Triiodothyronine induces lipid oxidation and mitochondrial biogenesis in rat Harderian gland

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Abstract

The rat Harderian gland (HG) is an orbital gland producing a copious lipid secretion. Recent studies indicate that its secretory activity is regulated by thyroid hormones. In this study, we found that both isoforms of the thyroid hormone receptor (*Trα* (*Thra*) and *Trβ* (*Thrb*)) are expressed in rat HGs. Although *Thra* is expressed at a higher level, only *Thrb* is regulated by triiodothyronine (T₃). Because T₃ induces an increase in lipid metabolism in rat HGs, we investigated the effects of an animal's thyroid state on the expression levels of carnitine palmitoyltransferase-1A (*Cpt1a*) and carnitine palmitoyltransferase-1B (*Cpt1b*) and acyl-CoA oxidase (*Acox1*) (rate-limiting enzymes in mitochondrial and peroxisomal fatty acid oxidation respectively), as well as on the mitochondrial compartment, thereby correlating mitochondrial activity and biogenesis with morphological analysis. We found that hypothyroidism decreased the expression of *Cpt1b* and *Acox1* mRNA, whereas the administration of T₃ to hypothyroid rats increased transcript levels. Respiratory parameters and catalase protein levels provided further evidence that T₃ modulates mitochondrial and peroxisomal activities. Furthermore, in hypothyroid rat HGs, the mitochondrial number and their total area decreased with respect to the controls, whereas the average area of the individual mitochondrion did not change. However, the average area of the individual mitochondrion was reduced by ~50% in hypothyroid T₃-treated HGs, and the mitochondrial number and the total area of the mitochondrial compartment increased. The mitochondrial morphometric data correlated well with the molecular results. Indeed, hypothyroid status did not modify the expression of mitochondrial biogenesis genes such as *Ppargc1a*, *Nrf1* and *Tfam*, whereas T₃ treatment increased the expression level of these genes.

Key Words

- ▶ Harderian gland
- ▶ triiodothyronine
- ▶ lipid oxidation
- ▶ mitochondrial biogenesis

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Introduction

The Harderian gland (HG) is a large tubuloalveolar gland present in the orbit of most terrestrial vertebrates (see Chieffi *et al.* 1996 for review). In the rat, HG excretes large amounts of characteristic lipids, and it also produces porphyrins and melatonin (see Chieffi *et al.* 1996 for review).

Although the function of HG is uncertain, its function is closely related to lipid secretion. Lipids act as lubricants and are most likely solvents for pheromones and other biologically active substances (Lin & Nadakavukaren 1981, Sakai 1981). Excretory lipids also act in thermoregulation

of gerbils (Thiessen 1988) and blind mole rats (Shanas & Terkel 1996).

HG activity is influenced by both exogenous factors (light and temperature) (Minucci *et al.* 1990, Buzzell *et al.* 1994, Santillo *et al.* 2011a) and endogenous factors (hormones). Androgens seem to be involved in the control of HG secretory activity (d'Istria *et al.* 1991, Chieffi Baccari *et al.* 1993, Santillo *et al.* 2011a), as well sexual dimorphism in the golden hamster gland (McMasters & Hoffman 1984, Santillo *et al.* 2008, Coto-Montes *et al.* 2009). Furthermore, significant evidence suggests that this gland is a target for thyroid hormones (TH). Nuclear receptors for triiodothyronine (T_3) have been observed in the HG of the golden hamster (Vilchis & Perez-Pelacios 1989). TH injections lead to an increase in porphyrin content in hamster HG, an effect that is reversed under conditions of TH deficiency (Hoffman *et al.* 1989, Buzzell & Menendez-Pelaez 1992). More recently, we demonstrated that hypothyroidism provoked a reduction in lipid secretion as well as apoptosis and autophagic phenomena in rat HG (Monteforte *et al.* 2008), whereas T_3 administration induced an increase in lipid secretion, hypertrophy of the mitochondrial compartment and an increase in the expression of uncoupling protein 3 (Chieffi Baccari *et al.* 2004, Santillo *et al.* 2011b), which is a mitochondrial protein with a function that is related to lipid metabolism (Cioffi *et al.* 2009). The presence of type II thyroxine 5'-deiodinase (5'D), an enzyme that converts thyroxine to T_3 , in both rat and hamster HGs provided further evidence that this gland is a target for TH (Delgado *et al.* 1988, Osuna *et al.* 1990). Following thyroidectomy, the activity of 5'D in rat HG increases and exhibits a marked circadian rhythm (Guerrero *et al.* 1987).

Because the physiological effects of TH are exerted at the level of transcription through interaction with specific TH receptors ($TR\alpha$ (THRA) and $TR\beta$ (THRB)), in this study, we investigated TH receptor expression in rat HG, as well as their regulation by T_3 . Further, as T_3 induced an increase in lipid metabolism in rat HG, we decided to investigate the effects of T_3 on the expression levels of carnitine palmitoyltransferase-1 (*Cpt1a* and *Cpt1b*) and acyl-CoA oxidase (AOX (*Acox1*)), which are rate-limiting enzymes in mitochondrial and peroxisomal fatty acid oxidation respectively. Next, because T_3 stimulates fat oxidation by acting at the mitochondrial and peroxisomal levels, we studied the effect of an animal's thyroid state on the mitochondria, correlating mitochondrial activity and biogenesis with morphological analysis, and on the peroxisomes, assessing catalase protein levels.

Materials and methods

Animals and T_3 determination

Male Wistar rats (*Rattus norvegicus albinus*), weighing 300–350 g, were maintained under regulated conditions of temperature (28 °C) and light (12 h light:12 h darkness cycles). The rats received commercial food pellets (Mil-Rat, Morini, Italy) and water and were allowed to feed and drink *ad libitum*. To induce hypothyroidism, rats ($n=30$) received i.p. injections of 6-*n*-propylthiouracil (PTU; 1 mg/100 g body weight) daily for 4 consecutive weeks. In addition, the rats received an i.p. injection of iopanoic acid (IOPA; 6 mg/100 g body weight) (Lanni *et al.* 1996) once a week. PTU blocks thyroidal hormone synthesis via inhibition of thyroid peroxidase activity, and it is also a strong inhibitor of type I 5'-D-deiodinase activity (Oppenheimer *et al.* 1972, Leonard & Visser 1986). IOPA inhibits all three types of deiodinase enzymes. Although the effect of IOPA is strong on type II and III, it is comparatively weak on type I (Kaplan & Utiger 1978, St Germain 1994). At the end of this treatment, 15 hypothyroid rats were killed and the remaining 15 hypothyroid rats received daily injections for 2 weeks of 15 µg T_3 /100 g body weight (Sigma–Aldrich Corp.). Control rats ($n=15$) received saline injections. Before killing, the animals were anaesthetised with an i.p. injection of chloral hydrate (40 mg/100 g body weight) and were decapitated. Trunk blood was collected, and the serum was separated and stored at –20 °C for subsequent T_3 determination. The HGs (weighing 100–150 mg) were removed through dissection, weighed and rapidly immersed in liquid nitrogen for molecular analysis. Pieces of gland were quickly immersed in fixative for electron microscopy, as described below. Total T_3 levels were determined in 50 µl samples of serum using reagents and protocols supplied by BD Biosciences (Franklin Lakes, NJ, USA). All experiments were performed in accordance with local and national guidelines governing animal experiments.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from rat HGs ($n=5$, for each experimental group) using a standard TRIzol protocol (Invitrogen Life Technologies) and later treated for 30 min at 37 °C with DNase I (10 U/sample) (Amersham Bioscience) to eliminate any genomic DNA contamination. Total RNA purity was determined by spectrophotometry at 260/280 nm, and RNA integrity was determined by electrophoresis on a denaturing formaldehyde agarose gel. One microgram of total RNA was reverse-transcribed using the SuperScript™ First-Strand Synthesis System kit (code 11904-018, Invitrogen Life Technologies).

Specific primer sets were designed for quantitative real-time RT-PCR (qRT-PCR) using Primer 3 (<http://frodo.wi.mit.edu/primer3>). The sequences of the primers used are reported in Table 1. As a reference gene, we used the ribosomal protein S12 (*Rps12*) oligonucleotide primers (Table 1). Each reaction contained 12.5 µl iQSYBR green Supermix (code 170-8882, Bio-Rad Laboratories), 2 µl cDNA template and 6 nmol/l primers. The expression of individual gene targets was analysed using the MyiQ2 Real-time PCR machine (Bio-Rad Laboratories). The thermocycle programme contained a step at 95 °C (3 min), 35 cycles at 95 °C (10 s) and a step with either 58 or 60 °C for 30 s (58 °C for *Thra*, *Thrb* and *Rps12* and 60 °C for *Cpt1a*, *Cpt1b*, *Acox*, *Ppargc1a*, *Nrf1* and mitochondrial transcription factor-A (*Tfam*)). Samples were subsequently subjected to a denaturation protocol consisting of 71 cycles that started at 55 °C and later increased 1 °C every 10 s to generate a dissociation curve to confirm the presence of a single amplicon. For every qRT-PCR assay, samples were run in duplicate along with a negative template control (RNase-free water instead of cDNA template). The relative standard curve method was used to interpolate relative mRNA abundance of target and reference genes within each sample. The standard curves were generated using equal parts of cDNA from each treatment. Reaction efficiencies were determined by the iQ5 Optical System Software (version 2.1, Bio-Rad Laboratories) using the slope of the standard curves, and for all the genes, the efficiencies were 90–110% with $R^2 \geq 0.990$. The mRNA levels of the reference gene *Rps12* did not change in the various experimental groups (data not shown). Therefore, transcript level data were normalised to *Rps12*.

Preparation of mitochondria and peroxisomes

Mitochondria and peroxisomes were isolated by homogenisation of rat HGs ($n=5$, for each experimental group)

in an isolation medium consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, 5 mM EGTA and 5 mM MgCl₂, pH 7.4, supplemented with the following protease inhibitors: 1 mM benzamidine, 4 µg/ml aprotinin, 1 µg/ml pepstatin, 2 µg/ml leupeptin, 5 µg/ml bestatin, 50 µg/ml *N*-tosyl-L-phenylalanine-chloromethyl ketone and 0.1 mM phenylmethylsulfonylfluoride. After homogenisation in this medium, samples were centrifuged at 700 *g* for 10 min. The supernatant was collected and transferred into new tubes for subsequent centrifugation at 3000 *g* for 10 min. The obtained mitochondrial pellet was washed twice, then resuspended in a minimal volume of isolation medium and used for the determination of respiratory parameters. The resulting supernatant fraction was subjected to centrifugation at 10 000 *g* for 10 min. The peroxisomal pellet was washed twice and resuspended in a minimal volume of isolation medium and used for the catalase expression analysis.

Respiratory parameters determination

Mitochondrial respiration was determined polarographically at 30 °C in a respiratory medium consisting of 80 mM KCl, 50 mM HEPES, 5 mM PBS, 10 nM sodium succinate, 3.75 µM rotenone and 1% free fatty acid BSA, pH 7.0. State 3 respiration was initiated by the addition of 300 µM ADP, and the method described by Estabrook (1967) was used to calculate the rates of state 4 and 3 respiration and the respiratory control ratio. The protein concentration was determined by the method of Hartree (1972).

Ultrastructure and morphometric analyses

For electron microscopy, pieces of HGs (3 mm³) were promptly immersed and left for 3 h in Karnovsky's fixative in cacodylate buffer (pH 7.4) and were postfixed for 2 h in

Table 1 Primers for real-time PCR

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	GenBank accession no.	Product size (bp)
<i>Thra</i>	GCTTCTTTCGCCGTACAATC	ACTGATTCGGGTGATCTTG	NM_001017960.1	96
<i>Thrb</i>	TTTCCTGTTGGCCTTTGAAC	AGGTCCGTCACCTTCATCAG	NM_012672.2	90
<i>Ppargc1a</i>	GTCAACAGCAAAAGCCACAA	GTGTGAGGAGGGTCATCGTT	NM_031347.1	79
<i>Nrf1</i>	CAACAGGGAAGAAACGGAAA	GTGGCTCTGAGTTTCCGAAG	NM_001100708.1	97
<i>Tfam</i>	CTGATGGCCATTACATGTGG	AAAGCCCGGAAGGTTCTTAG	NM_031326.1	98
<i>Cpt1a</i>	CGGAGCAGGGATACAGAGAG	TCAAAGCATCTTCCATGCAG	NM_031559.2	82
<i>Cpt1b</i>	ATCGAACGTGCTGCTTTCTT	ATTTGCCGTAGAGGCTGAGA	NM_013200.1	91
<i>Acox</i>	CTGATGAAATACGCCAGGT	GGTCCCATACGTCAGCTTGT	NM_017340.2	75
<i>Rps12</i>	AAATCGATCGAGAGGGGAAG	CTTGGCCTGAGATTCTTGC	NM_031709.3	86

Thra, thyroid hormone receptor α ; *Thrb*, thyroid hormone receptor β ; *Ppargc1a*, PPAR γ coactivator-1 α ; *Nrf1*, nuclear respiratory factor 1; *Tfam*, mitochondrial transcription factor-A; *Cpt1a*, carnitine palmitoyltransferase-1A; *Cpt1b*, carnitine palmitoyltransferase-1B; *Acox*, acyl-CoA oxidase; *Rps12*, ribosomal protein S12.

cacodylate buffer containing 0.01 g/ml osmium tetroxide. The samples were dehydrated with a graded ethanol series and later embedded in Epon 812 (Monteforte *et al.* 2009). Ultrathin sections, stained with 0.04 g/ml uranyl acetate and subsequently with 0.01 g/ml lead citrate, were examined using a Philips 301 transmission electron microscope (Philips Electronic Instruments, Rahway, NJ, USA).

Ultrastructural parameters (mitochondrial number per 100 μm^2 , mitochondrial total area per 100 μm^2 , and mitochondrial individual area) were measured on digitised electron micrographs using Image J Software (developed at the National Institutes of Health, Bethesda, MA, USA). Mitochondrial number per 100 μm^2 and mitochondrial total area per 100 μm^2 were measured in 20 different cells from each animal ($n=5$) in each experimental group. Mitochondrial individual area was measured in a total of 1000 mitochondria from each experimental group.

Western immunoblot analyses

The peroxisomal sample protein concentrations were estimated using the method of Hartree (1972). Thirty micrograms of peroxisomal protein extracts were boiled in Laemmli buffer for 5 min and then separated on a 13% SDS-PAGE gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane. The membrane was treated for 1 h with blocking solution (5% non-fat powdered milk in 25 mM Tris, pH 7.4; 200 mM NaCl; 0.5% Triton X-100 and TBS/Tween 20) and then incubated with a mouse anti-human MAB against catalase (Sigma-Aldrich) diluted 1:3000, or a goat anti-mouse polyclonal antibody against uricase (Santa Cruz Biotechnology) diluted 1:1000, over night at 4 °C. After washing with TBS/Tween, the membrane was incubated with the HRP-conjugated secondary antibody for 1 h at room temperature. The reactions were detected using an ECL system (Amersham Bioscience). Bands were scanned and then quantified by a Scan program, which converts optical density into numerical values. The protein levels of the uricase did not change in the various experimental groups. Therefore, catalase protein level data were normalised to uricase protein. Equal loading of the protein samples was confirmed by Ponceau staining.

Statistical analyses

ANOVA followed by a Student–Newman–Keuls's test was used to evaluate significant changes between experimental groups. Differences were considered statistically significant at $P<0.05$. All data were expressed as the mean $\pm$ s.d.

Results

The combined effect of PTU and IOPA produced hypothyroid rats (hypo) with significantly lower T₃ levels (0.15 ± 0.02 nmol/l) than control rats (0.89 ± 0.07 nmol/l); T₃ administration to hypothyroid rats produced significantly higher T₃ levels (1.91 ± 0.09 nmol/l).

Thra and Thrb expression

Thyroid receptors are members of the nuclear receptor superfamily and are derived from two genes located on two different chromosomes (Williams 2000). The *Thrb* gene encodes three T₃-binding TR β isoforms ($\beta 1$, $\beta 2$ and $\beta 3$) whereas the *Thra* gene encodes one T₃-binding TR $\alpha 1$ and two splicing variants (TR $\alpha 2$ and TR $\alpha 3$). These TR α variants have no T₃-binding activity (Mitsuhashi *et al.* 1988). We found that both isoforms *Thra1* and *Thrb1* were expressed in rat HG; *Thra1* mRNA levels were higher than *Thrb1* levels, given the much lower normalised Ct values (Fig. 1). *Thra1* mRNA levels were not influenced by the thyroid state (Fig. 1). *Thrb1* mRNA levels of hypothyroid rats were significantly lower when compared with those of control rats (Fig. 1). A significant increase in *Thrb1* expression compared with both the control and hypothyroid rats was observed in the HG of hypothyroid T₃-treated rats (Fig. 1).

Cpt1 and Acox gene expression

To investigate the effects of T₃ on HG fatty acid β -oxidation, the gene expression of both *Cpt1* (*a* and *b*) and *Acox* was measured with real-time PCR. Both isoforms of *Cpt1* (*a* and *b*) were expressed in rat HG; in particular, *Cpt1a* mRNA levels were not influenced by the thyroid

Figure 1

qRT-PCR analysis of *Thra1* and *Thrb1* mRNA in HG from control (C), hypothyroid (hypo) and hypothyroid T₃-treated (hypo + T₃) rats. The mRNA levels are normalised to ribosomal protein S12 (*Rps12*) mRNA levels. The values shown represent the means $\pm$ s.d. for five samples. *, *Thrb1* in hypo vs C; **, *Thrb1* in hypo + T₃ vs C and hypo.

Figure 2

qRT-PCR analysis of *Cpt1b* and *Acox* from control (C), hypothyroid (hypo) and hypothyroid T₃-treated (hypo + T₃) rat HGs. The mRNA levels are normalised to the *Rps12* mRNA levels. Values shown represent the means ± s.d. for five samples. *, hypo vs C; **, hypo + T₃ vs C and hypo.

state (data not shown). The *Cpt1b* mRNA levels of hypothyroid rats were significantly lower relative to those of control rats (Fig. 2). A significant increase in *Cpt1b* expression when compared with both control and hypothyroid rats was observed in the HG of hypothyroid T₃-treated rats (Fig. 2).

The *Acox* mRNA level of hypothyroid rats was significantly lower relative to that of control rats (Fig. 2). A significant increase in *Aox* expression when compared with both control and hypothyroid rats was observed in the HG of hypothyroid T₃-treated rats (Fig. 2).

Respiratory parameters

Mitochondrial respiratory rates are shown in Table 2. With succinate used as a substrate, both state 4 and state 3 oxygen consumption were significantly lower in hypothyroid rats than in control ones (by 31 and 30% respectively). T₃ administration to hypothyroid rats

induced a significant increase in both state 4 and state 3 oxygen consumption (by 25 and 21% respectively). The respiratory control ratio values were not different between these groups.

Mitochondrial ultrastructural and morphometric analyses

We performed mitochondrial ultrastructural and morphometric analyses in type B cells of rat HG. B cells represent the initial stage of the secretory cycle of rat HG epithelium (Chieffi Baccari *et al.* 2004, Monteforte *et al.* 2008). These cells have a smaller number of secretory vacuoles and a larger number of mitochondria than type A cells, which represent the final stage of the secretory cycle. As shown in Fig. 3B, a larger number of mitochondria were present in hypothyroid T₃-treated rat HG than in both the control (Fig. 3A) and hypothyroid rat HGs (not shown).

The morphometric data indicate that both the mitochondria number ($41 \pm 4/100 \mu\text{m}^2$) and the mean mitochondrial total area ($9 \pm 1/100 \mu\text{m}^2$) were significantly lower in hypothyroid than in control rats (59 ± 4 and $12.1 \pm 1.5/100 \mu\text{m}^2$ respectively) (Fig. 4). However, no significant differences in an average individual mitochondrial area were observed between hypothyroid HG (range, $0.022\text{--}0.946 \mu\text{m}^2$) and control HG (range, $0.016\text{--}1.13 \mu\text{m}^2$). In hypothyroid T₃-treated rats, both the mitochondria number ($88 \pm 6/100 \mu\text{m}^2$) and the mean mitochondrial total area ($17.3 \pm 1.5/100 \mu\text{m}^2$) were significantly greater relative to those of both hypothyroid and control rats (Fig. 4). The average individual mitochondrial area (range, $0.021\text{--}0.537 \mu\text{m}^2$) in the hypothyroid T₃-treated HG was about half of that found in the control HG.

Expression of genes involved in mitochondrial biogenesis

Pgc1 α , *Nrf1* and *Tfam* mRNA levels did not change in hypothyroid rats when compared with the controls (Fig. 5).

Table 2 Respiratory parameters in the HG of control (C), hypothyroid (hypo) and hypothyroid T₃-treated (hypo + T₃) rats. The values for states 4 and 3 respiration are expressed as nanoatoms of oxygen consumption per minute per milligram of protein. Results are expressed as the mean ± s.d. from five experiments in each group

Animals	State 4	State 3	RCR
C	6.2 ± 0.7	25.1 ± 1.6	4.0 ± 0.3
Hypo	4.3 ± 0.3*	17.7 ± 1.1*	4.1 ± 0.3
Hypo + T ₃	7.8 ± 0.2*	30.4 ± 1.9*	3.9 ± 0.1

RCR, respiratory control ratio (state 3 over state 4). **P* < 0.05.

Figure 3

Electron micrographs of type B cells from rat HG. (A) Type B from control rat HG is characterised by cytoplasmic unstained vacuoles and a moderate number of mitochondria (arrowheads). (B) Numerous mitochondria (arrowheads) and some secretory vacuoles are present in type B cell of hypothyroid T₃-treated rat HG. N, nucleus. Scale bar, 2 μm.

T₃ administration to hypothyroid rats induced a significant increase in *Ppargc1a*, *Nrf1* and *Tfam* expression (Fig. 5).

Catalase protein expression

Catalase protein level in hypothyroid rats was significantly lower compared with control rats (Fig. 6). A significant increase in catalase protein expression when compared with hypothyroid rats was observed in the HG of hypothyroid T₃-treated rats (Fig. 6).

Discussion

To our knowledge, this report is the first describing the expression and regulation of TH receptors in the rat HG.

Results indicated that both *Thra* and *Thrb* are present in rat HG, with levels of *Thra* being the highest. It is well known that *Thra* and *Thrb* mRNAs are expressed in most rat tissues, although their relative abundance varies (Cheng *et al.* 2010 for review). For example *Thra1* is the major isoform expressed in the heart (Falcone *et al.* 1992, Mai *et al.* 2004), whereas *Thrb1* predominates in the liver (Harvey & Williams 2002). In the brain, *Thra1* is the major foetal isoform, but at birth, there is a marked increase in the expression of *Thrb1*, which is later maintained during adult life (Harvey & Williams 2002). It is well known that the regulation of TH receptor mRNA is isoform- and cell-type dependent (Cheng *et al.* 2010, Flamant & Gauthier 2013 for reviews). We found that, although higher levels of *Thra1* are present in the rat HGs, only *Thrb1* is regulated by T₃. Experimentally induced hypothyroidism decreased HG *Thrb1* mRNA expression, whereas the administration of T₃ increased *Thrb1* transcript levels.

As it has been reported that the HG could play a role in thermoregulation and its activity is influenced by temperature (Chieffi *et al.* 1996 for review), we carried out the experiments under conditions of thermoneutrality, about 28 °C for rats. It is well known that an ambient temperature of 21 °C represents a mild cold stress to an individually housed rat (Brown *et al.* 1991). Indeed, total heat production over 24 h was ~25% higher for rats at 21 than 28 °C and underlying thermogenesis was ~20% higher for those in mild cold (Golozoubova *et al.* 2004, Feldmann *et al.* 2009).

In previous studies, we demonstrated that hypothyroidism provoked a reduction in lipid secretion in rat HG (Monteforte *et al.* 2008), whereas T₃ administration increased gland secretion with the accumulation of products in the lumina (Chieffi Baccari *et al.* 2004). In this study, we decided to investigate the effects of thyroid status on expression levels of *Cpt1* and *Acox*, which are

Figure 4

Both mitochondrial number and total area are significantly lower in hypothyroid (hypo) and higher in hypothyroid T₃-treated (hypo + T₃) rat HG compared with control (C). *, hypo vs C; **, hypo + T₃ vs C and hypo.

Figure 5
qRT-PCR analysis of genes involved in mitochondrial biogenesis from control (C), hypothyroid (hypo) and hypothyroid T₃-treated (hypo+T₃) rat HGs. The mRNA levels are normalised to the *Rps12* mRNA levels. Values shown represent the means $\pm$ s.d. for five samples. *, hypo+T₃ vs C and hypo.

rate-limiting enzymes in mitochondrial and peroxisomal fatty acid oxidation respectively. In particular, CPT1 catalyses the transfer of long-chain fatty acyl groups from CoA to carnitine for translocation across the mitochondrial inner membrane (see Bonnefont *et al.* 2004 for review), whereas AOX is the first enzyme of peroxisomal fatty acid β -oxidation, which catalyses the dehydrogenation of acyl-CoA thioesters to the corresponding trans-2-enoyl-CoA (Reddy & Mannaerts 1994). It has been demonstrated that TH induced an increase in *Cpt1b* mRNA levels in adipocytes (Lee *et al.* 2012), whereas TH induced *Cpt1a* in the liver, the predominant isoform in this tissue (Zhu & Cheng 2010). In this study, we demonstrated that both isoforms *Cpt1a* and *Cpt1b* were expressed in rat HG and that thyroid status did not affect the *Cpt1a* expression. On the contrary, *Cpt1b* transcripts significantly decreased in hypothyroid HG and increased following T₃ treatment. Furthermore, we found that the expression of *Aox* was strongly up-regulated by T₃, suggesting an involvement of peroxisomes in lipid catabolism in the rat HG. Peroxisomes mainly act in the β -oxidation of

Figure 6
The upper panel shows western blot detection of catalase protein in HG from control, hypothyroid (hypo) and hypothyroid T₃-treated (hypo+T₃) rat HGs. A specific band was observed at a size of 60 kDa. The lower panel shows the amount of catalase protein quantified using the Image J Program and normalised with respect to uricase (see upper panel). Values shown represent the means $\pm$ s.d. of five samples. *, hypo vs C; **, hypo+T₃ vs hypo.

very-long-chain fatty acids. Rat HG routinely secretes very long-chain fatty acids, saturated fatty acids up to C30 and even monoenoic acids up to C28 (Tvřickà *et al.* 1988, Seyama *et al.* 1992). Unlike in mitochondrial β -oxidation, fatty acid degradation in peroxisomes is accompanied by H₂O₂ production (Reddy & Mannaerts 1994). The involvement of the peroxisomes in rat HG lipid oxidation is supported by different activity, which these organelles showed in the experimental groups. In particular, we found that catalase expression decreased in hypothyroid rat HG and increased following T₃ treatment.

It is well documented that TH stimulate fat oxidation, mainly by acting at mitochondrial levels. Therefore, we thought it interesting to assess mitochondrial activity by studying respiratory parameters. We found that both state 4 and 3 of respiration were decreased in hypothyroid rats and increased after T₃ administration. This could be consistent with either increased activity or an increased number of mitochondria. The morphometric analysis revealed that the mitochondrial number and their total area significantly decreased in hypothyroid rat HG when compared with the controls, whereas the average area of individual mitochondria did not change. In keeping with these findings, autophagic degradation of mitochondria was the most notable effect caused by hypothyroidism in rat HG (Monteforte *et al.* 2008). Although the average area of the individual mitochondrion was reduced by ~50% in hypothyroid T₃-treated HG, the mitochondrial number and the total area of the mitochondrial compartment significantly increased. This result could be ascribed to an increased number of smaller mitochondria due to division of pre-existing mitochondria. Proliferation of mitochondria in rat liver (with an increased number of smaller mitochondria) after T₃ administration has been previously reported by Goglia *et al.* (1985). The present mitochondrial morphological and morphometric data correlated well with the molecular results. Indeed, we demonstrated that *Ppargc1a*, *Nrf1* and *Tfam* mRNA expression was induced by T₃ treatment. PPARGC1 α , which is predominantly expressed in brown adipose tissue and skeletal muscle, is currently considered to be the most important regulator of mitochondrial biogenesis (Puigserver & Spiegelman 2003) and activates the production of NRF1 and NRF2. These two factors, of which NRF1 is the most important, are potent stimulators of the expression of TFAM, which is a potent stimulator of mitochondrial DNA duplication (Puigserver *et al.* 1998). Thus, when active mitochondriogenesis is required, i.e. due to increased lipid excretion, PPARGC1 α is activated, leading to NRF1 activation and a subsequent increase in TFAM

synthesis, as well as mitochondria duplication. However, it has been demonstrated that T₃ has profound effects on mitochondrial biogenesis by modulating the expression of several mitochondrial respiratory genes (Sheehan *et al.* 2004).

Therefore, this study demonstrated that, in rat HG, T₃ affects mitochondrial and peroxisomal fat oxidation as well as mitochondrial biogenesis through *Thrb1*. Further studies will clarify whether the effects of T₃ on rat HG were a direct and/or indirect consequence of fatty acid overload.

Declaration of interest

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

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