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The Activity of Antioxidant Enzymes and the Content of Uncoupling Protein-1 in the Brown Adipose Tissue of Hypothyroid Rats: Comparison with Effects of Iopanoic Acid

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Summary

The activity of antioxidant enzymes, copper-zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD) and catalase (CAT), as well as that of the mitochondrial FAD-dependent α-glycerophosphate dehydrogenase (α-GPD) in the rat interscapular brown adipose tissue (IBAT) were studied after the treatment with methimazole (MMI) for three weeks or with iopanoic acid (IOP) for five days. Besides, the mitochondrial concentration of uncoupling protein-1 (UCP-1) and the activity of catecholamine degrading enzyme monoamine oxidase (MAO) in the IBAT as well as the activity of the catecholamine synthesizing enzyme, dopamine β-hydroxylase (DBH) in rat serum were examined. Judging by the significantly enhanced level of serum DBH, which is an index of sympathetic activity, and that of IBAT MAO, the increase in MnSOD and CAT activities in the IBAT of hypothyroid (MMI-treated) rats seems to be due to elevated activity of sympathetic nervous system (SNS). However, CuZnSOD activity is not affected by SNS. On the contrary, IOP, which is a potent inhibitor of T_4 deiodination into T_3 producing "local" hypothyroidism, did not change either SNS activity or activities of IBAT antioxidant enzyme. However, both treatments significantly decreased IBAT UCP-1 content and α-GPD activity suggesting that the optimal T_3 concentration in the IBAT is necessary for maintaining basal levels of these key mitochondrial parameters.

Key words
Brown adipose tissue • Hypothyroidism • Rat • Antioxidant enzymes • Uncoupling protein

Introduction

Brown adipose tissue (BAT) is a mammalian organ specialized for heat production. The inner membrane of BAT mitochondria contains an uncoupling protein (UCP), which represents the molecular marker of its metabolic activity. This protein has a unique function in the regulation of oxidative phosphorylation uncoupling in the tissue, resulting in heat production (Nicholls and Locke 1984). UCP is a member of the mitochondrial anion carrier family (Huang and Klingenberg 1996, Garlid *et al.* 1996) and its first uncovered member, UCP-1, is encoded by a nuclear gene, which is expressed only in the BAT (Bouillaud *et al.* 1985, Jacobsson *et al.* 1985). It is induced by cold exposure, β-adrenergic stimulation, and thyroid hormone action (Silva and Rabelo 1997).

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BAT functions as a metabolic buffer (Himms-Hagen 1990) in all situations when energy balance is changed. Its activity is under complex neural and hormonal control. The sympathetic nervous system (SNS) directly controls UCP synthesis by releasing noradrenaline (Ricquier and Cassard-Doulcier 1993) and stimulates BAT thermogenesis (Girardier and Seydoux 1986). However, the role of thyroid hormones on BAT facultative thermogenesis is not yet clear, although it is known that thyroid hormones are involved in cold-induced thermogenesis. Namely, the thyroid hormones increase obligatory thermogenesis and also play a role in the facultative thermogenesis interacting with the SNS or affecting BAT activity directly (Silva 1995). Thus, the concentration of plasma noradrenaline, which is the initiator of BAT thermogenesis, depends on the thyroid status, e.g. noradrenaline levels are increased in the hypothyroid and decreased in the hyperthyroid state (Premel-Cabic *et al.* 1986). At the same time, the density of adrenergic receptors in the BAT is also changed by thyroid status. Hypothyroidism markedly increases the density of α_1-adrenergic receptors (Dicker *et al.* 1992), decreases mRNA for β_1- and β_2-adrenergic receptors (Revelli *et al*. 1991), and increases the number of β_3-adrenergic receptors and β_3-adrenergic receptors mRNA. Besides, a postreceptor defect in the generation of cAMP was reported in hypothyroidism (Rubio *et al.* 1995, Carvalho *et al.* 1996). Hyperthyroidism produced by T_3 treatment increased the number of β- and α-adrenergic receptors, while the ratio of α-adrenergic receptors/β-adrenergic receptors was unaffected by T_3 treatment (Rothwell *et al.* 1985).

The effects of thyroid status on the BAT UCP concentration are still being disputed. Hypothyroidism may either decrease it (Bianco and Silva 1987) or maintain it at the same level (Mory *et al.* 1981, Triandafillou *et al.* 1982, Dicker *et al.* 1992). In contrast, hyperthyroidism, produced by T_3 administration, induces a significant increase in BAT UCP-1 content at thermoneutral temperature (Branco *et al.* 1999), while the same treatment or hypothyroidism did not change UCP-3 mRNA levels (Gong *et al.* 1997).

It is known that BAT contains a type II T_4 5'-deiodinase (DII), which is rapidly and markedly stimulated by the SNS *via* α_1-adrenergic receptors under both euthyroid and hypothyroid conditions (Silva and Larsen 1983, Silva 1995) and inhibited by T_4 (Silva 1995).

Since the thyroid hormones influence BAT metabolic activity, it is possible that they change the production of free radicals and hence alter the activity of the antioxidant protective system. The main enzyme, which dismutates superoxide anion radicals into hydrogen peroxide (H_2O_2) plus O_2, is superoxide dismutase (SOD). The rat IBAT contains a relatively high concentration of the two main SOD forms, cytosolic copper-zinc superoxide dismutase (CuZnSOD) and mitochondrial manganese superoxide dismutase (MnSOD) (Petrović *et al.* 1987), which protect adipocytes against toxic superoxide anion radicals. On the other hand, catalase (CAT) is also an antioxidant enzyme localized in the peroxisomes of brown adipocytes (Nicholls and Locke 1984), which scavenges H_2O_2 generated either during superoxide anion radicals dismutation or in the process of catecholamine deamination by mitochondrial enzyme monoamine oxidase (MAO) (Cohen 1985). Catalase also participates in the peroxisomal β-oxidation process (Crane and Masters 1984).

The activity of antioxidant enzymes in some rat tissues may be affected by different hormones. Thus, the activities of both SOD forms and MAO in the rat interscapular brown adipose tissue (IBAT) are differently influenced by synthetic glucocorticoids, such as dexamethasone, rather than by corticosterone, which is a natural glucocorticoid in the rat (Cvijić *et al.* 1994). We have also shown that insulin stimulates MnSOD activity in a dose-dependent manner *via* SNS, whereas it inhibits CuZnSOD activity and does not change that of CAT (Davidović *et al.* 1997).

However, the effects of thyroid hormones on BAT function, including the activity of antioxidant enzymes and UCP-1 content, still require further clarification. Therefore, in the present study, we have examined the effects of systemic and "local" hypothyroidism on the activities of rat IBAT antioxidant enzymes. Animals were either chemically thyroidectomized by methimazole (MMI), which produces systemic hypothyroidism, or were treated with iopanoic acid (IOP), potent DII inhibitor (Silva and Larsen, 1983), which depresses T_3 production in the BAT and induces "local" hypothyroidism. Besides, we have investigated IBAT UCP-1 content (a molecular marker of its metabolic activity), α–glycerophosphate dehydrogenase (α-GPD) activity (a sensitive marker of thyroid hormone action), as well as serum dopamine β-hydroxylase (DBH) and MAO activities in the IBAT to

evaluate the intensity of the SNS activity under these conditions.

Methods

Male rats of the Wistar strain (*Rattus norvegicus*), three months old at the time of sacrifice, were used for the experiments. The rats were kept at room temperature (22±1 °C), (means ± S.E.M.), in individual cages, at a 12:12 h light-dark cycle and had free access to a commercial rat food (Subotica, Yugoslavia) and tap water with or without MMI.

The rats were divided into two main groups. The first group (n=12) consisted of 6 animals, made hypothyroid with 0.02 % methimazole (MMI), (Sigma, St. Louis, USA) added into the drinking water for three weeks, and 6 controls which were drinking tap water over the same period. The other group (n=11) was treated for five days, with an alkaline ethanol solution, either supplemented with iopanoic acid (IOP) (Sigma, St. Louis, USA) 50 mg/kg, i.p. (5 animals) or without IOP (6 control animals).

MMI- and IOP-treated animals were killed by decapitation on days 21 and 6 of the treatment, respectively. Blood was then collected from the trunk and the serum frozen for the DBH determination, while the IBAT was rapidly excised, dissected (+4 °C), weighed and stored in a deep freeze at –20 °C prior to the measurement of enzyme activities and UCP-1 concentrations.

Antioxidant enzymes assays in the IBAT

IBAT was homogenized in a buffer, containing 0.25 M sucrose, 0.05 M TRIS and 1 mM EDTA, pH=7.4, sonicated (three times at 100 W for 20 s with 10 s pause in a Bronson model B-12 sonicator), as described by Takada *et al.* (1982), to release MnSOD. The samples were then centrifuged at 20 000 rpm in a Sorvall centrifuge for 120 min and used for the determination of CAT, CuZnSOD and MnSOD activities.

SOD activity was determined by the epinephrine method of Misra and Fridovich (1972), based on the spectrophotometrical measurement of the degree of epinephrine autooxidation inhibition by SOD contained in the examined samples. Total specific SOD activity and that of MnSOD (after CuZnSOD inhibition with KCN) was measured, and then the CuZnSOD activity was calculated. CAT activity was measured spectrophotometrically by the method of Beutler (1982) based on the rate of H_2O_2 degradation by the action of CAT contained in the examined samples.

DBH and MAO activities

DBH activity in the serum was determined by the method of Kato *et al.* (1974, 1978). This method is based on the hydroxylation of tyramine into octopamine (by DBH contained in the examined samples) and its oxidation to p-hydroxybenzaldehyde and formaldehyde, in the presence of $NaIO_4$. The concentration of the formed p-hydroxybenzaldehyde was measured spectrophotometrically.

MAO activity in the 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.

The assys for IBAT α-GDP and UCP-1

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

IBAT UCP-1 concentration was measured by Western blot analysis. Briefly, in the samples of solubilized mitochondrial fraction, which contained 10 μg of IBAT mitochondrial protein, the same volume of buffer was added consisting of 0.125 M TrisHCl, 0.14 M SDS, 20 % glycerol, 0.2 mM dithiothreitol and 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 nitrocellulose membrane (pore size 0.45 μm, LKB). After this transfer, the membrane was allowed to soak in Tris-buffered saline (50 mM TrisHCl, 150 mM sodium chloride, pH=7.5) twice for 5 min, followed by quenching of nonspecific binding (1 h at room temperature in 5 % nonfat dry milk, 0.2 % Tween 20 in Tris-buffered saline). After quenching, the membrane was incubated with a solution of rabbit antibody against rat UCP-1 (Alpha Diagnostic International) overnight at 4 °C. The primary antibody was detected with a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit (ICN)) and enhanced chemiluminescence (ECL, Amersham Life Science). The blots were exposed

to an X-ray film for autoradiography. After the film scanning, the intensity of signals was evaluated by Quant Image program.

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

The results are expressed as means ± S.E.M. Students t-test was employed for comparing the two groups and the level of significance was set at $p<0.05$.

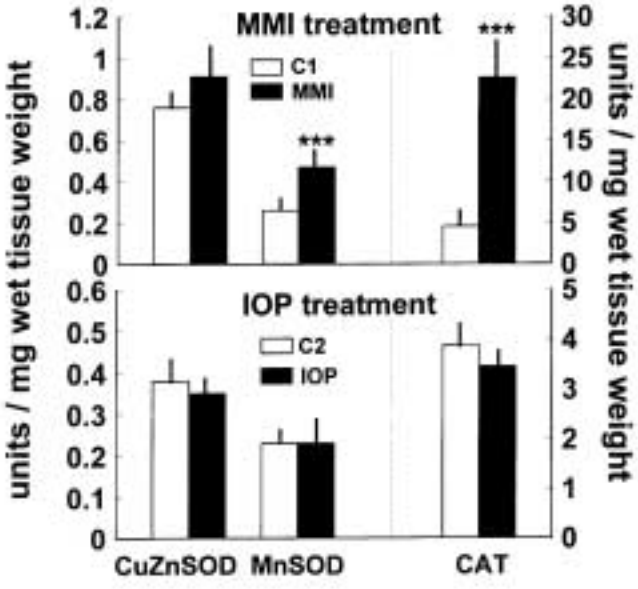

Fig. 1. *The activities of CuZnSOD, MnSOD and CAT in the IBAT of rats made hypothyroid by MMI treatment (0.02 % MMI solution in the drinking water for three weeks), or those treated with IOP (50 mg/kg body weight, i.p., for five days) and their corresponding controls. Values are expressed as units of SOD or CAT/mg wet tissue weight and represent the means ± S.E.M. of six (C1, C2 and MMI) or five (IOP) animals. *** $p<0.001$.*

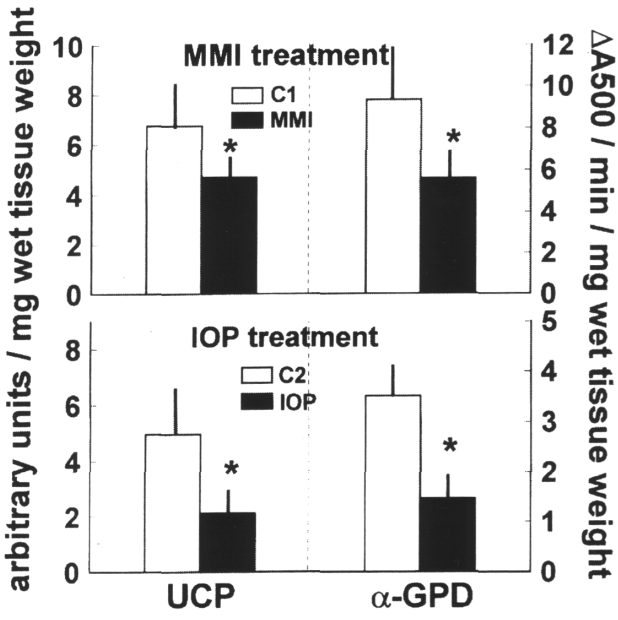

Fig. 2. *α-GPD activity and UCP-1 content in the IBAT of rats made hypothyroid by MMI treatment (0.02 % MMI solution in the drinking water for three weeks) or those treated with IOP (50 mg/kg body weight, i.p., for five days) and their corresponding controls. α-GPD activity is expressed as ΔA500/min/mg wet tissue weight. UCP-1 content is expressed in arbitrary units/mg wet tissue weight (10 μg of IBAT mitochondrial proteins was used for the analysis and the obtained number of pixels for control group represents one arbitrary unit. UCP-1 content of treated groups is expressed relative to control IBAT). Values are means ± S.E.M. of six (C1, C2 and MMI) or five (IOP) animals. *$p<0.05$.*

Results

It is clear from Figure 1 that the changes in IBAT antioxidant enzymes activities occurred only in MMI-treated animals, which resulted in systemic hypothyroidism. Thus, MnSOD and CAT activities were markedly increased ($p<0.001$), while CuZnSOD activity remained unchanged as compared to the controls. On the other hand, "local" hypothyroidism, induced by IOP, which is potent inhibitor of DII, did not cause any change in the IBAT antioxidant enzyme activities with respect to the corresponding controls.

Unlike the alterations in the IBAT antioxidant enzyme activities (Fig. 1), both systemic and "local" hypothyroidism changed the IBAT UCP-1 content and IBAT α-GPD activity in the same manner. Thus, both MMI and IOP treatment significantly reduced UCP-1 content and α-GPD activity ($p<0.05$) in the IBAT in relation to the corresponding controls (Fig. 2).

The results presented in Figure 3 show that activities of serum DBH (catecholamine synthesizing enzyme) and the IBAT MAO (catecholamine degrading and H_2O_2 generating enzyme) were significantly increased ($p<0.01$) under MMI-induced hypothyroid condition, while their activities remained unchanged after IOP treatment. These results are similar to those presented in Figure 1, from which it is evident that IOP treatment did not produce any significant effect on the IBAT antioxidant enzyme activities, while systemic hypothyroidism increased those of IBAT MnSOD and CAT.

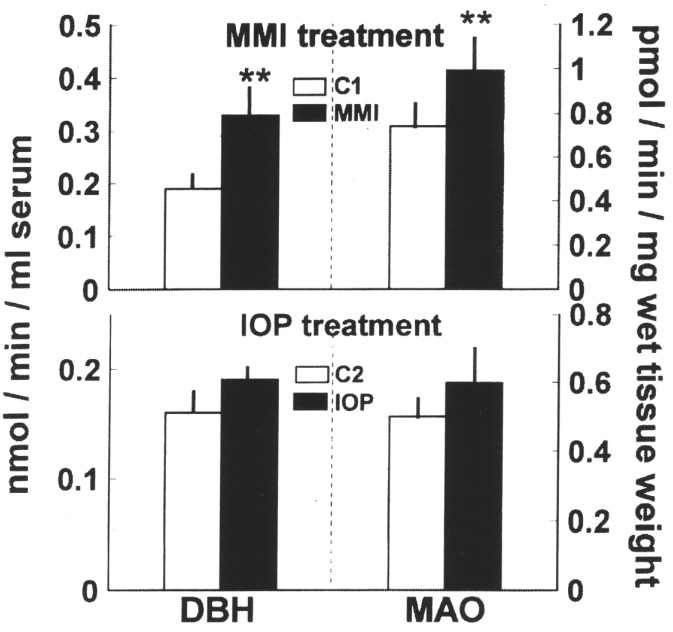

Fig. 3. *Serum DBH and IBAT MAO activities of rats made hypothyroid by MMI treatment (0.02 % MMI solution in the drinking water for three weeks) or those treated with IOP (50 mg/kg body weight, i.p., for five days) and their corresponding controls. DBH activity is expressed in nmol of octopamine/min/ml serum and that of MAO as pmol/min/mg wet tissue weight. Values are means ± S.E.M. of six (C1, C2 and MMI) or five (IOP) animals. **p<0.01.*

Discussion

Our present results clearly show that the IBAT antioxidant enzyme activities are significantly modified under hypothyroid conditions. Namely hypothyroidism, produced by 0.02 % MMI treatment for 3 weeks, the dose and duration of the treatment employed by Silva and Larsen (1986) and Obergon *et al.* (1987) for inducing severe hypothyroidism, markedly increased MnSOD and CAT activities (1.8 and 5 fold, respectively). To explain the present results one must bear in mind that obligatory thermogenesis in hypothyroidism is reduced (Rothwell *et al.* 1982, Liverini *et al.* 1992), while the BAT thermogenesis is increased (Mory *et al.* 1981), this being a compensatory mechanism. Given that SNS activity is also elevated, as indicated by the increased serum DBH activity, it may be supposed that the generation of superoxide anion radicals and that of H_2O_2 is increased in chemically induced hypothyroidism. Consequently, the activities of IBAT MnSOD and CAT are also enhanced. However, since we have also shown that the IBAT UCP-1 content is reduced, it seems that there is another pathway of heat production in peroxisomes, when it was disturbed in mitochondria. This pathway is exothermic

β-oxidation (Masters and Crane 1984) that plays a significant role in the oxidation of common fatty acids and may also eliminate fatty acids poorly oxidized by mitochondria. This is understandable since the density of α_1-adrenergic receptors on the brown adipocytes of hypothyroid rats is markedly increased (Dicker *et al.* 1992), the number of peroxisomes is increased in all such situations and fatty acid oxidation capacity is enhanced (Nedergaard *et al.* 1980, Giacobino *et al.* 1989). On the other hand, H_2O_2 production, which is a CAT substrate, seems to be significantly increased during elevated IBAT MnSOD and MAO activities, enhancing the CAT activity because it is directly proportional to the substrate level (Nedergaard *et al.* 1980).

It is also clear from the present findings that hypothyroidism increases sympathetic nerve activity, as evidenced by the elevation of serum DBH and IBAT MAO (catecholamine degrading and H_2O_2 generating enzyme) activities as well as by an increase in serum noradrenaline levels (Premel-Cabic *et al.* 1986). Therefore, it can be supposed that noradrenaline, through α_1-adrenoceptors, the number of which is markedly increased in hypothyroidism, also enhances the number of peroxisomes (Dicker *et al.* 1992) and consequently IBAT CAT activity. Bearing in mind the above mentioned data, we can conclude that the increased IBAT antioxidant enzymes (MnSOD, CAT) activities in hypothyroidism are probably mediated by sympathetic nerves. However, the activity of IBAT CuZnSOD was unchanged under hypothyroid condition, suggesting that the regulation of its activity is independent of SNS activity.

Unlike systemic hypothyroidism, "local" hypothyroidism produced by abrupt inhibition of T_4 deiodination into T_3 by IOP (potent inhibitor of DII), neither changed IBAT antioxidant enzymes activities nor those of serum DBH and MAO. Judging by these results, it might be supposed that IOP treatment did not modify SNS activity, the IBAT heat production, generation of free radicals and consequently the activity of antioxidant enzymes. However, both systemic and "local" hypothyroidism provoked the same changes in α-GPD activity and UCP-1 content. In fact, both treatments, which resulted in the decreased T_3 concentration in the BAT (Bianco and Silva 1987, Tuca *et al.* 1994), attenuated significantly these mitochondrial parameters. Our results are in agreement with those obtained by Bianco and Silva (1987) who found a marked decrease in the UCP content and α-GPD activity in the mitochondria of hypothyroid rats. In addition, Tuca *et al.* (1994) reported that the UCP content in rat BAT mitochondria after IOP treatment was also decreased. However, our results concerning the IBAT UCP content are contradictory to those of Mory *et al.* (1981), Park *et al.* (1989) and Dicker *et al.* (1992), who demonstrated the unchanged UCP content in hypothyroid brown adipocytes in comparison to the controls. The reason for this discrepancy is not clear yet, but it seems to arise from differences in the age of rats and in the dosage and duration of methimazole treatment. However, the decreased content of IBAT UCP-1 in hypothyroid rats may be explained as follows. Since noradrenaline, predominantly *via* β-adrenergic receptors and cAMP, increases UCP mRNA level (Himms-Hagen 1989, Trayhurn 1989) and T_3 also has stimulatory effect on UCP transcription (Ricquier *et al.* 1991), hence the decreased UCP-1 content in the IBAT of MMI-treated rats is probably the consequence of decreased IBAT T_3 level, lower density of β_1- and β_2-adrenergic receptors (Revelli *et al.* 1991), and decreased cAMP production (Rubio *et al.* 1995, Carvalho *et al.* 1996, Bronnikov *et al.* 1999).

Our results also demonstrate that the α-GPD activity in the IBAT mitochondria of MMI- and IOP-treated rats is markedly reduced due to the decreased T_3 level in this tissue.

As the CAT activity in the IBAT peroxisomes of hypothyroid rats is markedly increased, whereas the mitochondrial parameters, UCP-1 content, and α-GPD activity are decreased, it seems that hypothyroidism affects IBAT mitochondrial and peroxisomal enzyme activities in opposite directions. Since Bianco and Silva (1987) and Giacobino *et al.* (1989) showed that the mitochondrial β-oxidation in the BAT of hypothyroid animals is significantly decreased, it is probable that, under hypothyroid condition, the principal heat generating process is the exothermic peroxisomal β-oxidation. It is noteworthy that BAT is also functioning as a metabolic buffer under hypothyroid condition. Thus, despite the reduced T_3 levels, decreased IBAT UCP-1 content, attenuated α-GPD activity, and decreased mitochondrial fatty acid oxidation IBAT generate heat probably by peroxisomal β-oxidation, activated by SNS and mediated *via* α_1-adrenoreceptors.

In conclusion, the increased MnSOD and CAT activities in the IBAT of hypothyroid rats (systemic hypothyroidism) are probably due to elevated SNS activity, as judged by the significantly increased serum

DBH. In contrast, IOP treatment ("local" hypothyroidism) changed neither SNS activity nor IBAT antioxidant enzyme activities. However, both systemic and "local" hypothyroidism decreased IBAT UCP-1 content and α-GPD activity suggesting that the optimal T_3 concentration in the brown adipose tissue is necessary for maintaining basal levels of these key mitochondrial parameters.

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