Non-receptor-mediated actions are responsible for the lipid-lowering effects of iodothyronines in FaO rat hepatoma cells

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Abstract

Iodothyronines influence lipid metabolism and energy homeostasis. Previous studies demonstrated that 3,5-L-diiodothyronine (T_2), as well as 3,3′,5-L-triiodothyronine (T_3), was able to both prevent and reverse hepatic steatosis in rats fed a high-fat diet, and this effect depends on a direct action of iodothyronines on the hepatocyte. However, the involvement of thyroid hormone receptors (TRs) in mediating the lipid-lowering effect of iodothyronines was not elucidated. In this study, we investigated the ability of T_2 and T_3 to reduce the lipid overloading using the rat hepatoma FaO cells defective for functional TRs. The absence of constitutive mRNA expression of both *TRα1* and *TRβ1* in FaO cells was verified by RT-qPCR. To mimic the fatty liver condition, FaO cells were treated with a fatty acid mixture and then exposed to pharmacological doses of T_2 or T_3 for 24 h. Lipid accumulation, mRNA expression of the peroxisome proliferator-activated receptors (*PPAR-α, -γ, -δ*) the acyl-CoA oxidase (*AOX*), and the stearoyl CoA desaturase (*SCD1*), as well as fuel-stimulated O_2 consumption in intact cells, were evaluated. Lipid accumulation was associated with an increase in triacylglycerol content, *PPARγ* mRNA expression, and a decrease in *PPARδ* and *SCD1* mRNA expression. The addition of T_2 or T_3 to lipid-overloaded cells resulted in i) reduction in lipid content; ii) downregulation of PPARα, PPARγ, and AOX expression; iii) increase in PPARδ expression; and iv) stimulation of mitochondrial uncoupling. These data demonstrate, for the first time, that in the hepatocyte, the lipid-lowering actions of both T_2 and T_3 are not mediated by TRs.

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Introduction

Thyroid hormones (THs) play a major role in lipid metabolism, with the liver representing one of their main target tissues. Other iodothyronines display some thyromimetic activities; among them, 3,5-L-diiodothyronine (T_2) mimics several effects of 3,3′,5-L-triiodothyronine (T_3) on energy metabolism without inducing thyrotoxic effects (Cimmino *et al.* 1996, Lanni *et al.* 2005). Also T_2, as T_3, is able to stimulate both resting metabolic rate and mitochondrial activity in hypothyroid rats (Moreno *et al.* 1997). Moreover, when administered to rats receiving a high-fat diet, T_2 was able to both prevent (Lanni *et al.* 2005, Grasselli *et al.* 2008) and reduce (Mollica *et al.* 2009) the development of liver steatosis. Once entering the cell, THs mostly bind to specific nuclear receptors (TRs) acting as ligand-dependent transcription factors ('receptor-mediated' effects). In humans and rodents, TRs are encoded by two genes, *Thra* and *Thrb*, each encoding different isoforms (Izumo & Mahdavi 1988, Lazar 1993). Actions of iodothyronines that are not initiated by binding to TRs are termed 'non-receptor-mediated' mechanisms (Cheng *et al.* 2010), and could involve specific membrane-associated binding sites coupled with activation of rapid signaling pathways, involving MAP kinases, phosphoinositol-3-kinase (PI3K), or Ca^{2+} mobilization (Bergh *et al.* 2005).

Similar to THs, the peroxisome proliferator-activated receptors (PPARs) play important roles in lipid homeostasis. In humans and rodents, PPARs are encoded under three isoforms α, γ, and δ that bind to free fatty acids (FFAs; Viswakarma *et al.* 2010), but only PPARα binds to saturated FFAs (Xu *et al.* 1999). In the liver, PPARα is the master regulator of FFA oxidation, regulates the expression of acyl-CoA oxidase (AOX), and decreases FFA efflux (Martin *et al.* 1997). Also PPARδ is highly expressed in the liver, where it enhances FFA catabolism (Barish *et al.* 2006) and reduces steatosis (Shan *et al.* 2008). PPARγ is mainly expressed in adipose tissue (Schoonjans *et al.* 1996) but promotes lipid accumulation also in hepatocytes (Yu *et al.* 2003). Overall, PPARα and PPARδ function as catabolic regulators of energy, while PPARγ plays anabolic functions on lipid metabolism, even though an overlapping in their expression has been reported (Moreno *et al.* 1997).

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Recent findings provide evidence for a cross talk between TH and PPAR signaling (Lu & Cheng 2010) that occurs mainly via genomic actions (Chu *et al.* 1995). However, since PPAR transcription is affected by kinase/phosphatase pathways (Burns & Vanden Heuvel 2007, Moreno *et al.* 2010), the cross talk may also involve 'non-receptor-mediated' actions. Moreover, PPARγ (Yao-Borengasser *et al.* 2008) and PPARδ (Qin *et al.* 2008) as well as T_3 (Waters *et al.* 1997) regulate transcription of the lipogenic enzyme stearoyl CoA desaturase (SCD1) that controls FFA composition of lipid droplets (LDs).

The anti-steatosic effects of iodothyronines observed *in vivo* (Lanni *et al.* 2005, Grasselli *et al.* 2008) could not distinguish between their direct effects on the liver and the secondary effects due to upstream changes in endocrine or metabolic pathways. Subsequent *in vitro* experiments using primary rat hepatocytes overloaded with lipids demonstrated that T_2 and T_3 were able to directly reduce the excess fat (Grasselli *et al.* 2011). However, whether these effects were mediated by receptor-dependent or independent mechanisms has not been clarified yet.

In this study, we extended our *in vitro* model of steatosis to the rat hepatoma FaO cell line that is defective for functional TRs (Munoz *et al.* 1990). FaO cells have been widely employed for studies on FFA effects and PPAR activation (Konig & Eder 2006, Konig *et al.* 2008). In this study, FaO cells were used to verify whether the lipid-lowering effects of THs are mediated by TRs. In an attempt to investigate the possible molecular targets for the lipid-lowering effect of T_2 and T_3, the transcription of the different PPAR isoforms, as well as of the lipogenic (SCD1) and the lipolytic (AOX) enzymes, was assessed. Moreover, for both iodothyronines, the whole-cell respiration was evaluated in intact FaO cells.

Materials and Methods

Chemicals

All chemicals, unless otherwise indicated, were of analytical grade and were obtained from Sigma–Aldrich Corp.

Cell culture

Rat hepatoma cells (FaO) were supplied by European Collection of Cell Cultures (Sigma–Aldrich Corp.). FaO cell line is a well-differentiated liver cell line maintaining a number of hepatocyte-specific markers (Clayton *et al.* 1985, Lauris *et al.* 1986). Cells were grown in Coon's modified Ham's F12 medium supplemented with 10% FBS (Euroclone, Milan, Italy), 100 U/ml penicillin, and 0·1 mg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, FaO cells were seeded on collagenated Petri dishes (Grasselli *et al.* 2010) and used at 70–80% confluence. For treatments, cells were incubated in starvation medium supplemented with 0·25% BSA without FBS. Briefly, cells were treated with a mixture of oleate/palmitate (2:1 molar ratio, final concentration 0·75 mM, FFAs) for different periods of time (1, 3, and 6 h). After choosing the optimal conditions of exposure to FFAs, FFA-treated hepatocytes were incubated in the absence (FFAs) or in the presence of T_2 or T_3 at different concentrations (nominal concentrations ranging from 10^{-7} to 10^{-5} M) for 24 h (Demori *et al.* 1997). Both iodothyronines were added from stock solutions (10^{-3} M in 50 mM NaOH). As a negative control, hepatoma cells were cultured for the same time period with addition of the vehicle alone. At the end of treatment, hepatocytes were collected and stored at −80 °C until use. Cell viability, as assessed by trypan blue exclusion test, was not affected by exposure to FFAs or iodothyronines. For microscopical analyses, cells were grown and treated directly on collagen-coated glass slides (Falcon, BD, Milano, Italy).

To investigate whether the lipid-lowering effects of T_3 on FaO cells might depend on local, intracellular T_3-to-T_2 conversion, experiments with T_3 were repeated in the presence of 6-propylthiouracil (PTU), an inhibitor of type I deiodinase. PTU was prepared from stock solution (10^{-2} M in 50 mM NaOH) and was added to the medium (at the dose of 10^{-4} M) contemporarily to T_3 (Demori *et al.* 2004).

Lipid quantification

In intact cells, neutral lipids were visualized using the soluble selective oil-red-O (ORO) dye according to Koopman *et al.* (2001). After staining, slides were examined by Nikon Eclipse E80i light microscope (Nikon, Japan). Densitometric analysis was performed with a custom-made routine on the Optimas 6.5 image analysis system (Optimas, Washington, DC, USA).

Intracellular triacylglycerol (TAG) content was quantified using the commercial GPO–PAP kit (Roche), as described previously (Grasselli *et al.* 2010). After lysis of cells and lipid extraction according to the manufacturer's instructions, TAG content was assessed by spectrophotometric analysis. Values were normalized for the protein content determined by the bicinchoninic acid method using BSA as a standard (Wiechelman *et al.* 1988). Data are expressed as percent TAG content relative to controls.

Neutral lipids were also visualized by fluorescence microscopy using the selective Nile red (NR) dye (Koopman *et al.* 2001). Under non-saturating conditions, NR fluorescence is directly proportional to the amount of intracellular lipids. Cells grown on glass cover slips were rinsed twice with PBS and fixed with PBS containing 4% paraformaldehyde for 60 min at room temperature. After rinsing with PBS, cells were incubated with 1 μM NR in PBS for 15 min at room temperature. Then, cells were washed with PBS and mounted with 4′,6-diamidino-2-phenylindole (DAPI). Slides were examined by Nikon Eclipse E80i light microscope (Nikon) equipped with the standard epifluorescence filter set up for DAPI and Texas Red. Images were acquired with an exposure time of 100 ms.

RNA extraction and real-time RT-PCR

Total RNA was isolated from different hepatoma samples by the acid phenol:chloroform procedure using the TRIzol reagent according to the manufacturer's instructions (Chomczynski & Sacchi 1987). First-strand cDNA was synthesized from 1 μg total RNA (Grasselli *et al.* 2010). To differentiate between amplification of cDNA and that of contaminating genomic DNA, we performed a preliminary DNase I digestion of RNA samples. Quantitative RT-PCR was performed in quadruplicate in a final volume of 25 μl containing 0·3 μM of each primer, 10 ng cDNA, 1× SYBR Green PCR Master Mix and was analyzed in 96-well optical reaction by Chromo4 System PCR apparatus using the thermal protocol described elsewhere (Grasselli *et al.* 2008, 2011). The primer pairs employed in this study are listed in Table 1. The quantification cycle (C_q) represents the cycle number at which the amount of amplified target reaches the fixed threshold (Bustin *et al.* 2009). The relative quantity of target mRNA was calculated by using the comparative C_q method and was normalized for the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The normalized expression was expressed as relative quantity of mRNA (fold induction) with respect to controls (Pfaffl 2001).

Expression of TRα1 and TRβ1 was also evaluated in RNA samples isolated from six male Wistar rats (aged 8 weeks) purchased by Harlan Italy (S. Pietro al Natisone, Italy). Animal maintenance and treatment were carried out as described elsewhere (Grasselli *et al.* 2008) according to the guidelines of the European Community Council for animal care and use.

Oxygraphic measurements

O_2 consumption was assayed in intact native FaO cells permeabilized with 30 μg/ml digitonin by a thermostatically controlled oxygraph apparatus equipped with an amperometric electrode (Unisense-Microrespiration, Unisense A/S, Denmark) that determines the μM of O_2 of any solution. Oxymeter was connected to a PC running dedicated proprietary data logger software (MicOx, Unisense). An electromagnetic stirrer bar was used to mix the contents of the chamber. Additions were done by Hamilton syringes, through a rubber cup in a volume of no more than 0·05 ml. Electrode was equilibrated with the appropriate medium before each experiment, until the O_2 consumption remained constant.

In experiments with control FaO cells, samples were incubated, at 25 °C, in the following respiration solution: 120 mM NaCl, 2 mM $MgCl_2$, 1 mM KH_2PO_4, 50 mM Tris–HCl, pH 7·4, and 25 μg/ml ampicillin (final volume 1·7 ml; Ravera *et al.* 2009). After 2 min, the respiratory substrates and inhibitors were added in the following order: 10 mM pyruvate and 5 mM malate, 0·19 mM ADP, 40 μM rotenone, 20 mM succinate, 0·08 mM ADP, and 50 μM antimycin A. Pyruvate and malate were used as respiratory substrates to stimulate the pathway I+III+IV, while succinate was used for the pathway II+III+IV. To observe the ADP-stimulated respiration rates, ADP was added after pyruvate and malate or succinate addition. Rotenone (inhibitor of complex I) and antimycin A (inhibitor of complex III) were used as respiratory chain inhibitors. The respiratory rates were expressed in μM O_2/min per mg.

The oxidative phosphorylation efficiency (P/O ratio), measured as coupling O_2 consumption with ATP production,

Table 1 Characteristics of the primer pairs used for RT-qPCR analysis

Primer name	**Primer sequence** (5′→3′)	**Annealing temperature** (°C)	**Product length** (bp)	**Accession ID**	**References**
GAPDH	F: GACCCCTTCATTGACCTCAAC	60	136	DQ403053	Grasselli *et al.* (2008)
GAPDH	R: CGCTCCTGGAAGATGGTGATGGG				
PPARγ	F: CGGAGTCCTCCCAGCTGTTCGCC	60	116	Y12882	Grasselli *et al.* (2010)
PPARγ	R: GGCTCATATCTGTCTCCGTCTTC				
PPARα	F: CCCCACTTGAAGCAGATGACC	60	139	NM_013196	Grasselli *et al.* (2011)
PPARα	R: CCCTAAGTACTGGTAGTCCGC				
PPARδ	F: AATGCCTACCTGAAAAACTTCAAC	60	96	AJ306400.1	Grasselli *et al.* (2011)
PPARδ	R: TGCCTGCCACAGCGTCTCAAT				
TRα1	F: TGCTGTGTGTGGACAAGATC	66	174	NM_001017960.1	This work
TRα1	R: ACTTTCATGTGGAGGAAGCGGCT				
TRβ1	F: GCCCCATCCAGACCGAGGCC	64	82	AAA40916	This work
TRβ1	R: CCTCTCCACATGGCTCTTCCTG				
AOX	F: GCGCAAGGAGCGGGCCTCC	64	89	AAA40666	This work
AOX	R: CTCGACGGCGCCGGGTATTC				
SCD-1	F: CACACGCCGACCCTCACAACT	60	97	AF509569	This work
SCD-1	R: TCCGCCCTTCTCTTTGACAGCC				

was calculated as the ratio between the amount of consumed ADP (nmoles) and the amount of O_2 (nmoles) consumed during the ADP-induced respiration.

In experiments with iodothyronines, T_2 or T_3 (10^{-6} or 10^{-5} M) was added to control FaO cells directly in the chamber before the respiratory substrates. Experiments with T_3 were repeated in the presence of the deiodinase inhibitor PTU (10^{-4} M) that was added before addition of iodothyronines and respiratory substrate. The same analyses were also carried out on lipid-loaded FaO cells.

Statistical analysis

Data on RT-qPCR are means ± S.D. of at least three independent experiments, and each measurement was performed in quadruplicate. Data on TAG are means ± S.D. of five independent experiments. The significance of the difference between groups was evaluated by using ANOVA followed by Bonferroni's *post hoc* test. Significant differences were reported: C versus FFAs $^{\star}P \leq 0{\cdot}05$, $^{\star\star}P \leq 0{\cdot}01$, and $^{\star\star\star}P \leq 0{\cdot}001$; and FFAs versus THs $^{\#}P \leq 0{\cdot}05$, $^{\#\#}P \leq 0{\cdot}01$, and $^{\#\#\#}P \leq 0{\cdot}001$.

Results

Basal expression of TRα1 and TRβ1 in FaO cells

FaO cells have been reported to be deficient in functional TRα1 and TRβ1, and the absence of *TRβ1* mRNA was shown by northern blot analysis (Munoz *et al.* 1990). In this study, we verified the lack of constitutive expression of both TRα1 and TRβ1 in control FaO cells by RT-qPCR. Figure 1 shows the amplification curves of TRα1 and TRβ1 transcripts for both rat liver and FaO samples in comparison with the amplification curves of the reference gene *GAPDH*. The C_q value for TRα1 in the rat liver was about 20, and in FaO cells, it was about 24, with a ΔC_q over four cycles (Fig. 1A). Even larger difference was observed for TRβ1, the most abundant TR isoform in the hepatic cell. The C_q value for TRβ1 in the rat liver was about 19, while in FaO cells, it was about 28 with a ΔC_q of about nine cycles (Fig. 1B). Exposure to different iodothyronines did not affect the level of TRα1 and TRβ1 transcripts in FaO cells (data not shown).

Effects of exogenous FFA-induced lipid accumulation in FaO cells

We first verified the time course of lipid accumulation in rat hepatoma FaO cells exposed to a FFA mixture (oleate/palmitate 2:1 molar ratio), according to the model of 'cellular steatosis' described in human hepatoma HepG2 cells (Feldstein *et al.* 2004) and in primary rat hepatocytes (Feldstein *et al.* 2004, Grasselli *et al.* 2011). FFA exposure induced a rapid time-dependent increase in lipid accumulation, evaluated by densitometric analysis of ORO staining, reaching a plateau at 3–6 h (about threefold with respect to controls; $P \leq 0{\cdot}001$; Fig. 2A). FFA treatment did not result in

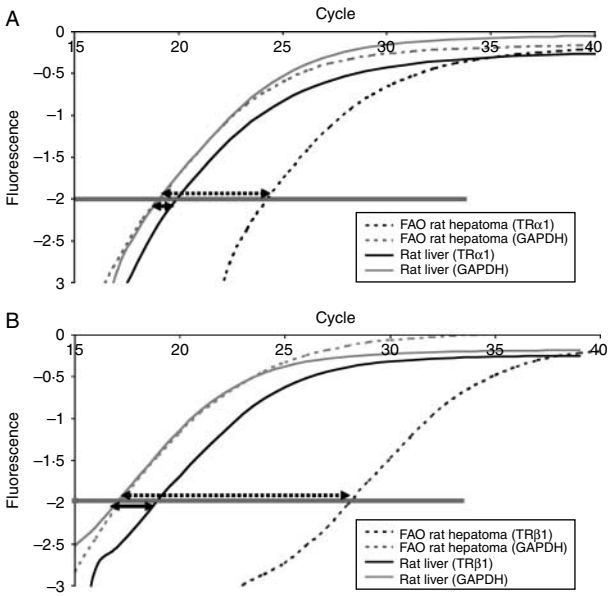


Figure 1 Relative abundance of TRα1 and TRβ1 in FaO cells. Basal expression of the thyroid hormone receptors quantified in FaO cells by RT-qPCR with respect to the rat liver. (A) Amplification curves of TRα1 and of the reference gene *GAPDH* in the rat liver and FaO cells. (B) Amplification curves of TRβ1 and of GAPDH in the rat liver and FaO cells. *X*-axis, amplification cycle number. *Y*-axis, normalized fluorescence signal.

lipotoxicity at any time point, as observed by trypan blue exclusion test (data not shown).

Figure 2B shows representative images of Nile red (NR)-stained FaO cells incubated for 3 h in the absence (C) or the presence of the FFA mixture (FFAs); NR fluorescence allowed us to appreciate the marked accumulation of neutral lipids under form of cytosolic LDs.

Effects of iodothyronines on lipid accumulation

Lipid-loaded FaO cells were then treated for 24 h with two different concentrations of T_2 or T_3 (10^{-6} and 10^{-5} M), as described previously for primary rat hepatocytes (Grasselli *et al.* 2011). As a preliminary experiment, the dose–response curves were assessed for both iodothyronines in the range of 10^{-7}–10^{-5} M, and no significant effect on the TAG content was observed at the lowest dose (10^{-7} M) of both T_2 and T_3 (data not shown). The incubation time and doses of iodothyronines were in the range of those typically employed for *in vitro* experiments on gene expression (Ball *et al.* 1997, Grasselli *et al.* 2011) or lipid metabolism (Giudetti *et al.* 2005). Moreover, several studies reported that a concentration of 10^{-6} M T_3 was required to trigger non-genomic effects (Cheng *et al.* 2010) such as to increase IP3 and Ca^{2+} levels in BDL cholangiocytes (Fava *et al.* 2007).

Exposure of FaO cells to FFAs for 3 h resulted in a significant increase in TAG content with respect to controls

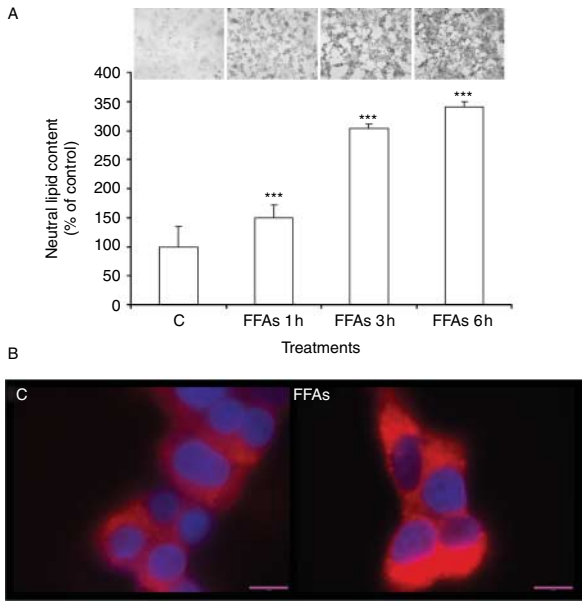


Figure 2 Lipid accumulation in FaO cells exposed to FFAs. (A) Neutral lipids were visualized by ORO staining in FaO cells incubated in the absence (C) or in the presence (FFAs) of the FFA mixture for 1, 3, and 6 h. Neutral lipid content was quantified by densitometric analysis of the optical microscopic images. Significant differences are denoted by symbols on bars (C versus FFAs, ***$P \leq 0{\cdot}001$). (B) Representative images of NR-stained FaO cells incubated for 3 h in the absence (C) or in the presence (FFAs) of the FFA mixture demonstrating the lipid accumulation under the form of cytosolic LDs. (magnification 10×; Bar, 100 μm).

(+104%; $P \leq 0{\cdot}001$) as evaluated by spectrophotometric assay (Fig. 3). In lipid-loaded FaO cells, treatment with both T_2 and T_3 resulted in a significant ($P \leq 0{\cdot}001$) reduction in TAG content (around −35% with respect to FFA cells) with similar effects of different concentrations of both THs.

In order to investigate whether the lipid-lowering effects of T_3 on FaO cells might depend on local, intracellular T_3-to-T_2 conversion via deiodinase activities, the experiments with T_3 were repeated in the presence of PTU, an inhibitor of type I deiodinase, that is known to be expressed in rat liver hepatocytes (Rooda *et al.* 1987). The effects of T_3 were unchanged by PTU (Fig. 3).

Effects of FFAs and iodothyronines on PPAR expression

The relative abundance of mRNA transcripts for PPARα, PPARγ, and PPARδ was assessed by RT-qPCR in control cells (Fig. 4A). The C_q values were about 22·3 for PPARα, 25·2 for PPARδ, and 27·7 for PPARγ, thus pointing at the following relative abundance of PPAR isoform mRNA in FaO cells: PPARα > PPARδ > PPARγ.

Lipid-loaded FaO cells did not show significant changes in *PPARα* mRNA expression (Fig. 4B). Treatment of lipid-loaded cells with the lowest dose of T_2 significantly reduced mRNA expression of *PPARα* (about 0·4-fold induction with respect to control, −74% with respect to FFA samples; $P \leq 0{\cdot}01$), whereas the decrease was not significant at the higher dose of T_2. A similar decrease was observed with both doses of T_3 (about 0·3-fold induction with respect to control at 10^{-6} M dose, −75% with respect to FFA samples; $P \leq 0{\cdot}01$ and about 0·6-fold with respect to control at 10^{-5} M dose, −59% with respect to FFA samples; $P \leq 0{\cdot}05$).

Different from *PPARα*, a significant increase in *PPARγ* mRNA expression was observed in FaO cells upon lipid loading (1·7-fold induction with respect to control; $P \leq 0{\cdot}05$; Fig. 4C). The FFA-induced upregulation of PPARγ was abolished upon treatment of lipid-loaded cells with both T_2 (about 0·6-fold induction at 10^{-6} M and about 0·8-fold at 10^{-5} M dose with respect to control; −64%, $P \leq 0{\cdot}01$, and −55%, $P \leq 0{\cdot}05$ respectively, with respect to FFA sample) or T_3 (about 0·5-fold induction with respect to control at both doses, −70% with respect to FFA samples; $P \leq 0{\cdot}01$; Fig. 4C).

An opposite trend was observed in the expression of PPARδ, whose mRNA level decreased upon exposure to FFAs (about 0·4-fold induction with respect to control; $P \leq 0{\cdot}001$). Treatment of lipid-loaded cells with the highest dose of both T_2 and T_3 significantly increased PPARδ expression (about 0·9-fold induction for T_2 and about 0·8-fold induction for T_3 with respect to control; +122%, $P \leq 0{\cdot}01$, and +103%, $P \leq 0{\cdot}01$ respectively, with respect to FFA samples; Fig. 4D).

When the same treatments with iodothyronines were performed in control FaO cells, no significant effects could be appreciated in *PPAR* gene expression (data not shown).

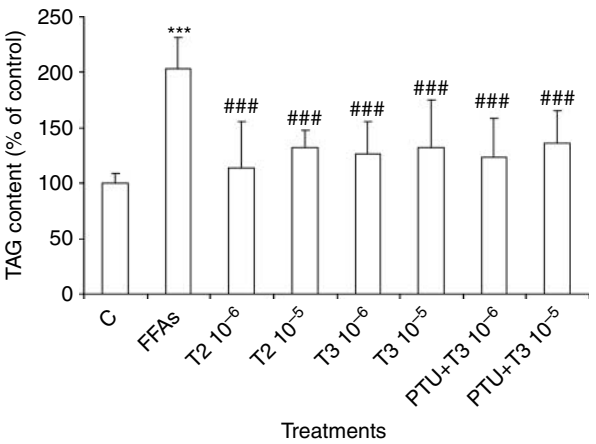


Figure 3 Lipid-lowering effects of iodothyronines in FaO cells. TAG content was quantified by spectrophotometric assay in control (C) and lipid loaded-FaO cells incubated in the absence (FFAs) or the presence of T_2 or T_3 at two different doses (10^{-6} and 10^{-5} M). The effects of PTU on the FaO cells treated with T_3 at both doses are also shown. Results are expressed as mean ± S.D. from five independent experiments. Significant differences are denoted by symbols on bars (C versus FFAs, ***$P \leq 0{\cdot}001$; FFAs versus THs, ###$P \leq 0{\cdot}001$).

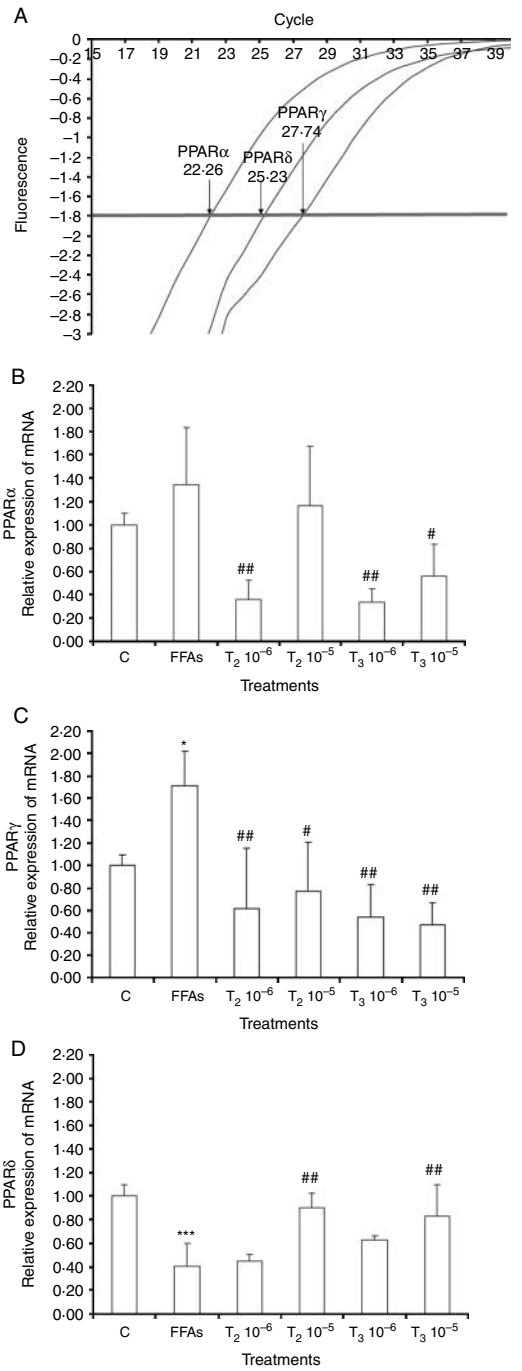


Figure 4 Effects of excess lipids and THs on PPAR mRNA expression. (A) Relative abundance of the three PPAR isoforms (PPAR-α, -γ, -δ) quantified in FaO control cells by RT-qPCR. X-axis, amplification cycle number. Y-axis, normalized fluorescence signal. Relative mRNA expression of *PPARα* (B), *PPARγ* (C) and *PPARδ* (D) quantified by RT-qPCR in control (C) and lipid-loaded FaO cells incubated in the absence (FFAs) or in the presence of T_2 or T_3 (10^{-6} and 10^{-5} M) for 24 h. Significant differences are denoted by symbols on bars (C versus FFAs, $^{*}P\leq0{\cdot}05$ and $^{***}P\leq0{\cdot}001$; FFAs versus THs, $^{\#}P\leq0{\cdot}05$ and $^{\#\#}P\leq0{\cdot}01$).

Effects of FFAs and iodothyronines on mRNA expression of lipid metabolizing enzymes

The effects of different experimental conditions on the mRNA expression of *AOX*, the main enzyme in the peroxisomal β-oxidation, and of SCD1, a lipogenic enzyme converting saturated to monounsaturated FFAs, were also assessed.

Lipid-loaded FaO cells did not show significant changes in *AOX* mRNA expression with respect to controls (Fig. 5A), but a significant reduction was observed in lipid-loaded cells exposed to the highest dose of T_2 or T_3 (about 0·8-fold induction with respect to control; −45% with respect to FFA samples; $P\leq0{\cdot}05$). No significant changes in the expression of *AOX* mRNA were observed with the lowest doses of both iodothyronines.

An opposite trend was observed in the expression of SCD1, whose mRNA level decreased upon exposure to FFAs (about 0·6-fold induction with respect to control; $P\leq0{\cdot}05$). Treatment of lipid-loaded cells with T_2 or T_3 did not modify significantly SCD1 expression with respect to FFA samples or controls (Fig. 5B).

The same treatments with iodothyronines were performed in control cells, but no significant effects could be appreciated (data not shown).

Influence of iodothyronines on hepatocyte O_2 consumption

Oxygen consumption in intact cells is mainly a reflection of mitochondrial respiration from glucose and FFAs to drive ATP production. To assess basal respiration of FaO cells, fuel-stimulated O_2 consumption in intact cells was evaluated. Figure 6 reports a typical amperographic tracing of ADP-stimulated respiration rate in permeabilized FaO cells (panel A). Resting respiration rate was assessed in the presence of complex I (malate/pyruvate) or complex II (succinate/rotenone) substrates, and maximal ADP-stimulated respiration was measured by adding saturating ADP concentration. As expected, in control hepatocytes, respiration rates decreased after addition of rotenone and antimycin A respectively. After ADP addition, the P/O ratio for pyruvate/malate showed a value of 2·54±0·21 and for succinate of 1·54±0·14 that are within the range of values reported for other cell types (Cocco *et al.* 2009).

Addition of T_2 or T_3 (at both 10^{-6} and 10^{-5} M doses) to control cells before the respiratory substrates prevented the ADP-induced transient stimulation of O_2 consumption (panels B and C), indicating that both iodothyronines effectively uncoupled previously well-coupled hepatocyte mitochondria. Oxygen consumption rates induced by the different respiratory substrates (panel E) confirm this apparent uncoupling.

Addition of PTU before the T_3 incubations did not modify the amperographic tracing of ADP-stimulated respiration rate (Table 2), thus demonstrating that the effect of T_3 on mitochondrial respiration does not depend on the conversion of T_3 into T_2.

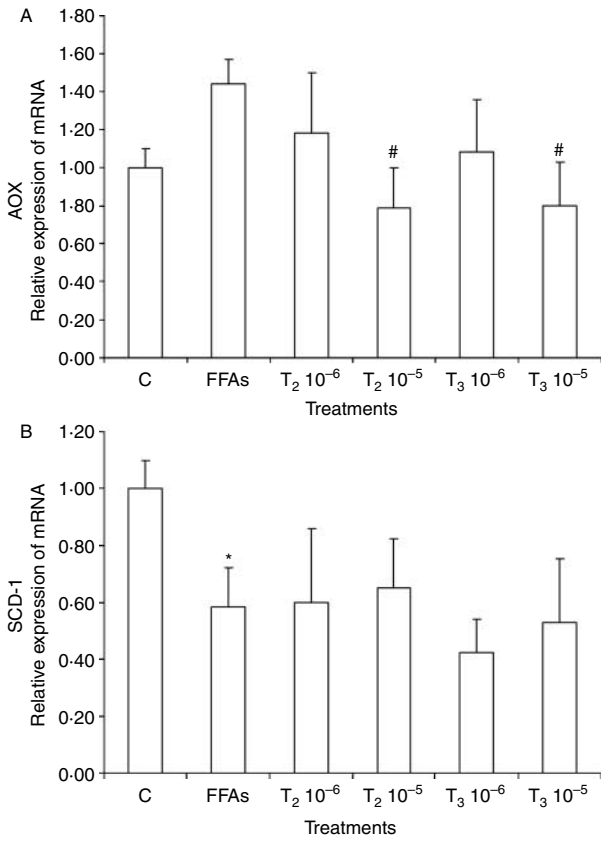


Figure 5 Effects of excess lipids and iodothyronines on AOX and SCD1 mRNA expression. Relative mRNA expression of *AOX* (A) and *SCD1* (B) quantified by RT-qPCR in control (C) and lipid-loaded FaO cells incubated in the absence (FFAs) or in the presence of T_2 or T_3 (10^{-6} and 10^{-5} M) for 24 h. Significant differences are denoted by symbols on bars (C versus FFAs, $^{*}P \leq 0{\cdot}05$; FFAs versus THs, $^{\#}P \leq 0{\cdot}05$).

The amperographic tracing of ADP-stimulated respiration rate in lipid-loaded FaO cells (panel D) showed that addition of ADP did not result in a significant increase in O_2 consumption. The lack of the ADP effect may reflect uncoupled mitochondrial respiration in lipid-loaded hepatocytes or a difficult access of ADP into the mitochondria of whole cells filled with LDs. With regard to this, a difficult access of all substrates into the mitochondria in the presence of TAG in excess was observed (not shown). Addition of T_2 or T_3 to lipid-loaded FaO cells resulted in a very high noise in the amperographic tracing that did not allow for the measurement of mitochondrial respiration (data not shown).

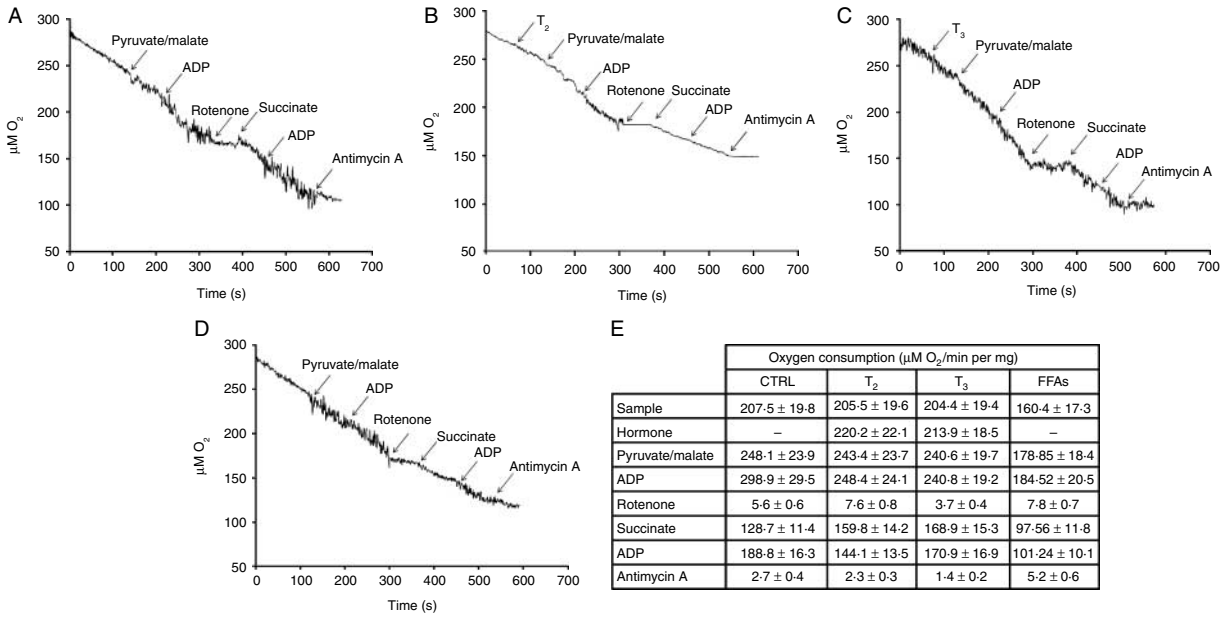
Discussion

In this study, a well-differentiated rat hepatoma cell line (FaO), previously reported to be deficient of functional TRs (Munoz *et al.* 1990), was used to investigate whether the direct effects of iodothyronines in reducing the excess lipid accumulation, previously observed in primary rat hepatocytes (Grasselli *et al.* 2011), can depend on 'non-receptor-mediated' mechanisms. This 'minimum deviation' hepatoma cell line has been shown to secrete albumin, to maintain a number of other hepatocyte-specific differentiation markers, and to respond to hormonal stimulation as adult primary hepatocytes (Clayton *et al.* 1985, Lauris *et al.* 1986).

In humans and rodents, TRs are encoded by two genes. The *Thra* gene encodes three isoforms, but only TRα1 binds T_3 (Izumo & Mahdavi 1988, Lazar 1993). The *Thrb* gene encodes two variants, TRβ1 and TRβ2 (Weiss *et al.* 1998, Ye *et al.* 2003). A previous study using the cDNA microarray analysis of *TRβ* knockout mice treated with T_3 demonstrated that the expression of a large number of genes was induced by THs also in the absence of TRβ1 (Flores-Morales *et al.* 2002). In the liver, the TRα1 levels are much lower than those of TRβ1, and most actions of THs are mediated by the TRβ1 isoform. Therefore, as a first step of this study, we verified the absence of constitutive mRNA expression of both *TRα1* and *TRβ1* in FaO cells by comparing their mRNA levels with those recorded in the rat liver. The primer pair for TRα utilized in this study recognizes also the two truncated forms p43 and p28 that are synthesized by alternative translational initiation (Wrutniak-Cabello *et al.* 2001). While p43 acts as a mitochondrial transcription factor (Casas *et al.* 2009), p28 seems to be involved in the early mitochondrial T_3 influence (Wrutniak-Cabello *et al.* 2001). Our results confirm that in FaO cells, the level of mRNA transcripts for TRs is negligible when compared with that of the rat liver.

According to the model of '*in vitro* steatosis' that we developed on primary rat hepatocytes, the lipid-loading protocol was extended with some modifications to FaO cells that were exposed for 3 h to an oleate/palmitate mixture (0·75 mM final concentration) that well mimics the plasmatic FFA levels of patients with the metabolic syndrome (Ferrannini *et al.* 1983, Vock *et al.* 2008). FFAs entering the hepatic cell are assembled in the cytosol into TAGs, representing the major component of neutral lipids (57% of total) that are stored under the form of LDs. When lipid-loaded FaO cells were treated with T_2 or T_3 for 24 h, the TAG content was reduced to values comparable with those observed in control cells. These results demonstrate that the lipid-lowering effect exerted by both T_2 and T_3 directly on the hepatic cell can occur also in the absence of TRs.

Many 'non-receptor-mediated' actions of THs have been described in a variety of cells involving different signal transduction systems and novel membrane-associated receptors (Davis *et al.* 2008, Davis 2010). A feature of 'non-receptor-mediated' mechanisms of iodothyronines is the plurality of TH derivatives, including T_2, or of functional analogs that may initiate specific actions and might have higher potency than T_3 (Cioffi *et al.* 2010*b*). The ability of THs to regulate energy utilization as well as their role in promoting mitochondrial uncoupling of substrate oxidation from ATP synthesis have been long recognized. T_2, as T_3, was shown to rapidly stimulate hepatic O_2 uptake when injected



	Oxygen consumption (µM O_2/min per mg)			
	CTRL	T_2	T_3	FFAs
Sample	207·5 ± 19·8	205·5 ± 19·6	204·4 ± 19·4	160·4 ± 17·3
Hormone	–	220·2 ± 22·1	213·9 ± 18·5	–
Pyruvate/malate	248·1 ± 23·9	243·4 ± 23·7	240·6 ± 19·7	178·85 ± 18·4
ADP	298·9 ± 29·5	248·4 ± 24·1	240·8 ± 19·2	184·52 ± 20·5
Rotenone	5·6 ± 0·6	7·6 ± 0·8	3·7 ± 0·4	7·8 ± 0·7
Succinate	128·7 ± 11·4	159·8 ± 14·2	168·9 ± 15·3	97·56 ± 11·8
ADP	188·8 ± 16·3	144·1 ± 13·5	170·9 ± 16·9	101·24 ± 10·1
Antimycin A	2·7 ± 0·4	2·3 ± 0·3	1·4 ± 0·2	5·2 ± 0·6

Figure 6 Influence of iodothyronines on hepatocyte O_2 consumption. Measurements of respiration rates in whole FaO hepatoma cells. O_2 consumption of fuel-stimulated FaO cells was evaluated in different experimental conditions. Each amperographic tracing is representative of at least four experiments. (Panel A) Amperographic tracing of basal respiration rates of control FaO cells upon the addition of different respiratory substrates. (Panels B and C) Amperographic tracings of respiration rates of FaO cells incubated in the presence of T_2 or T_3 (10^{-6} M). (Panel D) Amperographic tracing of respiration rates of lipid-loaded FaO cells (FFAs) upon the addition of different respiratory substrates. (Panel E) Changes in the angular coefficient values during the addition of several substrates in the different FaO samples. Values are representative of at least four experiments, and data are represented as mean ± S.D.

in perfused livers (Horst *et al.* 1989), or added to mitochondria isolated from hypothyroid rats (Moreno *et al.* 2002). Similar effects of T_3 have been observed in hepatocytes isolated from hypothyroid animals (Nobes *et al.* 1990).

In this study, the effects of T_2 and T_3 on the O_2 consumption in hepatic cells that do not express TRs were evaluated. The results clearly indicate that addition of both iodothyronines to whole hepatocytes rapidly induced uncoupling of previously well-coupled mitochondria. To our knowledge, these data represent the first report of the uncoupling effect of T_2 and T_3 in whole hepatocytes in the presence of adequate fuel and O_2 that confirm previous reports, demonstrating the uncoupling effects of THs on isolated mitochondria (Goglia *et al.* 1999). The short-term effects of T_3, and particularly of T_2, on mitochondrial respiration could be due to allosteric interaction with cytochrome *c* oxidase (COX) Va subunit, as shown in isolated rat liver mitochondria (Goglia *et al.* 1994, Arnold *et al.* 1998). The Kadenback group showed that in mitochondria, intrinsic and extrinsic uncoupling mechanisms of oxidative phosphorylation may take place through COX (Ramzan *et al.* 2010). Moreover, T_3 was reported to stimulate *in vitro* the mitochondrial adenine nucleotide translocase 1 that catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane (Sterling & Brenner 1995). Despite all these hypotheses, further investigations are needed to fully clarify the mechanisms involved in the uncoupling effect of T_2 and T_3.

In addition to the short-term effects on mitochondria, THs are known to exert pleiotropic effects on lipid metabolism. In the liver, THs stimulate enzymes regulating both lipogenesis and lipolysis. Since PPARs also play a crucial role in lipid metabolism, the transcription profiles of the three subtypes, PPARα, PPARγ, and PPARδ, were evaluated in FaO cells in different experimental conditions. Our results showed that all the three subtypes were constitutively expressed in rat FaO hepatoma cells, with higher expression of PPARα and PPARδ compared with PPARγ. Excess lipid accumulation in FaO cells resulted in upregulation of *PPARγ* mRNA, whereas that of *PPARα* was unaffected. These data are in line with the role of PPARγ in promoting lipid storage (Gavrilova *et al.* 2003, Schadinger *et al.* 2005). Moreover, in HepG2 cells, expression of PPARα was increased by palmitate (Ricchi *et al.* 2009). Therefore, the absence of PPARα upregulation in lipid-loaded FaO cells may depend on the excess oleate versus palmitate (2:1) in the FFA mixture, and this excess oleate may also explain the decrease in the transcription of SCD1 observed in lipid-loaded FaO cells, since this enzyme synthesizes oleic acid. On the other hand, the downregulation of PPARδ observed in lipid-loaded FaO cells fits well with previous reports showing an opposite regulation of PPARδ and PPARγ in ob/ob mice (Roberts *et al.* 2009).

Table 2 Effect of treatment with 6-propylthiouracil on O_2 consumption by whole FaO hepatoma cells incubated with 10^{-5} M T_3. The values shown are from a representative experiment of at least three performed

	Oxygen consumption (μM O_2/min per mg protein)	
	T_3 10^{-5} M	T_3 10^{-5} M+PTU
Sample	205·43	215·54
Hormone	216·78	224·84
Pyruvate/malate	228·95	240·35
ADP	229·86	241·02
Rotenone	8·6	9·1
Succinate	132·58	148·71
ADP	131·29	149·52
Antimycin A	2·3	3·6

Indeed, PPARδ was shown to increase the synthesis of high-density lipoproteins, to inhibit LD formation in the liver, and to enhance FFA catabolism and energy uncoupling in adipose tissue and muscle (Wang *et al.* 2003, Nagasawa *et al.* 2006). Therefore, the concomitant downregulation of PPARδ and upregulation of PPARγ in lipid-loaded FaO cells may sustain inhibition of FFA oxidation and secretion, and promote their storage into LDs. Overall, the differential transcription pattern of the three PPAR subtypes suggests that, in our *in vitro* model of hepatic steatosis, the hepatocyte did not activate markedly β-oxidation pathways (through PPARα- and PPARδ-mediated mechanisms), as confirmed by the lack of significant changes in the expression of their downstream lipolytic gene *AOX*, but induced the storing of excess fat in LDs (probably through PPARγ-mediated mechanisms).

Treatment of lipid-enriched FaO cells with iodothyronines leads to a decrease in TAG content. Accordingly, the FFA-induced upregulation of PPARγ was abolished following incubation with either T_2 or T_3 that also decreased the mRNA expression of *PPARα* and upregulated the expression of PPARδ. In the light of the role of PPARδ in promoting synthesis of high-density lipoproteins, FFA catabolism and energy uncoupling, and inhibiting LD formation, our data indicate that in lipid-loaded hepatocytes, both iodothyronines may act by stimulation of FFA catabolism and energy uncoupling, at the same time decreasing LD formation. Moreover, the decrease in intracellular lipid content induced by both T_2 and T_3 could be coupled with changes in the synthesis of lipoproteins and in the rate of lipid exocytosis, and experiments are in progress to investigate this mechanism.

We wish to underline that our study showing that T_2 is as effective as T_3 in reducing the fat excess in lipid-loaded FaO cells may be of great interest since it supports the possible utilization of T_2 as a pharmacological tool in the treatment of dysmetabolic syndromes such as NAFLD, also in the light of its lack of thyrotoxic effects (Cimmino *et al.* 1996, Cioffi *et al.* 2010*a*).

In conclusion, this study demonstrates that the lipid-lowering effect of iodothyronines in FaO rat hepatoma cells occurs via non-receptor-mediated mechanisms that seem to involve both a short-term action by stimulation of mitochondrial O_2 consumption and a long-term action by differential transcriptional effects on PPARs that could in turn activate pathways promoting mitochondrial oxidation and/or lipid exocytosis.

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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Author contribution statement

All authors contributed to this work significantly. E G followed all the experimental activities and carried out the RT-qPCR experiments; A V and L C followed the activities of hepatoma cell culture and treatments, supervised all the experimental activities, and revised the manuscript; G G and F G supported the experimental design and revised the manuscript; S R and I P performed the oxygraphic measurements; L V carried out the planning of the study, analyzed the data, and wrote the manuscript; G G provided funding, laboratory facilities, and project oversight.

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References

Arnold S, Goglia F & Kadenbach B 1998 3,5-Diiodothyronine binds to subunit Va of cytochrome-*c* oxidase and abolishes the allosteric inhibition of respiration by ATP. *European Journal of Biochemistry* **252** 325–330. (doi:10.1046/j.1432-1327.1998.2520325.x)

Ball SG, Sokolov J & Chin WW 1997 3,5-Diiodo-L-thyronine (T_2) has selective thyromimetic effects *in vivo* and *in vitro*. *Journal of Molecular Endocrinology* **19** 137–147. (doi:10.1677/jme.0.0190137)

Barish GD, Narkar VA & Evans RM 2006 PPAR delta: a dagger in the heart of the metabolic syndrome. *Journal of Clinical Investigation* **116** 590–597. (doi:10.1172/JCI27955)

Bassett JH, Harvey CB & Williams GR 2003 Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions. *Molecular and Cellular Endocrinology* **213** 1–11. (doi:10.1016/j.mce.2003.10.033)

Bergh JJ, Lin HY, Lansing L, Mohamed SN, Davis FB, Mousa S & Davis PJ 2005 Integrin alphaVbeta3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. *Endocrinology* **146** 2864–2871. (doi:10.1210/en.2005-0102)

Burns KA & Vanden Heuvel JP 2007 Modulation of PPAR activity via phosphorylation. *Biochimica et Biophysica Acta* **1771** 952–960. (doi:10.1016/j.bbalip.2007.04.018)

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL *et al.* 2009 The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* **55** 611–622. (doi:10.1373/clinchem.2008.112797)

Casas F, Pessemesse L, Grandemange S, Seyer P, Baris O, Gueguen N, Ramonatxo C, Perrin F, Fouret G, Lepourry L *et al.* 2009 Overexpression of the mitochondrial T_3 receptor induces skeletal muscle atrophy during aging. *PLoS ONE* **4** e5631. (doi:10.1371/journal.pone.0005631)

Cheng SY, Leonard JL & Davis PJ 2010 Molecular aspects of thyroid hormone actions. *Endocrine Reviews* **31** 139–170. (doi:10.1210/er.2009-0007)

Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159. (doi:10.1016/0003-2697(87)90021-2)

Chu R, Lin Y, Rao MS & Reddy JK 1995 Cooperative formation of higher order peroxisome proliferator-activated receptor and retinoid X receptor complexes on the peroxisome proliferator responsive element of the rat hydratase–dehydrogenase gene. *Journal of Biological Chemistry* **270** 29636–29639. (doi:10.1074/jbc.270.30.17917)

Cimmino M, Mion F, Goglia F, Minaire Y & Geloen A 1996 Demonstration of *in vivo* metabolic effects of 3,5-di-iodothyronine. *Journal of Endocrinology* **149** 319–325. (doi:10.1677/joe.0.1490319)

Cioffi F, Lanni A & Goglia F 2010*a* Thyroid hormones, mitochondrial bioenergetics and lipid handling. *Current Opinion in Endocrinology, Diabetes, and Obesity* **17** 402–407. (doi:10.1097/MED.0b013e32833cf354)

Cioffi F, Zambad SP, Chhipa L, Senese R, Busiello RA, Tuli D, Munshi S, Moreno M, Lombardi A, Gupta RC *et al.* 2010*b* TRC150094, a novel functional analog of iodothyronines, reduces adiposity by increasing energy expenditure and fatty acid oxidation in rats receiving a high-fat diet. *FASEB Journal* **24** 3451–3461. (doi:10.1096/fj.10-157115)

Clayton DF, Weiss M & Darnell JE Jr 1985 Liver-specific RNA metabolism in hepatoma cells: variations in transcription rates and mRNA levels. *Molecular and Cellular Biology* **5** 2633–2641.

Cocco T, Pacelli C, Sgobbo P & Villani G 2009 Control of OXPHOS efficiency by complex I in brain mitochondria. *Neurobiology of Aging* **30** 622–629. (doi:10.1016/j.neurobiolaging.2007.08.002)

Davis P 2010 New insights into thyroid hormone actions and lipid metabolism. *Current Opinion in Endocrinology, Diabetes, and Obesity* **17** 395. (doi:10.1097/MED.0b013e32833e2f0f)

Davis PJ, Leonard JL & Davis FB 2008 Mechanisms of nongenomic actions of thyroid hormone. *Frontiers in Neuroendocrinology* **29** 211–218. (doi:10.1016/j.yfrne.2007.09.003)

Demori I, Bottazzi C, Voci A, Gallo G, Scharf JG & Fugassa E 1997 Tri-iodothyronine increases insulin-like growth factor binding protein-4 expression in rat hepatocytes. *Journal of Endocrinology* **154** 155–165. (doi:10.1677/joe.0.1540155)

Demori I, Gerdoni E, Fugassa E & Voci A 2004 3,5-Diiodothyronine mimics the effect of triiodothyronine on insulin-like growth factor binding protein-4 expression in cultured rat hepatocytes. *Hormone and Metabolic Research* **36** 679–685. (doi:10.1055/s-2004-826017)

Fava G, Ueno Y, Glaser S, Francis H, Demorrow S, Marucci L, Marzioni M, Benedetti A, Venter J, Vaculin B *et al.* 2007 Thyroid hormone inhibits biliary growth in bile duct-ligated rats by PLC/IP(3)/Ca(2+)-dependent downregulation of SRC/ERK1/2. *American Journal of Physiology. Cell Physiology* **292** C1467–C1475. (doi:10.1152/ajpcell.00575.2006)

Feldstein AE, Werneburg NW, Canbay A, Guicciardi ME, Bronk SF, Rydzewski R, Burgart LJ & Gores GJ 2004 Free fatty acids promote hepatic lipotoxicity by stimulating TNF-alpha expression via a lysosomal pathway. *Hepatology* **40** 185–194. (doi:10.1002/hep.20283)

Ferrannini E, Barrett EJ, Bevilacqua S & DeFronzo RA 1983 Effect of fatty acids on glucose production and utilization in man. *Journal of Clinical Investigation* **72** 1737–1747. (doi:10.1172/JCI111133)

Flores-Morales A, Gullberg H, Fernandez L, Stahlberg N, Lee NH, Vennstrom B & Norstedt G 2002 Patterns of liver gene expression governed by TRbeta. *Molecular Endocrinology* **16** 1257–1268. (doi:10.1210/me.16.6.1257)

Gavrilova O, Haluzik M, Matsusue K, Cutson JJ, Johnson L, Dietz KR, Nicol CJ, Vinson C, Gonzalez FJ & Reitman ML 2003 Liver peroxisome proliferator-activated receptor gamma contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass. *Journal of Biological Chemistry* **278** 34268–34276. (doi:10.1074/jbc.M300043200)

Giudetti AM, Leo M, Geelen MJ & Gnoni GV 2005 Short-term stimulation of lipogenesis by 3,5-L-diiodothyronine in cultured rat hepatocytes. *Endocrinology* **146** 3959–3966. (doi:10.1210/en.2005-0345)

Goglia F, Lanni A, Barth J & Kadenbach B 1994 Interaction of diiodothyronines with isolated cytochrome *c* oxidase. *FEBS Letters* **346** 295–298. (doi:10.1016/0014-5793(94)00476-5)

Goglia F, Moreno M & Lanni A 1999 Action of thyroid hormones at the cellular level: the mitochondrial target. *FEBS Letters* **452** 115–120. (doi:10.1016/S0014-5793(99)00642-0)

Grasselli E, Canesi L, Voci A, De Matteis R, Demori I, Fugassa E & Vergani L 2008 Effects of 3,5-diiodo-L-thyronine administration on the liver of high fat diet-fed rats. *Experimental Biology and Medicine* **233** 549–557. (doi:10.3181/0710-RM-266)

Grasselli E, Voci A, Pesce C, Canesi L, Fugassa E, Gallo G & Vergani L 2010 PAT protein mRNA expression in primary rat hepatocytes: effects of exposure to fatty acids. *International Journal of Molecular Medicine* **25** 505–512. (doi:10.3892/ijmm_00000370)

Grasselli E, Voci A, Canesi L, De Matteis R, Goglia F, Cioffi F, Fugassa E, Gallo G & Vergani L 2011 Direct effects of iodothyronines on excess fat storage in rat hepatocytes. *Journal of Hepatology.* In press (doi:10.1016/j.jhep.2010.09.027)

Horst C, Rokos H & Seitz HJ 1989 Rapid stimulation of hepatic oxygen consumption by 3,5-di-iodo-L-thyronine. *Biochemical Journal* **261** 945–950.

Izumo S & Mahdavi V 1988 Thyroid hormone receptor alpha isoforms generated by alternative splicing differentially activate myosin HC gene transcription. *Nature* **334** 539–542. (doi:10.1038/334539a0)

Konig B & Eder K 2006 Differential action of 13-HPODE on PPARalpha downstream genes in rat Fao and human HepG2 hepatoma cell lines. *Journal of Nutritional Biochemistry* **17** 410–418. (doi:10.1016/j.jnutbio.2005.08.011)

Konig B, Koch A, Giggel K, Dordschbal B, Eder K & Stangl GI 2008 Monocarboxylate transporter (MCT)-1 is up-regulated by PPARalpha. *Biochimica et Biophysica Acta* **1780** 899–904. (doi:10.1016/j.bbagen.2008.03.002)

Koopman R, Schaart G & Hesselink MK 2001 Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochemistry and Cell Biology* **116** 63–68. (doi:10.1007/s004180100297)

Lanni A, Moreno M, Lombardi A, de Lange P, Silvestri E, Ragni M, Farina P, Baccari GC, Fallahi P, Antonelli A *et al.* 2005 3,5-Diiodo-L-thyronine powerfully reduces adiposity in rats by increasing the burning of fats. *FASEB Journal* **19** 1552–1554. (doi:10.1096/fj.05-3977fje)

Lauris V, Crettaz M & Kahn CR 1986 Coordinate roles of insulin and glucose on the growth of hepatoma cells in culture. *Endocrinology* **118** 2519–2524. (doi:10.1210/endo-118-6-2519)

Lazar MA 1993 Thyroid hormone receptors: multiple forms, multiple possibilities. *Endocrine Reviews* **14** 184–193. (doi:10.1210/edrv-14-2-184)

Lu C & Cheng SY 2010 Thyroid hormone receptors regulate adipogenesis and carcinogenesis via crosstalk signaling with peroxisome proliferator-activated receptors. *Journal of Molecular Endocrinology* **44** 143–154. (doi:10.1677/JME-09-0107)

Martin G, Schoonjans K, Lefebvre AM, Staels B & Auwerx J 1997 Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARalpha and PPARgamma activators. *Journal of Biological Chemistry* **272** 28210–28217. (doi:10.1074/jbc.272.45.28210)

Mollica MP, Lionetti L, Moreno M, Lombardi A, De Lange P, Antonelli A, Lanni A, Cavaliere G, Barletta A & Goglia F 2009 3,5-Diiodo-L-thyronine, by modulating mitochondrial functions, reverses hepatic fat accumulation in rats fed a high-fat diet. *Journal of Hepatology* **51** 363–370. (doi:10.1016/j.jhep.2009.03.023)

Moreno M, Lanni A, Lombardi A & Goglia F 1997 How the thyroid controls metabolism in the rat: different roles for triiodothyronine and diiodothyronines. *Journal of Physiology* **2** 529–538. (doi:10.1111/j.1469-7793.1997.529bb.x)

Moreno M, Lombardi A, Beneduce L, Silvestri E, Pinna G, Goglia F & Lanni A 2002 Are the effects of T_3 on resting metabolic rate in euthyroid rats entirely caused by T_3 itself? *Endocrinology* **143** 504–510. (doi:10.1210/en.143.2.504)

Moreno M, Lombardi A, Silvestri E, Senese R, Cioffi F, Goglia F, Lanni A & de Lange P 2010 PARs: nuclear receptors controlled by, and controlling, nutrient handling through nuclear and cytosolic signaling. *PPAR Research* **2010**, Article ID 435689. (doi:10.1155/2010/435689)

Munoz A, Hoppner W, Sap J, Brady G, Nordstrom K, Seitz HJ & Vennstrom B 1990 The chicken c-erbA alpha-product induces expression of thyroid hormone-responsive genes in 3,5,3′-triiodothyronine receptor-deficient rat hepatoma cells. *Molecular Endocrinology* **4** 312–320. (doi:10.1210/mend-4-2-312)

Nagasawa T, Inada Y, Nakano S, Tamura T, Takahashi T, Maruyama K, Yamazaki Y, Kuroda J & Shibata N 2006 Effects of bezafibrate, PPAR pan-agonist, and GW501516, PPARdelta agonist, on development of steatohepatitis in mice fed a methionine- and choline-deficient diet. *European Journal of Pharmacology* **536** 182–191. (doi:10.1016/j.ejphar.2006.02.028)

Nobes CD, Brown GC, Olive PN & Brand MD 1990 Non-ohmic proton conductance of the mitochondrial inner membrane in hepatocytes. *Journal of Biological Chemistry* **265** 12903–12909.

Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29** e45. (doi:10.1093/nar/29.9.e45)

Qin B, Anderson RA & Adeli K 2008 Tumor necrosis factor-alpha directly stimulates the overproduction of hepatic apolipoprotein B100-containing VLDL via impairment of hepatic insulin signaling. *American Journal of Physiology. Gastrointestinal and Liver Physiology* **294** G1120–G1129. (doi:10.1152/ajpgi.00407.2007)

Ramzan R, Staniek K, Kadenbach B & Vogt S 2010 Mitochondrial respiration and membrane potential are regulated by the allosteric ATP-inhibition of cytochrome *c* oxidase. *Biochimica et Biophysica Acta* **1797** 1672–1680. (doi:10.1016/j.bbabio.2010.06.005)

Ravera S, Panfoli I, Calzia D, Aluigi MG, Bianchini P, Diaspro A, Mancardi G & Morelli A 2009 Evidence for aerobic ATP synthesis in isolated myelin vesicles. *International Journal of Biochemistry and Cell Biology* **41** 1581–1591. (doi:10.1016/j.biocel.2009.01.009)

Ricchi M, Odoardi MR, Carulli L, Anzivino C, Ballestri S, Pinetti A, Fantoni LI, Marra F, Bertolotti M, Banni S *et al.* 2009 Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *Journal of Gastroenterology and Hepatology* **24** 830–840. (doi:10.1111/j.1440-1746.2008.05733.x)

Roberts LD, Hassall DG, Winegar DA, Haselden JN, Nicholls AW & Griffin JL 2009 Increased hepatic oxidative metabolism distinguishes the action of peroxisome proliferator-activated receptor delta from peroxisome proliferator-activated receptor gamma in the ob/ob mouse. *Genome Medicine* **1** 115. (doi:10.1186/gm115)

Rooda SJ, van Loon MA & Visser TJ 1987 Metabolism of reverse triiodothyronine by isolated rat hepatocytes. *Journal of Clinical Investigation* **79** 1740–1748. (doi:10.1172/JCI113014)

Schadinger SE, Bucher NL, Schreiber BM & Farmer SR 2005 PPARgamma2 regulates lipogenesis and lipid accumulation in steatotic hepatocytes. *American Journal of Physiology. Endocrinology and Metabolism* **288** E1195–E1205. (doi:10.1152/ajpendo.00513.2004)

Schoonjans K, Staels B & Auwerx J 1996 The peroxisome proliferator activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation. *Biochimica et Biophysica Acta* **1302** 93–109. (doi:10.1016/0005-2760(96)00066-5)

Shan W, Nicol CJ, Ito S, Bility MT, Kennett MJ, Ward JM, Gonzalez FJ & Peters JM 2008 Peroxisome proliferator-activated receptor-beta/delta protects against chemically induced liver toxicity in mice. *Hepatology* **47** 225–235. (doi:10.1002/hep.21925)

Sterling K & Brenner MA 1995 Thyroid hormone action: effect of triiodothyronine on mitochondrial adenine nucleotide translocase *in vivo* and *in vitro*. *Metabolism* **44** 193–199. (doi:10.1016/0026-0495(95)90264-3)

Sterling K, Campbell GA & Brenner MA 1984 Purification of the mitochondrial triiodothyronine (T_3) receptor from rat liver. *Acta Endocrinologica* **105** 391–397. (doi:10.1530/acta.0.1050391)

Viswakarma N, Jia Y, Bai L, Vluggens A, Borensztajn J, Xu J & Reddy JK 2010 Coactivators in PPAR-regulated gene expression. *PPAR Research* **2010**, Article ID 250126. (doi:10.1155/2010/250126)

Vock C, Gleissner M, Klapper M & Doring F 2008 Oleate regulates genes controlled by signaling pathways of mitogen-activated protein kinase, insulin, and hypoxia. *Nutrition Research* **28** 681–689. (doi:10.1016/j.nutres.2008.06.010)

Wang YX, Lee CH, Tiep S, Yu RT, Ham J, Kang H & Evans RM 2003 Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. *Cell* **113** 159–170. (doi:10.1016/S0092-8674(03)00269-1)

Waters KM, Miller CW & Ntambi JM 1997 Localization of a negative thyroid hormone-response region in hepatic stearoyl-CoA desaturase gene 1. *Biochemical and Biophysical Research Communications* **233** 838–843. (doi:10.1006/bbrc.1997.6550)

Weiss RE, Murata Y, Cua K, Hayashi Y, Seo H & Refetoff S 1998 Thyroid hormone action on liver, heart, and energy expenditure in thyroid hormone receptor beta-deficient mice. *Endocrinology* **139** 4945–4952. (doi:10.1210/en.139.12.4945)

Wiechelman KJ, Braun RD & Fitzpatrick JD 1988 Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. *Analytical Biochemistry* **175** 231–237. (doi:10.1016/0003-2697(88)90383-1)

Wrutniak C, Cassar-Malek I, Marchal S, Rascle A, Heusser S, Keller JM, Flechon J, Dauca M, Samarut J, Ghysdael J *et al.* 1995 A 43-kDa protein related to c-Erb A alpha 1 is located in the mitochondrial matrix of rat liver. *Journal of Biological Chemistry* **270** 16347–16354. (doi:10.1074/jbc.270.27.16347)

Wrutniak-Cabello C, Casas F & Cabello G 2001 Thyroid hormone action in mitochondria. *Journal of Molecular Endocrinology* **26** 67–77. (doi:10.1677/jme.0.0260067)

Xu HE, Lambert MH, Montana VG, Parks DJ, Blanchard SG, Brown PJ, Sternbach DD, Lehmann JM, Wisely GB, Willson TM *et al.* 1999 Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Molecular Cell* **3** 397–403. (doi:10.1016/S1097-2765(00)80467-0)

Yao-Borengasser A, Rassouli N, Varma V, Bodles AM, Rasouli N, Unal R, Phanavanh B, Ranganathan G, McGehee RE Jr & Kern PA 2008 Stearoyl-coenzyme A desaturase 1 gene expression increases after pioglitazone treatment and is associated with peroxisomal proliferator-activated receptor-gamma responsiveness. *Journal of Clinical Endocrinology* **93** 4431–4439. (doi:10.1210/jc.2008-0782)

Ye L, Li YL, Mellstrom K, Mellin C, Bladh LG, Koehler K, Garg N, Garcia Collazo AM, Litten C, Husman B *et al.* 2003 Thyroid receptor ligands. 1. Agonist ligands selective for the thyroid receptor beta1. *Journal of Medicinal Chemistry* **46** 1580–1588. (doi:10.1021/jm021080f)

Yu S, Matsusue JK, Kashireddy P, Cao WQ, Yeldandi V, Yeldandi AV, Rao MS, Gonzalez FJ & Reddy JK 2003 Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor gamma1 (PPARgamma1) overexpression. *Journal of Biological Chemistry* **278** 498–505. (doi:10.1074/jbc.M210062200)

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