-1 and do not supply information on the protective efficacy of the antioxidant system of the tissues. On the other hand, even determination of the concentrations of all ROS scavengers and the activities of all antioxidant enzymes should supply only a partial evaluation of such an efficacy, which greatly depends on the system's ability to co-ordinate and integrate the function of the various antioxidants.

However, we obtained some information on the effectiveness of the antioxidant system by a method able to evaluate tissue sensitivity to oxidative processes (Venditti et al., 1999). In this method the extent of oxidative changes occurring in conditions leading to increased *in vivo* ROS production is obtained by measuring the levels of light emission resulting from *in vitro* exposure to H_2O_2 of tissue homogenates.

Because the level of light emission, and particularly the emission maximum, can be considered an index of the susceptibility of the preparations to oxidative challenge (Di Meo et al., 1996), our results reveal an increased susceptibility following both treatments, but in smaller measure with GC-1.

Analysis of the parameters affecting the emission levels shows that the lower levels found in H+GC-1 rats are due mainly to lower values of the parameter *a*, whereas a marginal contribution is supplied by changes in the value of parameter *b*. It has been shown that the values of parameters *a* and *b* are dependent on the concentration of substances able to induce and inhibit, respectively, the H_2O_2-promoted luminescence reaction (Venditti et al., 1999). It has also been shown that substances able to induce the luminescence reaction include Fe^{2+} complexes, such as the haemoproteins, which promote the conversion of H_2O_2 into reactive $^{\bullet}OH$ radicals *via* the Fenton reaction (Halliwell and Gutteridge, 1990), whereas substances inhibiting the luminescence reaction include antioxidants able to prevent the formation of or interact with $^{\bullet}OH$ radicals.

Thus, the aforementioned results supply indirect evidence that a smaller increase in tissue levels of respiratory mitochondrial chain components is responsible for both a lower oxidative capacity and a lower degree of oxidative damage induced by GC-1 in rat tissues. Conversely, the changes in antioxidant capacity are too little to distinguish the oxidative effects of T_3 and GC-1 treatments. However, further studies are necessary to establish whether a lower generation of ROS contributes to make the extent of oxidative damage smaller in H+GC-1 than in H+T_3 preparations.

In summary, the results reported in the present paper indicate that GC-1 induces some beneficial effects of T_3, such as a lowering of the plasma cholesterol level, and causes a moderate increase in basal metabolic rate due to an enhancement of target tissue aerobic metabolism lower than that elicited by T_3. It is important that GC-1 can elicit these effects at doses that are able to limit the increase in heart rate and other detrimental effects, such as an enhancement of tissue oxidative damage and susceptibility to oxidants, which accompany treatment with equimolar doses of T_3. Thus, our paper shows that GC-1 has promising properties for a possible therapeutic utilization. However, further studies are necessary to establish whether GC-1 can have unforeseen toxic effects for mammalian species and in particular for humans, in which it has never been tested.

This work was supported by grants from the Italian Ministry of University and Scientific and Technological Research.

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