3,5-Diiodo-L-thyronine modulates the expression of genes of lipid metabolism in a rat model of fatty liver

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Abstract

Recent reports demonstrated that 3,5-diiodo-L-thyronine (T₂) was able to prevent lipid accumulation in the liver of rats fed a high-fat diet (HFD). In this study, we investigated how the rat liver responds to HFD and T₂ treatment by assessing the transcription profiles of some genes involved in the pathways of lipid metabolism: oxidation, storage and secretion. The mRNA levels of the peroxisome proliferator-activated receptors (PPAR α , PPAR γ and PPAR δ), and of their target enzymes acyl-CoA oxidase and stearoyl-CoA desaturase were evaluated by real-time RT-PCR. Moreover, the expression of the adipose triglyceride lipase involved in lipid mobilisation, of the main PAT proteins acting in lipid droplet (LD) turnover, and of apoprotein B (apo B), the major

protein component of very low-density lipoproteins (VLDLs) were analysed. Overall, our data demonstrated that T₂ administration to HFD rats counteracts most of the hepatic transcriptional changes that occurred in response to the excess exogenous fat. In particular, our results suggest that T₂ may prevent the pathways leading to lipid storage in LDs, promote the processes of lipid mobilisation from LDs and secretion as VLDL, in addition to the stimulation of pathways of lipid oxidation. In conclusion, our findings might give an insight into the mechanisms underlying the anti-steatotic ability of T₂ and help to define the potential therapeutic role of T₂ for preventing or treating liver steatosis.

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Introduction

Hepatic steatosis refers to an excess accumulation of lipids, primarily triglycerides (TAGs) and it is a major element of non-alcoholic fatty liver disease (NAFLD; Unger *et al.* 2010). Lipid metabolism is mainly regulated by the peroxisome proliferator-activated receptors (PPARs), a subfamily of lipid-activated transcription factors, consisting of three members, PPAR α , PPAR γ and PPAR δ , with distinct functional roles (Viswakarma *et al.* 2010, Wanders *et al.* 2010). In the liver, PPAR α is the master regulator of free fatty acid (FFA) oxidation systems, and decreases FFA efflux (Reddy 2001, Pyper *et al.* 2010). Also PPAR δ induces expression of genes required for FFA oxidation (Evans *et al.* 2004, Pyper *et al.* 2010). Conversely, PPAR γ is mainly expressed in adipose tissue, but it promotes lipid accumulation also in hepatocytes where it plays anti-inflammatory effects by both reducing inflammatory cytokine and chemokine expression and preventing the activation of pro-inflammatory transcription factors (Delerive *et al.* 2001, Straus & Glass

2007, Kapadia *et al.* 2008). In summary, PPAR α and PPAR δ mainly participate in energy burning, whereas PPAR γ is critical in regulating energy storage (Musso *et al.* 2009), even though an overlapping in their function has been widely reported.

Excess TAGs are stored under form of cytosolic lipid droplets (LDs) regulating storage and traffic of lipids (for a review see Ducharme & Bickel (2008) and Olofsson *et al.* (2009)). Typically, LDs exhibit a core of TAGs and cholesteryl esters surrounded by a phospholipid monolayer that contains numerous proteins including the ‘PAT proteins’ with structural, regulatory or enzymatic functions (Bickel *et al.* 2009). A previous study of our group (Grasselli *et al.* 2010) showed that, in rat hepatocytes, lipid overload was associated with altered expression of three PAT proteins: the adipocyte differentiation-related protein (ADRP, also called PLIN2), the oxidative tissue-enriched PAT protein (OXPAT or PLIN5) and the tail-interacting protein (TIP47 or PLIN3; Kimmel *et al.* 2010), which are thought to be involved in LD turnover together with the endoplasmic reticulum (ER)

resident protein fat-inducing transcript 2 (FIT2; Kadereit *et al.* 2008, Sun *et al.* 2010).

In the liver, TAGs stored in LDs may be mobilised by lipases that trigger FFAs either for oxidation or for secretion as very low-density lipoproteins (VLDLs). The relative abundance of PAT proteins on LDs is decisive for the regulation of the action of lipases (Zimmermann *et al.* 2009). Recently, discovered adipose triglyceride lipase (ATGL) selectively performs the first step in TAG hydrolysis (Duncan *et al.* 2007, Zechner *et al.* 2009), and is constitutively expressed in the liver of mammals (Reid *et al.* 2008).

To be secreted by the liver through exocytosis, TAGs must be packaged together with apoprotein B (apo B), the major protein component of VLDL (Vance & Vance 1990). Recent studies have pointed out that hepatic secretion of VLDL is increased during NAFLD, even though it seems to be inadequate to match the increased TAG availability (Zivkovic *et al.* 2007).

Thyroid hormones (THs) are major modulators of energy homeostasis, and their role in lipid metabolism has supported their potential use as drugs to promote weight loss. Several evidences suggested that 3,5-diiodo-L-thyronine (T₂), a naturally occurring iodothyronine, mimics several effects of 3,3',5-L-triiodothyronine (T₃) on energy metabolism without inducing thyrotoxic effects (Lanni *et al.* 1994, Cimmino *et al.* 1996, Lombardi *et al.* 1998). Also T₂, like T₃, is able to stimulate both resting metabolic rate and mitochondrial activity in hypothyroid rats (Moreno *et al.* 1997). However, although it is likely that iodothyronines regulate energy metabolism by both short- and long-term actions, the molecular mechanisms are still unclear (Goglia 2005). Moreover, long-term administration of T₂ to rats fed high-fat diet (HFD) was able to counteract the body weight (BW) and fat mass gain, as well as lipid accumulation and oxidative stress in the liver (Lanni *et al.* 2005, Grasselli *et al.* 2008). T₂ affects liver metabolism by increasing mitochondrial FFA oxidation and mitochondrial uncoupling (Lanni *et al.* 2005, Grasselli *et al.* 2008), but exerts metabolic effects also in skeletal muscle by increasing mitochondrial oxidative capacity (Lombardi *et al.* 2007). In a recent study (Grasselli *et al.* 2011b), we demonstrated that the lipid-lowering effect of both T₂ and T₃ occurs via non-receptor-mediated mechanisms that seem to involve both a short-term action by stimulation of mitochondrial O₂ consumption, and a long-term action by differential transcriptional effects on PPARs.

In the attempt to clarify the mechanisms underlying the anti-steatotic ability of T₂ better, in this study we analysed the mRNA expression of several genes involved in three processes of lipid metabolism in the rat liver: oxidation, storage and secretion. Using a validated *in vivo* model of fatty liver, we show that HFD induces the expression of genes of lipid storage and catabolism, whereas T₂ prevents these changes. At the same time, T₂ stimulates mechanisms of FFA oxidation and lipid secretion thus ameliorating the status of hepatic steatosis as well as the pre-inflammatory condition induced by HFD.

Materials and Methods

Chemicals

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

Animals and treatments

Animal maintenance and treatment were carried out according to the guidelines of the European Community Council for animal care and use, as described elsewhere (Grasselli *et al.* 2008). Briefly, 24 male Wistar rats (Harlan, San Pietro al Natisone, Italy), housed in individual cages, were randomly divided into four groups (each group, six animals). In each group, BW was normally distributed, and BW means were similar for all animals. The first group (C) received a standard diet (15.88 kJ gross energy/g); the second group (D) was fed a HFD (19.85 kJ gross energy/g); the third group (DT) received the same HFD and a simultaneous daily i.p. injection of T₂ (25 µg/100 g BW); and the fourth group (CT) received a standard diet and a simultaneous daily i.p. injection of T₂ (25 µg/100 g BW). C and D rats were daily i.p. injected with the same volume of physiological saline solution. After 30 days of treatment, rats were anaesthetised and killed by cervical dislocation. Before blood and tissue sampling, rats were not fasted to avoid possible interference with the effects of HFD. Blood was collected and serum concentrations of TAGs, cholesterol and glucose were measured using a Hitachi 7170 clinical analyser (Hitachi) by following standard procedures. Immediately after death, livers were rapidly dissected, weighed, cut into small pieces, quickly frozen in liquid nitrogen and stored at -80 °C until use.

Liver morphological analysis and lipid staining

To detect fat accumulation in the livers, frozen tissue sections were stained with Oil Red O (ORO) according to the method described by Koopman *et al.* (2001). Briefly, liver cryosections were fixed in 10% formalin at room temperature for 15 min, then dipped in 70% isopropanol for 3 min. Afterwards, slides were immersed in 1% ORO solution for 15 min, washed first in 70% isopropanol then in distilled water, and mounted using aqueous mounting medium.

Haematoxylin-eosin staining of liver sections was done using standard protocols (Grasselli *et al.* 2011a). Livers were fixed, dehydrated, embedded in paraffin, sectioned (8 µm) and stained with haematoxylin-eosin. Slides were examined by Nikon Eclipse E80i light microscope (Nikon, Japan).

Immunohistochemical staining for ADRP

Paraffin-embedded liver sections (4 µm) were incubated with anti-ADRP polyclonal antibody (clone GP40-mN1, Fitzgerald Industries International, Concord, MA, USA). The immunoreaction was detected with the avidin-biotin-peroxidase

complex (ABC) method (Hsu *et al.* 1981) using ABC Vectastain-Elite Kit (Vectastain ABC Elite Kit, Vector Labs, Burlingame, CA, USA), as described previously (Grasselli *et al.* 2011a). The liver sections were counterstained with haematoxylin to reveal nuclei and mounted in Eukitt (Kindler, Freiburg, Germany). Omission of the primary antibody served as negative control.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from aliquots of frozen pooled tissues from each experimental group by Trizol Reagent (Sigma-Aldrich) according to the manufacturer's instructions. First-strand cDNA was synthesised from 2 µg of total RNA using 200 RevertAid H-Minus M-MuLV Reverse Transcriptase (Fermentas, Hannover, MD, USA) 200 ng oligo(dT)18-mer, 1 mM dNTPs (Invitrogen), 100 U RNase inhibitor (Fermentas), as described elsewhere (Grasselli *et al.* 2011b). Quantitative RT-PCR (RT-qPCR) was performed in quadruplicate in a final volume of 25 µl containing 0.3 µM of each primer, 10 ng cDNA, 1× SybrGreen PCR Master Mix and were analysed in 96-well optical reaction by 7900 HT fast real-time PCR system (Applied Biosystems, Monza, Italy) by the thermal protocol described previously (Vergani *et al.* 2011). Primers were synthesised by TibMolBiol custom oligosynthesis service (Genova, Italy). Primers (Table 1) were designed *ad hoc* starting from the coding sequences of *Rattus*

norvegicus available on the GenBank database (<http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html>). A melting curve of RT-PCR products (55–94 °C) was also performed to ensure the absence of artefacts. The quantification cycle (C_q) represents the cycle number at which the amount of amplified target reaches the fixed threshold (Bustin 2010). The relative quantity of target mRNA was calculated by the comparative C_q method and was normalised for the expression of Gapdh gene (Pfaffl 2001). The normalised expression was thus expressed as relative quantity of mRNA (fold induction) with respect to controls (C).

Statistical analysis

Data on RT-qPCR are means ± s.d. of three independent RNA extractions performed in quadruplicate. Enzyme activities are means ± s.d. of three independent experiments performed in triplicate. Statistical analysis was performed by ANOVA followed by Bonferroni *post-hoc* test (Instat Software, GraphPad Software, Inc., San Diego, CA, USA).

Results

Liver lipid accumulation, histology and inflammation

Table 2 summarises BW gain and serum values of TAGs, cholesterol and glucose in control rats (C), HFD-fed rats (D),

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

Primer names (gene names)	Primer sequences (5' → 3')	Annealing temperature (°C)	Product length (bp)	Accession ID	References
GAPDH (<i>Gapdh</i>)	F: GACCCCTTCATTGACCTCAAC R: CGCTCCTGGAAGATGGTGATGGG	60	136	DQ403053	Grasselli <i>et al.</i> (2008)
IL1β (<i>Il1b</i>)	F: AGGCATAACAAGCTCATCTGGG R: CATCTGGACAGCCCAAGTCAAGG	56	176	NM_031512	This work
PPARα (<i>Ppara</i>)	F: CCCCACTTGAAGCAGATGACC R: CCCTAAGTACTGGTAGTCCGC	60	139	NM_013196	Grasselli <i>et al.</i> (2011a)
PPARδ (<i>Ppard</i>)	F: AATGCCTACCTGAAAACTTCAAC R: TGCCTGCCACAGCGTCTCAAT	60	96	AJ306400.1	Grasselli <i>et al.</i> (2011a)
PPARγ (<i>Pparg</i>)	F: CGGAGTCTCCAGCTGTTCGCC R: GGCTCATATCTGTCTCCGTCTTC	60	116	Y12882	Grasselli <i>et al.</i> (2011a)
SCD-1 (<i>Scd1</i>)	F: CACACGCCGACCCTCACAACT R: TCCGCCCTTCTCTTTGACAGCC	60	97	AF509569	Grasselli <i>et al.</i> (2011b)
AOX (<i>Acox</i>)	F: GCGCAAGGAGCGGGCCTCC R: CTCGACGGCGCGGGGTATTC	64	84	AAA40666	This work
ATGL (<i>Pnpla2</i>)	F: CGGTGGATGAAGGAGCAGACA R: TGGCACAGACGGCAGAGACT	60	137	NM_001108509	Romero <i>et al.</i> (2009)
ADRP (<i>Plin2</i>)	F: CCGAGCGTGGTGACGAGGG R: GAGGTCACGGTCTCACTCCC	60	148	AAH85861	Grasselli <i>et al.</i> (2010)
OXPAT (<i>Plin5</i>)	F: GGATGTCCGGTGATCAGAC R: GTGCACGTGGCCCTGACCAG	60	96	XM_576698	Grasselli <i>et al.</i> (2010)
TIP47 (<i>Plin3</i>)	F: GGAAGTGGTGTCATCAACAG R: GGTCACATCCACTGCTCCTG	60	108	316130	Grasselli <i>et al.</i> (2010)
Fit2 (<i>Fitm2</i>)	F: AAGGGGGCTCTGGTACGGCT R: TGGCTGCGATCTGTCTTACC	60		NM_001107799.1	This work
Apo B (<i>Apob</i>)	F: CGTGGGCTCCAGATTCTA R: TCACCAGTCATTCTGCCTTTG	60	71	NM_019287.2	Spann <i>et al.</i> (2006)

F, forward; R, reverse.

Table 2 Effects of high-fat diet (HFD) and 3,5-diiodo-L-thyronine on body weight gain and serum values

Treatments	Body weight gain	Triglycerides	Cholesterol	Glucose
C	20.3±4.3	174±26	70±7	167±15
D	33.7±2.6**	266±38**	74±19	181±25
DT	24.3±4.9 [‡]	191±57	56±19	166±17
CT	25.8±4.6 [‡]	129±14	73±7	155±10

Twenty-four male Wistar rats were divided into four groups; serum concentrations of triglycerides, cholesterol and glucose were measured using a Hitachi 7170 mean serum values±s.d. of body weight gain (%) triglycerides, cholesterol and glucose (mg/dl) in standard diet-fed rats (C), HFD (D), HFD+T₂ (DT) and C+T₂ (CT) rats. Significant differences are reported: C vs D (**P≤0.01) and D vs DT or D vs CT ([‡]P≤0.05).

HFD-fed rats treated with T₂ (DT) and standard diet-fed rats treated with T₂ (CT; Grasselli *et al.* 2008). D rats showed a significant increment in mean BW with respect to control (C) rats (+13%; P≤0.01). Such an increase was prevented by T₂ administration to D rats (−28% with respect to D rats; P≤0.01). A marked increase in TAG levels (+52%; P≤0.01) was observed in D rats with respect to C rats, while in DT and CT rats the values were similar to those of C rats. No changes in cholesterol, and glucose levels were detected in all experimental groups.

In this study, ORO staining of liver sections confirmed that HFD feeding (D rats) increased hepatic lipid accumulation with respect to controls (C), and this effect was prevented by simultaneous T₂ administration (DT; Fig. 1A).

When liver histology was assessed by haematoxylin–eosin staining, the tissue of control rats (C) showed an array of wheel-shaped cells along the centrilobular vein, with a clear lobular structure, well-arranged hepatic cords and cells with round and central nuclei (Fig. 1B). No significant histological changes could be appreciated in different experimental conditions, including the HFD group, despite the increase in lipid accumulation.

Since fatty liver could be associated with inflammatory condition, the expression of interleukin 1β (IL1β) mRNA was assessed as a marker of liver inflammation. In liver of D rats, a significant increase in IL1β mRNA expression was detected (about 2.2-fold induction with respect to C; P≤0.001; Fig. 1C). Administration of T₂ to D rats prevented the up-regulation of IL1β expression (about 1.2-fold induction with respect to C, −50% with respect to D rats; P≤0.001). No changes in IL1β expression were observed in CT rats with respect to C.

Expression of PPARs

The relative abundance of mRNA transcripts for PPARα, PPARγ and PPARδ was assessed by RT-qPCR in the liver of control rats (Fig. 2A). The C_q values were about 23.6 for PPARα, 25.1 for PPARγ and 25.6 for PPARδ, thus pointing at the following relative abundance of PPAR isoform mRNA

in rat liver: PPARα>PPARγ≅PPARδ, the last two differing only for 0.5 C_q.

The liver of D rats exhibited a significant increase in the level of PPARγ transcripts (Fig. 2B) in comparison to controls (about 1.8-fold induction, P≤0.05), and T₂ administration to D rats (DT) resulted in a further increase in PPARγ mRNA expression (about 3.2-fold induction with respect to C, +205% with respect to D rats; P≤0.001). By contrast, PPARδ expression did not change significantly in the liver of D rats (Fig. 2B), but it was reduced in DT rats (about 0.6-fold induction with respect to C, −41% with respect to D rat; P≤0.01).

Figure 1 Effects of high-fat diet (HFD) and T₂ on rat liver. (A) Representative image of ORO-stained liver cryosections from standard diet-fed rats (C), HFD (D) and HFD+T₂ (DT). Magnification 100×; bar=10 μm (cv, centrilobular vein). (B) Haematoxylin–eosin staining of rat liver sections of standard diet-fed rats (C), HFD (D) and HFD+T₂ (DT). Light microscopic images were acquired on the paraffin-embedded tissues. Magnification 40×; bar=30 μm; arrows indicate lipid droplets. (C) Relative mRNA expression of IL1β quantified by RT-qPCR in HFD (D), HFD+T₂ (DT) and C+T₂ (CT) rats with respect to standard diet-fed rats (C). Data (mean±s.d.) are reported as fold induction with respect to controls after normalisation for GAPDH mRNA. Significant differences are reported: C vs D (***) and D vs DT (###P≤0.001).

Figure 2 Effects of high-fat diet (HFD) and T_2 on hepatic expression of PPARs. (A) Relative abundance of the PPAR isoforms in the liver of control rats. The amplification curves of PPAR α , PPAR δ and PPAR γ were acquired by RT-qPCR. X-axis, amplification cycle number and Y-axis, normalised fluorescence signal. (B) Relative mRNA expression of PPAR α , PPAR δ , and PPAR γ , and quantified by RT-qPCR in HFD (D), HFD + T_2 (DT) and C + T_2 (CT) rats with respect to standard diet-fed rats (C). Data (mean $\pm$ s.d.) are reported as fold induction with respect to controls after normalisation for GAPDH mRNA. Significant differences are reported: C vs D or vs CT (*** $P \leq 0.001$, ** $P \leq 0.01$ and * $P \leq 0.05$) and D vs DT (*** $P \leq 0.001$, ** $P \leq 0.01$ and * $P \leq 0.05$).

Similar effects on PPAR expression were observed when T_2 was administered to control rat (CT). In fact, T_2 treatment resulted in up-regulation of PPAR γ transcripts (about 1.8-fold induction with respect to C; $P \leq 0.05$) and down-regulation of PPAR δ expression (about 0.5-fold induction with respect to C; $P \leq 0.01$).

The transcription profile of PPAR γ showed a trend distinct from that previously observed for the lipolytic transcription factor PPAR α (inset in Fig. 2A; Grasselli *et al.* 2008). PPAR α transcription was in fact up-regulated in D rats (about 0.3-fold induction with respect to C; $P \leq 0.001$), and T_2 administration (DT) prevented this up-regulation (1.7-fold induction with respect to C, -45% with respect to D rats; $P \leq 0.05$), but, differently from PPAR γ , expression of PPAR α did not change in CT rats.

Expression of genes involved in lipid metabolism

Regarding the lipogenic pathways, transcription of the stearoyl-CoA desaturase (SCD-1), a desaturase that is required for the TAG synthesis, showed a significant decrease in the livers of D rats (about 0.4-fold induction with respect to C; $P \leq 0.001$; Fig. 3A). Such a decrease was prevented by T_2 administration (DT; about 1.0-fold induction with respect to C, +164% with respect to D; $P \leq 0.001$). T_2 *per se* did not affect SCD-1 transcription in control rats (CT).

With regards to lipolytic pathways, the mRNA expression of the peroxisomal enzyme acyl-CoA oxidase (AOX) did not show a significant increase in D rats compared with controls (Fig. 3B). On the other hand, T_2 administration to D rats induced a large increase in hepatic AOX mRNA levels (about 2.8-fold induction with respect to C, +119% with respect to D; $P \leq 0.01$), whereas T_2 *per se* did not affect AOX expression in CT rats.

In the liver of D rats, a dramatic increase in mRNA expression of ATGL was detected (about 7.7-fold induction with respect to C; $P \leq 0.001$; Fig. 3C). Administration of T_2 to D rats prevented the up-regulation of ATGL expression (about 0.70-fold induction with respect to C, -91% with respect to D rats; $P \leq 0.001$). No changes in ATGL expression were observed in CT rats.

Expression of proteins involved in LD turnover and lipoprotein secretion

The liver of D rats showed a significant up-regulation of both ADRP and OXPAT mRNA expression (about 1.9-fold induction with respect to C, $P \leq 0.01$, for OXPAT; about 1.6-fold induction with respect to C, $P \leq 0.001$, for ADRP; Fig. 4A). This diet-induced up-regulation was prevented by T_2 administration (DT; about 1.1-fold induction with respect to C, for OXPAT; about 0.8-fold induction with respect to C, for ADRP; -43% $P \leq 0.01$ and -50% $P \leq 0.001$, respectively, with respect to D). Conversely, neither HFD nor T_2 , nor their combination significantly affected the mRNA expression of the other PAT protein TIP47.

As reported in Fig. 4B, the expression of FIT2, an ER resident protein involved in LD biogenesis, was not modified in D rats. However, FIT2 expression was significantly down-regulated both in DT (about 0.6-fold induction with respect to C, -46% with respect to D; $P \leq 0.001$) and in CT (about 0.7-fold induction in CT with respect to C; $P \leq 0.05$) rats.

To better investigate alterations of hepatic LDs in the different experimental conditions, possible changes in their size and number were assessed by ADRP immunostaining (Fig. 4C). In liver of C rats, little or no ADRP staining was present in the hepatic lobules. By contrast, the liver of D rats exhibited numerous and large ADRP-positive vesicles that were preferentially located at the periphery of the cell. T_2 administration to D rats prevented this accumulation of positive vesicles.

Figure 3 Effects of high-fat diet (HFD) and T₂ on hepatic expression of genes involved in lipid metabolism. Relative mRNA expression of stearoyl-CoA desaturase (SCD-1) (A), acyl-CoA oxidase (AOX) (B) and adipose triglyceride lipase (ATGL) (C) quantified by RT-qPCR in HFD (D), HFD + T₂ (DT) and C + T₂ (CT) rats with respect to standard diet-fed rats (C). Data (mean ± s.d.) are reported as fold induction with respect to controls after normalisation for GAPDH mRNA. Significant differences are reported: C vs D or vs CT (****P* ≤ 0.001) and D vs DT (###*P* ≤ 0.001 and ##*P* ≤ 0.01).

With regards to lipid secretion, the liver of D rats did not show significant changes in mRNA expression of apo B, the major protein component of VLDL (Fig. 4D). Administration of T₂ to D rats up-regulated mRNA expression of apo B (about 1.7-fold induction with respect to C, +79% with respect to D; *P* ≤ 0.001). No changes in apo B mRNA levels were observed in CT animals with respect to C.

Discussion

In previous studies (Lanni *et al.* 2005, Grasselli *et al.* 2008), T₂ administration was shown to prevent the HFD-induced BW gain and hepatic steatosis, as well as the oxidative stress associated with the fatty liver condition, thus suggesting the therapeutic potential role of T₂ for preventing or treating steatosis (Mollica *et al.* 2009). In this study, the effects of T₂ on the liver of rat fed HFD were examined by assessing the transcription profiles of some important genes involved in lipid metabolism.

As previously reported (Grasselli *et al.* 2008), histologically evident hepatic microvesicular steatosis was induced in HFD rats and the effect was prevented by concomitant T₂ administration. Typically, the excess fat accumulation stimulates FFA oxidation likely leading to oxidative stress that may induce the release of several pro-inflammatory cytokines

(such as IL1β and IL6) by Kupffer cells and hepatocytes (Day 2006). Even if in our experimental model the increase in hepatic lipid accumulation was not associated with marked changes in the physiological morphology of the liver, a significant increase in transcription of IL1β was observed, indicating the initiation of inflammatory processes induced by diet. T₂ administration to HFD rats not only prevented lipid accumulation, but also IL1β up-regulation.

In the liver of control rats, all PPAR isoforms are constitutively expressed, but PPARα transcripts are more abundant than their PPARγ and PPARδ counterparts. Associated with fat accumulation, the liver of D rats showed a large increase in expression of PPARα, and a smaller increase in that of PPARγ. This can be explained by the role of PPARα in stimulating the activities of the FFA oxidation systems (Duval *et al.* 2007, Pyper *et al.* 2010) and of PPARγ in promoting fat accumulation in LDs (Gavrilova *et al.* 2003, Browning & Horton 2004). By contrast, the excess fat did not significantly alter the hepatic expression of PPARδ.

Despite the increase in PPARα and PPARγ expression induced by diet, we observed a slight, but not significant increment in mRNA expression of the peroxisomal enzyme AOX, which is a target of both PPARs (Rogue *et al.* 2011). However, an increase in both activity (Grasselli *et al.* 2008) and protein level (Silvestri *et al.* 2010) of catalase in D rats was previously reported, suggesting that peroxisomal β-oxidation is stimulated as a response to HFD. Besides, other reports indicated that mitochondrial FFA oxidation is also widely involved in fat liver condition (Serviddio *et al.* 2008). The presence of an excess of fat in the liver induced a down-regulation of the lipogenic enzyme SCD-1 that is target of PPARs. Therefore, it is likely that the rat liver responds to the excess fat both by activating mechanisms of lipid storage and FFA oxidation, as well as by inhibiting pathways of lipid synthesis.

In association with its anti-steatotic effects, T₂ prevented the HFD-induced up-regulation of PPARα, further up-regulated expression of PPARγ and down-regulated expression of PPARδ. Moreover, T₂ *per se* up-regulated PPARγ and down-regulated PPARδ expression in the liver of CT rats, but it did not affect PPARα expression. These results indicate that in rat liver, PPARγ and PPARδ are direct targets for the action of T₂.

The increased expression of PPARγ in liver of DT rats requires some further considerations. Although PPARγ is typically considered a key factor in regulation of lipogenesis, it is a pleiotropic transcription factor that regulates a variety of genes involved in virtually all pathways of lipid metabolism, including local FFA release from circulating lipoproteins (Schoonjans *et al.* 1996), FFA uptake (Motojima *et al.* 1998, Chui *et al.* 2005), FFA synthesis (Castelein *et al.* 1994, Jitrapakdee *et al.* 2005), LD stabilisation (Arimura *et al.* 2004, Schadinger *et al.* 2005) and also FFA oxidation (Bogacka *et al.* 2005). A recent study of comparative global gene expression profiles induced by PPARγ and dual PPARα/γ agonists in rat hepatocytes showed that PPARγ shares many

Figure 4 Effects of high-fat diet (HFD) and T_2 on expression genes involved in LD turnover and lipoprotein secretion. Relative mRNA expression of ADRP, OXPAT, TIP47 (A) and FIT2 (B), quantified by RT-qPCR in HFD (D), HFD + T_2 (DT) and C + T_2 (CT) rats with respect to standard diet-fed rats (C). Data (mean $\pm$ s.d.) are reported as fold induction with respect to controls after normalisation for GAPDH mRNA. Significant differences are reported: C vs D or vs CT (***) $P \leq 0.001$, ** $P \leq 0.01$ and * $P \leq 0.05$ and D vs DT (### $P \leq 0.001$ and ## $P \leq 0.01$). (C) Immunohistochemical staining for ADRP of liver sections from standard diet-fed rats (C), HFD (D) and HFD + T_2 (DT). Nuclear staining with haematoxylin is also shown. For each image, higher magnification of the framed area was presented below. Upper images: magnification 40 $\times$; bar = 30 μ m. Lower images: magnification 100 $\times$; bar = 10 μ m. (D) Relative mRNA expression of apo B quantified by RT-qPCR in HFD (D), HFD + T_2 (DT) and C + T_2 (CT) rats with respect to standard diet-fed rats (C). Data (mean $\pm$ s.d.) are reported as fold induction with respect to controls after normalisation for GAPDH mRNA. Significant differences are reported: D vs DT (### $P \leq 0.001$). Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-11-0288>.

target genes with PPAR α , including those involved in FFA oxidation (Rogue *et al.* 2011). Besides, PPAR γ is the molecular target for anti-diabetic agents (that are synthetic PPAR γ agonists) that lower the hepatic TAG content (Balasubramanian *et al.* 2010, Cheatham 2010). In conclusion,

it is likely that PPAR γ may increase both TAG hydrolysis and lipid synthesis, with the latter effecting predominating under most, but not all, conditions. Finally, PPAR γ may play also a protective effect through its ability to reduce hepatic inflammatory responses in rats fed a high-cholesterol fructose diet (Collino *et al.* 2010).

T_2 administration to HFD rats increased AOX expression also, but this effect was not observed in CT rats. This indicates that a stimulation of peroxisomal FFA oxidation occurs upon T_2 administration, in addition to stimulation of the mitochondrial oxidative pathways (Mollica *et al.* 2009, Silvestri *et al.* 2010), in the attempt to reduce the excess fat. Moreover, the increased PPAR γ levels are in accordance with an action of T_2 in preventing the pro-inflammatory condition associated with the diet.

To further investigate the possible pathways supporting the anti-steatotic effects of T_2 , we assessed the expression of genes regulating lipid accumulation within LDs and lipid secretion. Lipid overload is associated with modulation of expression of PAT proteins. ADRP, a well-known marker of steatosis, promotes the incorporation of lipids in LDs (Bickel *et al.* 2009); ADRP-containing large LDs represent, in fact, a relatively inert pool for long-term TAG storage (Wang *et al.* 2010). By contrast, TIP47 preferentially labels nascent LDs (Zimmermann *et al.* 2009) and binds LDs in response to lipid loading (Wolins *et al.* 2001), and the nascent TIP47-containing small LDs may represent a metabolically active pool (Wang *et al.* 2010). On the other hand, OXPAT is postulated to regulate lipid storage for short-term utilisation through oxidative pathways and moves to the LD surface during lipid loading (Yamaguchi *et al.* 2006, Dalen *et al.* 2007, Bickel *et al.* 2009). Our results show a significant up-regulation of both ADRP and OXPAT mRNA expression in liver of D rats, and this up-regulation was prevented by T_2 administration; these results are in accordance with the anti-steatotic effects above described for T_2 . No significant changes were instead observed in TIP47 expression.

To visualise LD size and number in the different experimental conditions, ADRP immunostaining of liver sections was performed. As expected, ADRP-positive LDs were increased in number and size in D rats in comparison with C rats. T_2 administration to D rats partially counteracted accumulation of ADRP-positive LDs.

Lipid stored in LDs can be mobilised by lipases as a response to external or internal stimuli. ATGL is considered the rate-limiting lipolytic enzyme in mammals (Haemmerle *et al.* 2006), and is a major TAG lipase in the liver where it acts, likely through PPAR α , to channel hydrolyzed FFAs towards oxidative pathways as opposed to VLDL secretory pathways (Ong *et al.* 2011). In adipocytes, ATGL levels are increased by PPAR γ agonists (Kershaw *et al.* 2007), while, in hepatocytes, ATGL overexpression is associated with increased catabolism of FFAs and stimulated PPAR α activity (Ong *et al.* 2011). In our study, we observed a large increase in ATGL mRNA expression in liver of D rats that indicates a stimulation of

lipolytic pathways as a response to excess fat. Concomitant administration of T₂ to D rats prevented the up-regulation of ATGL expression. On the other hand, T₂ *per se* did not affect ATGL expression and this suggests that the reduction in ATGL mRNA levels in DT rats may be a consequence of the reduced fat accumulation.

In addition to oxidation, in the liver the excess TAGs can be packaged into VLDL, which is exported in serum (Vance & Vance 1990). In this regard, a crucial role is played by apo B, a lipid-binding protein that is used to assemble and stabilise the VLDL. In D rats, apo B expression did not change with respect to controls, but it was up-regulated upon T₂ administration (DT animals). Since in CT rats apo B expression was not affected, we can hypothesise that the effects of T₂ on apo B are strictly dependent on the excess fat in the liver, and that T₂ may promote pathways leading to TAG mobilisation from LDs and secretion as VLDL. Studies are in progress to confirm an increment in VLDL secretion in hepatocytes overloaded of lipids and exposed to T₂. Noteworthy, the modulation of apo B transcription fits well the pattern of ADRP expression that exhibits an opposite trend. In fact, the increase in ADRP expression detected in D rats may reduce the assembly of VLDL (Magnusson *et al.* 2006), while the lower ADRP expression in DT rats could be permissive for VLDL packaging driven by apo B overexpression.

In DT and CT rats, we also observed a decrease in the expression of FIT2, a membrane protein located in the ER, the site of TAG biosynthesis, and of LD biogenesis. Overexpression of FIT2 in mouse liver was reported to increase the LD number (Kadereit *et al.* 2008). Our results, showing a reduction in FIT2 expression upon T₂ administration, support a role for T₂ in promoting lipid secretion rather than accumulation.

Taken together, all these data suggest that T₂, in addition to induction of oxidative pathways, stimulates lipoprotein secretion to reduce the hepatic fat excess. However, serum TAGs, which are high in HFD-fed rats, appear to be normalised by T₂ treatment, as previously reported by us and others (Lanni *et al.* 2005, Grasselli *et al.* 2008), so that an increase in triglyceridemia by T₂ is not apparent, despite the possible stimulation of VLDL secretion. The VLDL receptor number is increased in the muscle of hyperthyroid rats (Jokinen *et al.* 1994), and TH administration increases serum levels of T₂ (Nishikawa *et al.* 1983). Therefore, it could be hypothesised that the increased rate of VLDL secretion induced by T₂ might be balanced by an increase in plasma VLDL removal rate by skeletal muscle, or other tissues. This point needs further investigation.

In conclusion, this study demonstrates that T₂ administration to HFD rats prevents most of the transcriptional changes of those genes that were modulated in response to the excess exogenous fat. In particular, T₂ prevents the pathways leading to the long-term TAG storage in LDs that were induced by HFD, and at the same time stimulates mechanisms of FFA oxidation and lipid secretion.

In summary, this study identifies some possible mechanisms underlying the improvement of the liver steatosis induced by T₂.

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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