Thyroxine and tri-iodothyronine differently affect uncoupling protein-1 content and antioxidant enzyme activities in rat interscapular brown adipose tissue

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Abstract

The activity of the antioxidant enzymes copper–zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD) and catalase (CAT), as well as mitochondrial glyceraldehyde-3-phosphate dehydrogenase (mGPDH) activity, uncoupling protein-1 (UCP1) content, catecholamine-degrading enzyme monamine oxidase (MAO) activity and malonyl dialdehyde (MDA) concentration were studied in rat interscapular brown adipose tissue (IBAT). Rats were treated with either thyroxine (T4) or tri-iodothyronine (T3) for five days and then exposed to cold (4 °C, 24 h) or housed at room temperature (22 °C). Under basal conditions, T3 treatment significantly increased UCP1 content and MnSOD activity whereas CuZnSOD, CAT and MAO activities were significantly decreased. Thyroxine treatment significantly decreased IBAT CAT activity while MDA levels markedly increased. Cold exposure induced a significant augmentation of UCP1 content and MnSOD and mGPDH activities only in animals that were rendered hyperthyroid by T4 treatment. In T3-treated animals acutely exposed to cold stress, MDA concentration, an indicator of lipid peroxidation, was significantly higher compared with that of T3-treated animals housed at room temperature. However, in T4-treated animals, MDA concentrations were markedly lower. These results show that T4 and T3 differentially affect BAT parameters studied not only under basal but also under cold-stimulated conditions.


Introduction

Brown adipose tissue (BAT) is an organ highly specialized for heat production by a mechanism referred to as ‘non-shivering’ thermogenesis (Nicholls & Locke 1984). BAT mitochondria contain uncoupling protein-1 (UCP1) which allows protons to re-enter the mitochondrial matrix without coupling to ATP synthesis (Cannon & Lindberg 1979). UCP1 transforms electrochemical energy into heat (Nicholls & Locke 1984), enabling small mammals to tolerate cold exposure (Nedergaard et al. 1999).

The thermogenic activity of BAT and UCP1 gene expression are mainly regulated by the sympathetic nervous system (SNS), which innervates the tissue (Cassard-Doulcier et al. 1993). Although it is known that thyroid hormones are involved in cold-induced thermogenesis, their role in BAT thermogenesis is not yet clear. Thyroid hormones increase obligatory thermogenesis and play a role in the facultative thermogenesis interacting with the SNS or may affect BAT activity directly (Silva 1995). The presence of type 2 thyroxine 5'-deiodinase (D2) in BAT (Leonard et al. 1983) and its manifold stimulation by noradrenaline (NA) (Silva & Larsen 1983) have led to a better understanding of the active role of thyroid hormones in BAT thermogenesis. Nevertheless, the action of thyroid hormones on BAT is complex and is dependent on tri-iodothyronine (T3) content (Bianco & Silva 1987), a physiologically active thyroid hormone. Since T3 concentration in BAT is strongly influenced by D2 that is inhibited by its substrate thyroxine (T4) (Silva & Larsen 1986), it might be argued that BAT of T4-hyperthyroid rats may lack T3 (Ablena & Puerta 1992). Otherwise, nuclear and mitochondrial thyroid receptors in BAT of T3-hyperthyroid rats can be occupied fully by excessive T3. Given that response elements for thyroid hormone receptors have been identified in the UCP1 gene promoter (Silva & Rabelo 1997), it is not surprising that alterations in BAT nuclear thyroid receptor occupancy levels produce substantial changes in UCP1 concentration. Thus, hyperthyroidism produced by T3 treatment significantly increased UCP1 mRNA levels (Masaki et al. 1997), UCP1 content (Branco et al. 1999), and guanosine 5'-diphosphate (GDP) binding to brown-adipocyte mitochondria (Woodward & Saggerson 1989). On the other
hand, the effects of T₄ on BAT UCP1 levels are still controversial. It is reported that T₄ treatment may either increase GDP binding to brown-adipocyte mitochondria (Nedregaard et al. 1997) or maintain it at the same level (Sundin 1981).

However, Echtay et al. (2002) recently postulated that UCP1 evolved a thermogenic role in mammals as a side pathway of an original, more general function of protection against the cold-induced production of reactive oxygen species (ROS). They substantiated this opinion by the fact that a mild uncoupling decreases mitochondrial production of ROS (Papa & Skulachev 1997, Brand 2000) and that superoxide anion increases mitochondrial conductance through effects on uncoupling proteins (UCPs). In accordance with this presumption are the results of Nègre-Salvayre et al. (1997), who showed that the inhibition of UCP1 by GDP induced a rise in mitochondrial membrane potential and hydrogen peroxide production. Since extensive changes in the BAT mitochondrial compartment occur in response either to thyroid hormones or to cold exposure (Triandafillou et al. 1982), and since thyroid hormones exert profound effects on the energy metabolism and influence BAT oxygen consumption (Saha et al. 1998), it is likely that thyroid hormones alter ROS production in BAT and the activity of its antioxidant protective system.

The aim of the present study was to examine the content of UCP1 in terms of its thermogenic and possible ROS protective function and its relationship with key antioxidant enzymes – cytosolic copper–zinc superoxide dismutase (CuZnSOD), mitochondrial manganese superoxide dismutase (MnSOD) and peroxisomal catalase (CAT) in interscapular brown adipose tissue (IBAT) of rats rendered hyperthyroid either by T₄ or T₃ treatment under basal or cold-stimulated conditions. We have also studied the activities of two mitochondrial enzymes involved in the hydrogen peroxide production – mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) and monoamine oxidase (MAO). MAO, a catecholamine degrading enzyme localized in the outer mitochondrial membrane, produces hydrogen peroxide. Recent results of Drahota et al. (2002) indicate that hydrogen peroxide is also produced directly by mGPDH, aFAD-linked, thyroid hormone responsive enzyme, localized in the inner mitochondrial membrane. Finally, to evaluate possible damage of IBAT membranes as a consequence of altered ROS production and/or ROS degradation, we have measured the concentration of tissue lipid peroxides.

Materials and Methods

Animals

Male rats of the Wistar strain (*Rattus norvegicus*), three months old when killed, were used for experiments. Rats were kept at room temperature (22 ± 1 °C) (mean ± S.E.M.), in a 12 h light:12 h darkness cycle and had free access to commercial rat food (Subotica, Yugoslavia) and tap water.

Animals were randomly assigned to one of three groups: euthyroid (treated with 9 mM NaOH, which is the solvent for T₄ and T₃, in a volume of 1 ml/kg body weight, i.p. for five days), T₄-treated (300 µg/kg body weight, i.p. for five days) and T₃-treated (200 µg/kg body weight, i.p. over five days). After the last injection the animals of all three groups were subdivided into two groups: one group remained at room temperature for 24 h and the other was exposed to cold (4 °C) for 24 h. Animals were killed by decapitation with a guillotine (Harvard-Apparatus, Holliston, MA, USA). The IBAT, heart and liver were rapidly excised, dissected (4°C), weighed and stored at −20 °C prior to enzyme activity and UCP1 concentration measurements. Experiments were approved by the local ethics committee for animal research.

**Analytical procedure**

All chemicals used were purchased from Sigma (St Louis, MO, USA) unless otherwise specified.

**Antioxidant enzymes assays in IBAT** IBAT was homogenized (Sorwall omni-mixer, Norwall, CT, USA) in a buffer containing 0·25 M sucrose, 0·05 M Tris–HCl and 1 mM EDTA, pH=7·4 and sonicated three times at 100 W for 20 s with 10 s pause in a Bronson model B–12 sonicator to release MnSOD. The samples were then centrifuged at 20 000 r.p.m. in a Beckman centrifuge, JA-20, for 120 min and used for CAT, CuZnSOD and MnSOD determination.

SOD activity was determined by the adrenaline method of Misra and Fridovich (1972), based on the spectrophotometrical measurement of the degree of adrenaline auto-oxidation inhibition by SOD, contained in the examined samples. Total specific SOD activity and that of MnSOD (after CuZnSOD inhibition with potassium cyanide (KCN)) were measured, and then CuZnSOD activity was calculated. CAT activity was measured spectrophotometrically by the method of Beutler (1982), based on the rate of hydrogen peroxide degradation by the action of CAT contained in the examined samples.

**Lipid peroxidation measurement** The extent of the peroxidative reactions in IBAT was determined by measuring malonyl dialdehyde (MDA) concentration in IBAT homogenates (prepared in 50 mM Tris–HCl buffer, pH=7·4 in a ratio of 1:50) by the modified thiobarbituric acid method without stimulation of peroxidative processes with Fe²⁺ and ascorbate (Rehncrona et al. 1980).

**MAO activity** MAO activity in IBAT was determined by the method of Wurtman and Axelrod (1963), based on
the measurement of radioactivity of $^{14}$C-indol-3-acetic acid, which is generated during incubation of $^{14}$C-tryptamine bissucinate with the IBAT homogenate.

Assay for mGPDH in IBAT and liver The assay of mGPDH is based on the ability of iodonitrotetrazolium to directly accept electrons from the dehydrogenase (contained in the solubilized mitochondrial fraction) with its reduction to iodoformazan, the concentration of which was determined spectrophotometrically at 500 nm (Gardner 1974, Bianco & Silva 1987).

UCP1 assay UCP1 protein levels were measured by Western blot analysis. Samples of solubilized mitochondrial fraction (containing 10 µg of the IBAT mitochondrial protein) were added to an equal volume of buffer (consisting of 0·125 M Tris–HCl, 0·14 M SDS, 20% glycerol, 0·2 mM dithiothreitol, 0·03 mM bromphenol blue, pH=6·8). After denaturation by heating to 100 °C for 5 min, samples were separated on a 12.5% polyacrylamide gel and electrotransferred to a PVDF membrane (pore size 0·45 µm, LKB, Belgrade, Yugoslavia). After transfer of proteins, the membrane was soaked in Tris-buffere (containing 0·2% Tween 20, 0·2% dithiothreitol, 0·2% horseradish peroxidase-conjugated goat anti-rabbit antibody (ICN, Belgrade, Yugoslavia) and enhanced chemiluminescence (ECL, Amersham Life Science). The blots were exposed to X-ray film for autoradiography. The intensity of signals was evaluated by the Image Quant program (Molecular Dynamics, Amersham Biosciences).

The obtained number of pixels for the control group (10 µg mitochondrial proteins) represents one arbitrary unit. The UCP1 content of treated groups is expressed relative to control IBAT.

Protein content of the tissues was measured by the method of Lowry et al. (1951).

The results are expressed as means ± S.E.M. Student’s $t$-test was employed for comparing the two groups and the level of significance was set at $P<0.05$.

Results

The effectiveness of both T$_4$ and T$_3$ administration in producing hyperthyroidism, expressed in body weight gain and relative heart weight, as well as liver mGPDH activity reflecting T$_3$ tissue availability during the treatment, is presented in Table 1.

Both T$_4$ and T$_3$ treatment produced a marked IBAT hypertrophy in terms of significant increase in absolute and relative IBAT weight (Table 2). However, it is important to point out that only T$_3$ treatment produced a marked decrease in total IBAT protein concentration and significant increase in the rat rectal temperature regardless of ambient temperature.

UCP1 content and mGPDH activity in IBAT of T$_4$- or T$_3$-treated rats under basal or cold-stimulated conditions

Given that thyroid hormone treatment significantly changed IBAT weight and/or its protein concentration (Table 2), UCP1 concentration is expressed as total UCP1 content (arbitrary units (AU)/tissue). Under basal conditions, only T$_3$ treatment significantly increased UCP1 content in brown fat mitochondria (Fig. 1). However, after 24 h of cold exposure, UCP1 content of T$_3$-treated rats, in contrast to the control and T$_4$-treated groups, was not

### Table 1 Assessment of hyperthyroid state in terms of changes in body weight gain, relative heart weight and the liver mGPDH activity of rats treated with either T$_4$ or T$_3$ and thereafter exposed to 4 °C for 24 h or housed at 22 °C. Values are means ± S.E.M. of six animals

<table>
<thead>
<tr>
<th>Condition</th>
<th>Body weight gain (g)</th>
<th>Heart weight/body weight$^1$ (mg/g)</th>
<th>Liver mGPDH activity (A500/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Housed at 22 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euthyroid</td>
<td>26·09 ± 20·7</td>
<td>2·93 ± 0·08</td>
<td>0·032 ± 0·003</td>
</tr>
<tr>
<td>T$_4$-treated</td>
<td>19·25 ± 2·21</td>
<td>3·36 ± 0·08</td>
<td>0·073 ± 0·013</td>
</tr>
<tr>
<td>T$_3$-treated</td>
<td>4·91 ± 2·15***</td>
<td>3·96 ± 0·18**</td>
<td>0·145 ± 0·025**</td>
</tr>
<tr>
<td>Exposed to 4 °C for 24 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euthyroid</td>
<td>15·50 ± 2·58$^b$</td>
<td>3·42 ± 0·08$^{b}$</td>
<td>0·035 ± 0·003</td>
</tr>
<tr>
<td>T$_4$-treated</td>
<td>16·67 ± 6·04</td>
<td>3·84 ± 0·11$^s$</td>
<td>0·103 ± 0·013$^{***}$</td>
</tr>
<tr>
<td>T$_3$-treated</td>
<td>4·77 ± 3·62$^a$</td>
<td>4·13 ± 0·12$^{**}$</td>
<td>0·167 ± 0·007$^{***}$</td>
</tr>
</tbody>
</table>

$^1$Heart weight (mg)/body weight (g).

* $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus the corresponding euthyroid group; $^s$ $P<0.05$, $^{**}P<0.01$ versus the group treated in the same way but housed at 22 °C.
significantly enhanced relative to the basal content in T3-treated rats. Unlike the increment of the liver mGPDH activity (Table 1), hyperthyroidism, produced with either T4 or T3 treatment, had no effect on its activity in IBAT of animals housed at room temperature. In our present experiment, acute cold exposure produced similar changes in IBAT mGPDH and UCP1. Thus, after a 24 h cold exposure, IBAT mGPDH activity and UCP1 content increased significantly only in the control and T4-treated groups.

MAO activity in IBAT of hyperthyroid animals under basal and cold-stimulated conditions

The activity of the catecholamine degrading and hydrogen peroxide producing enzyme, MAO, decreased significantly following T3 treatment (Fig. 2) irrespective of the ambient temperature. Cold stress produced a significant increase in IBAT MAO activity in all groups studied, indicating the activation of the sympathoadrenal system.

Activity of antioxidant enzymes in IBAT of hyperthyroid rats under basal and cold-stimulated conditions

Under basal conditions, SODs activities in IBAT were changed only in T3-treated animals (Fig. 3). Thus, CuZn-SOD activity markedly decreased while that of MnSOD significantly increased in the IBAT of animals rendered hyperthyroid by T3 administration. However, the IBAT CAT activity was markedly decreased in both T4- and T3-treated animals. After cold stress, each of the IBAT antioxidant enzymes studied was affected differently. Thus, CuZnSOD activity was increased in IBAT of both T4- and T3-treated animals (no effect on control animals) while that of CAT was significantly increased in all groups studied relative to basal activities. After cold exposure,
MnSOD activity changed in a manner similar to that of other mitochondrial parameters (UCP1 and mGPDH) studied (cold stress increased MnSOD activity in IBAT of control and T4-treated animals but not T3-treated animals).

**Lipid peroxidation in IBAT of hyperthyroid rats under basal and cold-stimulated conditions**

Figure 4 clearly shows that the extent of peroxidative processes in IBAT of hyperthyroid animals depends on whether T4 or T3 is used for inducing hyperthyroidism. Thus, under basal conditions, T4 treatment, but not T3 treatment, produced a significant increase in IBAT MDA concentration. Interestingly, exposure to cold induced opposite effects in MDA levels in IBAT of T4- and T3-treated rats as compared with animals exposed to 22 °C. Cold stress produced a marked decrease in IBAT MDA concentration of T4- and T3-treated rats as compared with animals exposed to 22 °C. Cold stress produced a marked decrease in IBAT MDA concentration of T4- and T3-treated rats as compared with animals treated in the same way but housed at room temperature. Cold exposure had no effect on MDA concentrations in the control animal group.

**Discussion**

Our present results clearly show that UCP1 content, lipid peroxide level, antioxidant enzyme activities and MAO and mGPDH activities in IBAT of hyperthyroid rats are changed differently depending on whether T4 or T3 is used for inducing hyperthyroidism. To explain these results one must bear in mind that in the hyperthyroid state, obligatory thermogenesis is elevated (Silva 1995) and SNS activity is significantly attenuated (Matsukawa et al. 1993). Hence, at subthermoneutral temperatures the thyroid hormone-induced heat production would partly compensate for the need for extra heat production in the cold. Thus, at any subthermoneutral temperature an animal would require less BAT-derived heat to counteract heat loss to the surroundings (Nedergaard et al. 1997). On the other hand, BAT itself is a target for thyroid hormones and has a large number of α1 and β1 thyroid hormone receptors (Hernández & Obregón 1996). Since high plasma levels of T4 inhibit D2 (Silva & Larsen 1986) it could be argued that the BAT of T4-hyperthyroid rats is
really lacking in T₃ and, therefore, results obtained in T₃-hyperthyroid rats cannot be attributed to an excess of T₃ in BAT (Ablenda & Puerta 1992). Knowing that UCP1 was predominantly affected by the occupancy of BAT nuclear T₃ receptors (Branco et al. 1999), it is understandable why T₄ treatment, under basal conditions, does not enhance IBAT UCP1 content. This finding is not in agreement with the results reported by Branco et al. (1999). This discrepancy may result from different doses applied and different acclimation temperature. The maximum T₄ dose applied by Branco et al. (1999) was 12.5 times compared with 37.5 times as much as the physiological replacement daily dose in this study. Since the SNS plays a much more significant role in nonshivering thermogenesis at any temperature below thermoneutral than at thermoneutral temperature, it may be supposed that the decrement in the SNS activity produced by T₄ treatment influences UCP1 expression to a lesser degree at thermoneutral than at subthermoneutral temperature. On the other hand, in BAT, T₃ has little ability, if any, of inhibiting D2 activity and even 2-fold increases are reported (Silva & Larsen 1986). Tri-iodothyronine–induced hyperthyroidism increases D2 mRNA levels (Croteau et al. 1996) and cultured rat brown adipocytes require T₃ for the adrenergic stimulation of D2 mRNA expression and D2 activity (Martinez-deMena et al. 2002). Hence, T₃ application enables enough T₃ to directly occupy nuclear T₃ receptors in IBAT so as to significantly increase UCP1 content. Similar results were obtained by Branco et al. (1999), who showed augmented UCP1 content with increasing T₃ doses, and by Masaki et al. (1997) and Gong et al. (1997), who showed increased IBAT UCP1 mRNA levels after T₃ treatment.

When SNS activity was stimulated by exposing animals to 4 °C, IBAT UCP1 content increased only in control and T₄-treated rats. It is noteworthy that mGPDH and MnSOD activities and IBAT mitochondrial protein concentrations were changed in a similar manner. To theorize why IBAT mitochondrial parameters, investigated in this study, were not increased further after cold exposure of T₃-treated rats, it is necessary to point out that (1) D2 activity in IBAT is increased by cold stimulation (Silva & Larsen 1983, 1986, Guerra et al. 1998) and (2) in addition to T₄, T₃, reverse T₃ and other iodothyronines are substrates for deiodinase enzymes (Goglia et al. 1999). It is conceivable that some of the effects observed following T₄ or T₃ administration might be due, at least in part, to some of their deiodinated products. Recently, Moreno et al. (2002) showed that when T₃ is injected into euthyroid animals, not all the effects on resting metabolic rate are attributable to T₃ itself but could be largely due to its in vivo deiodination to 3,5-di-iodothyronine (3,5-T₂) (there were increased 3,5-T₂ levels in serum and liver 12–24 h after T₃ injection). Taking into account these facts, it could be rationalized that, after 24 h cold exposure, T₃ concentrations in IBAT of T₃-treated animals increase due to an increased deiodination of T₄, while T₃ concentration in IBAT of T₃-treated animals probably decreases due to T₃ deiodination to di-iodothyronines. Since T₃ nuclear receptors occupancy is dependent on IBAT T₃ concentration, it is understandable why UCP1 content, mGPDH and MnSOD activities as well as mitochondrial protein concentrations have not significantly increased after exposure of T₃-treated rats to low ambient temperature. Measurement of D2 activity under the described experimental regimes would clarify this issue.

Despite the lower UCP1 content, rectal temperature of T₃-treated and cold-exposed rats was still significantly higher than that of control cold-exposed animals. Since di-iodothyronines, or at least 3,5-T₂, are considered as active iodothyronines and not as simple products of T₄ or T₃ metabolism (Goglia et al. 1999), it is likely that they can directly affect mitochondrial metabolism in IBAT. Di-iodothyronines can enhance the resting metabolic rate independently of protein synthesis (Moreno et al. 1997) and improve the cold tolerance of hypothyroid rats (Lanni et al. 1998).

Mitochondrial UCPs are of interest for controlling the level of ROS. Nègre-Salvayre et al. (1997) demonstrated that UCP1 inhibition activated hydrogen peroxide generation in brown fat mitochondria, and Echtay et al. (2002) demonstrated that superoxide anion activates mitochondrial UCPs and suggested that superoxide–UCP interaction may be a mechanism for decreasing ROS concentrations inside mitochondria. This indicates that changes not only in oxidative metabolism, but also in UCP1 expression will provoke marked alterations in free radical synthesis, and, consequently, in the control mechanisms and limitations of these ions that have deleterious effects on cellular processes. Our results correlate well with this observation. In IBAT of rats made hyperthyroid by T₄

Figure 4 MDA concentration in rat IBAT. Rats were made hyperthyroid by either T₄ or T₃ treatment and thereafter exposed to cold (4 °C for 24 h) or housed at room temperature (22 °C). MDA concentration is expressed as pmol of MDA formed/mg wet tissue weight and represents the means ± s.e.m. of six animals. *P<0.05 versus the corresponding euthyroid group; †P<0.05 versus the group treated in the same way housed at 22 °C.
treatment, UCP1 content and SOD activity were not changed whereas MDA levels were significantly increased as compared with the euthyroid group. In contrast, after T3 treatment both IBAT UCP1 content and MnSOD activity significantly increased but MDA concentrations were unaltered. Similarly, cold exposure of hyperthyroid rats induced different changes in MDA levels, depending on whether T4 or T3 was used for provoking hyperthyroidism. Thus, in cold-exposed T4-treated rats, IBAT UCP1 content as well as CuZnSOD, MnSOD and CAT activities were markedly increased. Consequently, the IBAT MDA concentration was significantly decreased in respect to that in animals treated in the same way but housed at room temperature (although MAO and mGPDH (hydrogen peroxide producing enzymes) activities were also increased). Despite the fact that cold exposure of animals rendered hyperthyroid by T3 treatment induced significant increases in IBAT CuZnSOD and CAT activities, MDA levels were also increased relative to basal values. Hence, it might be postulated that both mitochondrial proteins, UCP1 – the proton conductance of which is activated by superoxide anion and which, by decreasing mitochondrial membrane potential, decreases superoxide anion production (Echtay et al. 2002) – and MnSOD, the main scavenger of superoxide anion in mitochondria, probably play a crucial role in the brown adipocyte protection against ROS.

In conclusion, our results demonstrate that T4 and T3 differently influence the IBAT UCP1 content, antioxidant enzyme, mGPDH and MAO activities as well as MDA levels not only under basal but also under cold-stimulated conditions (4°C for 24 h). These findings, at least in part, could be explained by different IBAT T3 concentrations resulting from changed IBAT D2 activity and D2 substrate availability (as a consequence of performed treatments). Our results clearly show that the ROS-stimulated damage of IBAT membranes, evaluated by MDA concentration, depends not only on antioxidant enzyme activities but also on UCP1 content.

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