Effects of 3,5-Diiodo-L-Thyronine Administration on the Liver of High Fat Diet-Fed Rats

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In rats fed a high fat diet (HFD), long-term administration of 3,5-diiodo-L-thyronine (T_2), a naturally occurring iodothyronine, was shown to reduce body-weight gain, fat mass, and hepatic lipid accumulation. This work was aimed at investigating the mechanisms of T_2 action in the liver of HFD rats. The results show that HFD induces liver lipid peroxidation and stimulates the activity of enzymes involved in hydrogen peroxide (H_2O_2) metabolism, catalase in particular. Moreover, quantitative RT-PCR revealed HFD-induced upregulation of the transcription factor PPARα, as well as of metallothionein isoforms (MT-1 and MT-2). T_2 administration prevented the HDF-induced lipid peroxidation, as well as the increase in H_2O_2 metabolism, and reduced the upregulation of both PPARα and MT-2. These data demonstrate that in the liver of HFD rats, T_2 prevents both lipid accumulation and oxidative stress associated with increased fat metabolism. Exp Biol Med 233:549–557, 2008

Key words: Wistar rats; oxidative stress; lipid peroxidation; metallothioneins (MT-1 and MT-2)

Introduction

Recently, attention has been focused on the excessive accumulation of lipids within the liver (i.e., fatty liver or steatosis) as a part of the metabolic syndrome or of nonalcoholic fatty liver disease (NAFLD). NAFLD is a clinicopathological condition of emerging importance that is characterized by an association between visceral obesity and cardiovascular risk factors (1, 2). Simple steatosis can progress to steatohepatitis, advanced fibrosis, cirrhosis, and ultimately to hepatocellular carcinoma (3). Obesity is one of the most significant risk factors for the development of fatty liver in industrialized countries.

Fat accumulation, resulting from the imbalance in the input/output/oxidation of fatty acids (FAs), leads to altered energy metabolism. Fatty liver is characterized by altered lipid metabolism, increased oxidative stress, and an abnormal pattern of cytokine production (4). In rodent liver, lipid metabolism is mainly regulated by the peroxisome proliferator-activated receptor alpha (PPARα), which acts as a fat sensor and transcription factor for a number of genes involved in FA uptake and metabolism (5, 6). It has been shown that a high fat diet (HFD) increases PPARα expression and activation, leading to induction of PPARα target genes involved in FA oxidation (7). Elevated rates of FA oxidation lead to an increased production of reactive oxygen species (ROS) at the peroxisomal, mitochondrial, and microsomal levels (8). The hepatic cell attempts to counteract the increased oxidative stress by incrementing the activity/expression of antioxidant enzymes and/or by inducing antioxidant molecules such as glutathione and metallothioneins (9).

Metallothioneins (MTs) are highly-conserved, low-molecular weight (6–7 kDa), cysteine-rich proteins with high affinity for divalent metals (10, 11). MTs are ubiquitous proteins involved in essential functions, including homeostatic regulation of zinc and copper, detoxification of heavy metals, and scavenging of free radicals (12). The mobilization of zinc from MTs by oxidant agents may explain the antioxidant activity of MTs *in vivo* (13). A number of stimuli induce MT expression, including acute phase response, cold, heat, stress, and hormones (11). Moreover, it has been demonstrated that MT-overexpressing transgenic mice are significantly protected from hepatic oxidative stress associated with alcoholic liver injury (14). In rodents, four MT isoforms (MT-1 to MT-4) encoded by

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four functional genes have been identified. The different MT isoforms seem to play distinct functions in various physiological and pathological conditions (10). The highly dynamic nature of the metallothionein/thionein (MT/T) system, and the observation that mice whose MT I/II genes have been disrupted become mildly obese, point to a role of MTs as modulators of energy metabolism (15–17).

Thyroid hormones (THs; thyroxine [T_4] and 3,3′,5-triiodo-L-thyronine [T_3]) are known as major modulators of energy metabolism, and their role in lipid metabolism has supported their potential use as drugs to promote weight loss. The natural compound 3,5-diiodo-L-thyronine (T_2), which is produced by peripheral deiodination of T_3 and T_4, has significant thyromimetic activity without inducing the thyrotoxic effects that can be associated with long-term administration of T_3 (18). Long-term administration of T_2 to rats fed a HFD has been shown to reduce body-weight gain, fat mass, and lipid accumulation in the liver. The effects of T_2 on liver metabolism were shown to involve increased mitochondrial FA oxidation with a concomitant decrease in efficiency of utilization of lipid substrates and mitochondrial uncoupling (19). However, the mechanisms of T_2 action on the liver remain partially unexplored.

The present study was aimed at investigating whether T_2-induced reduction in fat accumulation in the liver of HFD-fed rats may be associated with changes in oxidative stress and in MT expression. Body weight, serum triglycerides, liver lipid accumulation, and expression of PPARα were assessed in HFD-fed rats treated or not treated with T_2 and compared with these assessments for control rats. The effects of HFD and T_2 on the levels of hepatic lipid peroxidation (thiobarbituric acid-reactive substances [TBARS]) and specific activities of the antioxidant enzymes responsible for hydrogen peroxide (H_2O_2) detoxification (i.e., catalase and glutathione peroxidases) were evaluated. Transcription of the main liver MT isoforms (MT-1 and MT-2) was also quantified by quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

Materials and Methods

Chemicals. All chemicals, unless otherwise indicated, were of analytical grade and were obtained from Sigma-Aldrich Corp. (Milano, Italy).

Animals and Treatments. Twenty-four male Wistar rats (aged 8 weeks) were purchased from Harlan Italy (S. Pietro al Natisone, Italy) and housed in individual cages in a temperature-controlled room at 28°C with a 12:12-hr light:dark cycle. The animals had free access to water and to a commercial mash (Charles Rivers; Lecco, Italy). Animal maintenance and treatment were carried out according to the guidelines of the European Community Council for animal care and use. After 1 week of acclimatization, rats were randomly divided into four groups ($n = 6$ for each group). In each group, body weight was normally distributed, and body weight means were similar for all animals (about 300 g). The first group (C) received a standard diet (total metabolizable percentage of energy: 60.4 carbohydrates, 29 proteins, 10.6 fats [J/J]; 15.88 kJ gross energy/g); the second group (D) was fed a HFD (total metabolizable percentage of energy: 21 carbohydrates, 29 proteins, 50 fats [J/J]; 19.85 kJ gross energy/g); the third group (DT) received the same HFD and a simultaneous daily intraperitoneal (ip) injection of T_2 (25 μg/100 g body wt); the fourth group (CT) received a standard diet and a simultaneous daily ip injection of T_2 (25 μg/100 g body wt; Ref. 19). C and D rats were injected daily ip with the same volume of physiological saline solution. Rat body weight and food intake were recorded every 2 days for each animal. Food intake was not significantly affected by the diet composition nor by T_2 treatment.

After 30 days of treatment, rats were anesthetized and killed by cervical dislocation. Blood was collected and allowed to clot, and serum was stored at –80°C; serum concentrations of triglycerides, cholesterol, and glucose were measured using a Hitachi 7170 clinical analyzer (Hitachi; Milano, Italy) by following standard procedures. Immediately after rats were killed, their livers were rapidly dissected, weighed, cut into small pieces, quickly frozen in liquid nitrogen, and stored at –80°C until use.

Morphological Analysis. Small fragments of liver were fixed by immersion in 2% glutaraldehyde-2% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4, for 4 hrs, postfixed in 0.1% osmium tetroxide, and embedded in an Epon-Araldite (Epon: Multilab Supplies, Fetcham, UK; Araldite: Fluka Chemie AG, Buchs, Switzerland) mixture. Semithin sections (2 μm) were stained with toluidine blue.

Lipid Peroxidation, Antioxidant Enzyme Activities, and Mitochondrial H_2O_2 Production. Lipid peroxidation in aliquots of liver homogenates was evaluated by the TBARs assay using malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) as a standard (20).

For enzymatic assays, aliquots of pooled tissues from each experimental group were homogenized in 3 vol of 20 m*M* Tris-HCl pH 7.6, 1 m*M* EDTA, 1 m*M* DTT, 500 m*M* sucrose, 0.1 m*M* PMSF, and 2 μg/ml leupeptin. After a first centrifugation at 500 *g* for 20 mins, the supernatant was centrifuged at 20,000 *g* for 40 mins, and the resulting supernatant and pellet were utilized for determination of enzyme activities.

Catalase activity was evaluated in aliquots of both 20,000 *g* supernatant and pellet, suitably diluted in 50 m*M* potassium phosphate buffer (pH 7.0) following the consumption of H_2O_2 at 240 nm ($\varepsilon_{240nm} = 40\ M^{-1}\cdot cm^{-1}$). Catalase specific activity was expressed as micromoles of decomposed H_2O_2 per minute per milligram of sample protein (21).

Both Se-dependent and Se-independent glutathione peroxidase (GPx) activities were evaluated using either hydrogen peroxide or cumene hydroperoxide as a substrate, respectively. The 20,000 *g* supernatants were further centrifuged at 105,000 *g* for 1 hr, and aliquots of the resulting supernatant and pellet were assayed following

NADPH consumption at 340 nm ($\varepsilon_{340\ nm} = 6.22\ M^{-1} \cdot cm^{-1}$). GPx activity was expressed as nanomoles of consumed NADPH per minute per milligram of sample protein (22).

Spectrophotometric analyses were carried out at 25°C using a Varian Cary 50 spectrophotometer (Varian; Torino, Italy).

For mitochondrial H_2O_2 production determination, livers were homogenized (10% w/v) in 220 m*M* mannitol, 70 m*M* sucrose, 20 m*M* Tris-HCl, 1 m*M* EDTA, 5 m*M* EGTA, 5 m*M* $MgCl_2$, pH 7.4, supplemented with protease inhibitors (23). Homogenates were freed from debris and nuclei by centrifugation at 500 *g* for 10 mins at 4°C, and the resulting supernatants were centrifuged at 3000 *g* for 10 mins at 4°C. The mitochondrial pellets were washed three times before final resuspension in the homogenization buffer. The mitochondrial H_2O_2 production was measured by fluorimetric assay as previously described (24, 25). Fluorescence was measured using a PerkinElmer LS 50B spectrophotofluorimeter (PerkinElmer-Italia; Monza, Italy).

Protein content was determined by the bicinchoninic acid (BCA) method using bovine serum albumin as a standard (26).

RNA Extraction and Quantitative RT-PCR. Total RNA was extracted from aliquots of frozen pooled tissues from each experimental group by using Trizol Reagent (Sigma-Aldrich) according to the manufacturer's instructions. First-strand cDNA was synthesized in 1× transcriptase buffer from 2 μg of total RNA using 200 U RevertAid H-Minus M-MuLV Reverse Transcriptase (Fermentas; Hannover, MD), 200 ng of oligo(dT)18-mer, 1 m*M* dNTPs (Invitrogen; Milano, Italy), 100 U RNase inhibitor (Fermentas) in a final volume of 20 μl. Quantitative RT-PCR reactions were performed in quadruplicate in a final volume of 25 μl containing 0.3 μ*M* of each primer, 10 ng cDNA, 1× SybrGreen PCR Master Mix (Applied Biosystems, Monza, Italy) and were analyzed in a 96-well optical reaction using a 7900 HT fast real time PCR system (Applied Biosystems; Monza, Italy). Primers were synthesized by TibMolBiol custom oligosynthesis service (Genova, Italy). Primers for peroxisome proliferator-activated receptor α (PPARα; PPARa_FWD 5′-CCCCACTTGAAGCAGATGACC-3′ and PPARa_REV 5′-CCCTAAGTACTGGTAGTCCGC-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GAPDH_FWD 5′-GACCCCTTCATTGACCTCAAC-3′ and GAPDH_REV 5′-CGCTCCTGGAAGATGGTGATGGG-3′) 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). Primers for MT-1 (MT1_FWD 5′-CTGCTCCACCGGCGG-3′ and MT1_REV 5′-GCCCTGGGCACATTTGG-3′) and MT-2 (MT2_FWD 5′-TCCTGTGCCACAGATGGATC-3′ and MT2_REV 5′-GTCCGAAGCCTCTTTGCAGA-3′) were obtained from the literature (27). The thermal protocol consisted of 3 mins initial denaturation at 95°C, followed by 40 cycles of amplification (15 secs at 95°C; 30 secs at 60°C). A melting curve of RT-PCR products (55–94°C) was also performed to ensure the absence of artifacts. The threshold cycle (C_T) represents the cycle number at which the amount of amplified target reaches the fixed threshold. The relative quantity of target mRNA was calculated by using the comparative C_T method and was normalized for the expression of the GAPDH gene (28). The normalized expression was thus expressed as relative quantity of mRNA (fold induction) with respect to controls (C).

Statistics. Data on quantitative RT-PCR are means ± SD of three independent RNA extractions performed in quadruplicate. Enzyme activities are means ± SD of three independent experiments performed in triplicate. Statistical analysis was performed by using ANOVA followed by the Bonferroni *post hoc* test (Instat software; GraphPad Software, Inc.; San Diego, CA).

Results

Body-Weight Gain, Serum Values, PPARα Expression. The effects of HFD and/or T_2 treatments on rat body weight are shown in Figure 1A. When we compared the mean body weight gains measured at the end of treatments (Day 30), HFD-fed rats (D) showed a significant increment in the mean body weight with respect to control (C) rats (+13%; $P < 0.01$). Such an increase was not observed in HFD-fed rats treated with T_2 (DT). T_2 did not alter the body weight gain in rats fed a standard diet (CT). The body weight gain of both the DT and CT rats statistically differed from that of the D rats ($P < 0.05$).

HFD feeding increased visceral fat depots, as well as hepatic lipid accumulation, detected as numerous large brown droplets in histological sections of liver stained with toluidine blue. This indicated microvesicular steatosis typical of NAFLD (Fig. 1B). Administration of T_2 to HFD-fed rats prevented the increase in fatty droplet accumulation (DT), and only some hepatocytes showed a few small lipid droplets, similar to what was observed in the liver of standard diet-fed rats (C).

Table 1 summarizes the mean serum values of triglycerides, cholesterol, and glucose for all experimental groups. A marked increase in triglyceride 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.

The expression of PPARα was evaluated by quantitative RT-PCR on all experimental groups (Fig. 2). In the liver of D rats, a significant increase in the level of PPARα transcripts was observed with respect to controls (3-fold; $P < 0.001$). The HFD-induced upregulation of PPARα in DT rats was reduced with respect to D rats (1.7-fold; $P < 0.05$), whereas no changes in PPARα expression were observed in CT rats.

Liver Oxidative Stress. Liver lipid peroxidation was evaluated by the TBARs assay. In D rats, a large increase in the MDA level was observed with respect to C rats (+200%; $P < 0.001$) (Fig. 3). No changes in lipid peroxidation were observed in DT and CT rats.

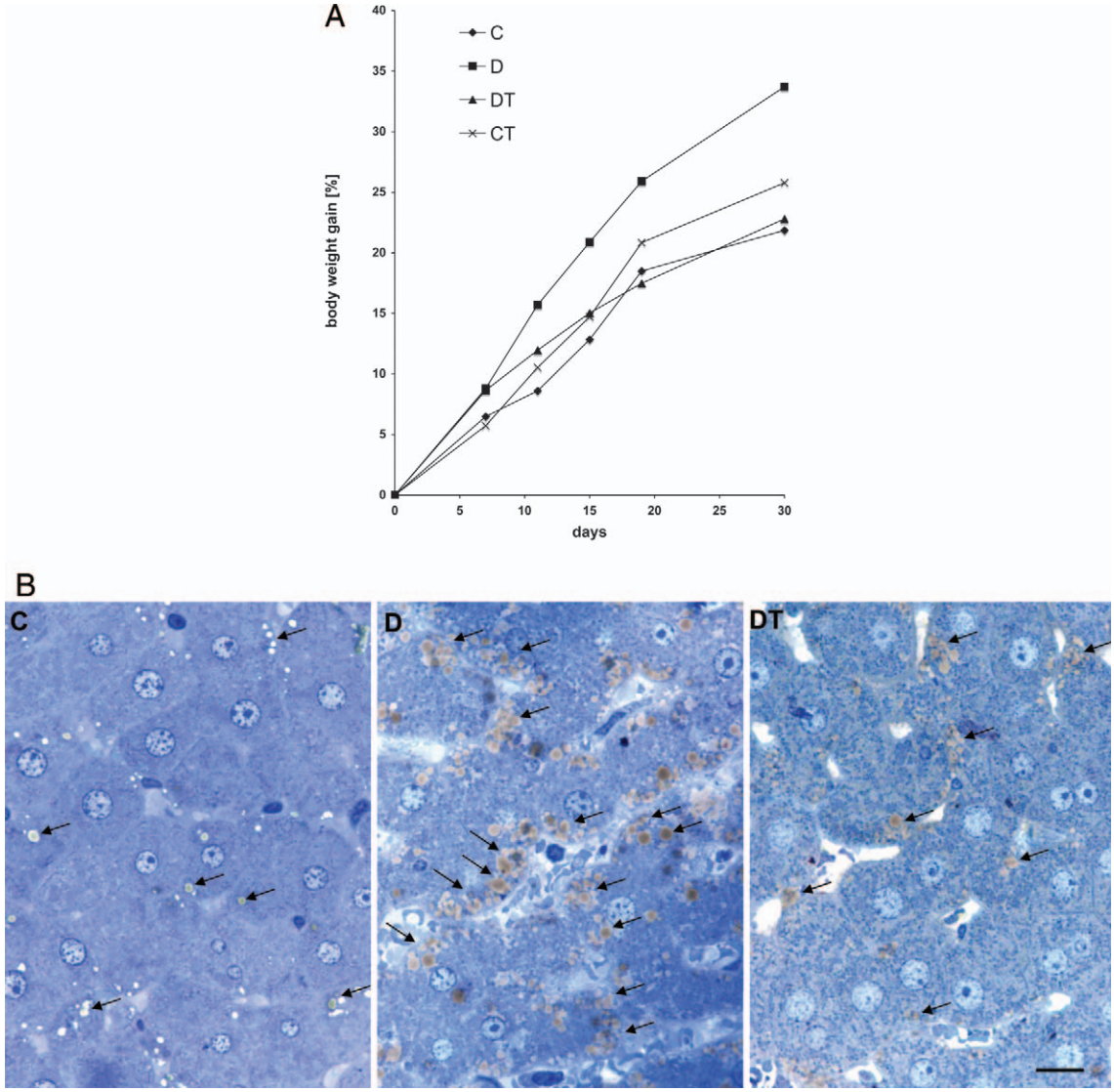


Figure 1. Effects of HFD and T_2 on body weight gain and hepatic morphology. (A) Body weight gain measured throughout the experimental period (30 days) for the four experimental groups ($n = 6$ for each group): standard diet-fed rats (C); HFD (D); HFD + T_2 (DT) and C + T_2 (CT). Values are expressed as means ± SD of body-weight gain (%). (B) Hepatic tissue morphology assessed in standard diet-fed rats (C), HFD (D), and HFD + T_2 (DT). Numerous fatty droplets (brown stain, indicated by arrows) were observed within hepatocytes in D rats. T_2 prevented this increase in fatty droplet accumulation, and only some hepatocytes showed a few small lipid droplets as in the normal-diet animal liver. Light microscopic images were acquired on the resin-embedded tissue. Bar, 13 μm. A color version of this figure is available in the online version of the journal.

Specific activities of catalase (CAT) and GPx, were also evaluated. Total CAT activity was increased in D rats with respect to C rats (+100%; $P < 0.01$; Fig. 4A). The HFD-induced increase in CAT activity was prevented by T_2 administration in DT rats, whereas T_2 did not affect CAT activity in CT rats.

Also, total Se-dependent GPx specific activity showed a small but significant increase in D rats with respect to C rats (+35%; $P < 0.01$); such an increase was not observed in DT rats (Fig. 4B). The activities of the Se-independent GPx form, which was evaluated utilizing cumene hydroperoxide as a substrate, and of glutathione S-transferase (29) were not affected by either diet or T_2 (data not shown).

Evaluation of mitochondrial H_2O_2 production revealed an increase, although not significant, in D rats (+39%), and a significant increase (+79%; $P < 0.05$) in DT rats with respect to C rats (Fig. 4C).

Metallothionein Expression. The transcription profile of the two main liver isoforms of metallothioneins, MT-1 and MT-2, was evaluated by quantitative RT-PCR using specific primer pairs. In line with previous reports (30), more abundant transcripts for the MT-1 isoform than for the MT-2 isoform were observed in the liver of C rats, with MT-1 and MT-2 genes showing ΔCt values of about three cycles (Fig. 5A). In D rats, both MT isoforms showed a significant upregulation that was higher for MT-2

Table 1. Effects of HFD and T_2 on Serum Values[a]

Treatments	Triglycerides	Cholesterol	Glucose
C	174 ± 26	70 ± 7	167 ± 15
D	266* ± 38	74 ± 19	181 ± 25
DT	191 ± 57	56 ± 19	166 ± 17
CT	129 ± 14	73 ± 7	155 ± 10

[a] Mean serum values ± SD of triglycerides, cholesterol, and glucose (mg/dl) for each experimental group ($n = 6$ for each group): standard diet-fed rats (C), HFD (D), HFD + T_2 (DT), and C + T_2 (CT). Data were analyzed by ANOVA followed by the Bonferroni *post hoc* test. Significant differences are reported in the table: * C vs. D, $P < 0.01$. Other pairwise comparisons not indicated on the table are D vs. DT ($P < 0.05$) and D vs. CT ($P < 0.01$).

(about 4.5-fold induction with respect to C; $P < 0.001$) than for MT-1 (about 3.5-fold induction; $P < 0.001$). The HFD-induced upregulation of MT-2 was reduced by T_2 administration in DT rats, even though it remained higher than in

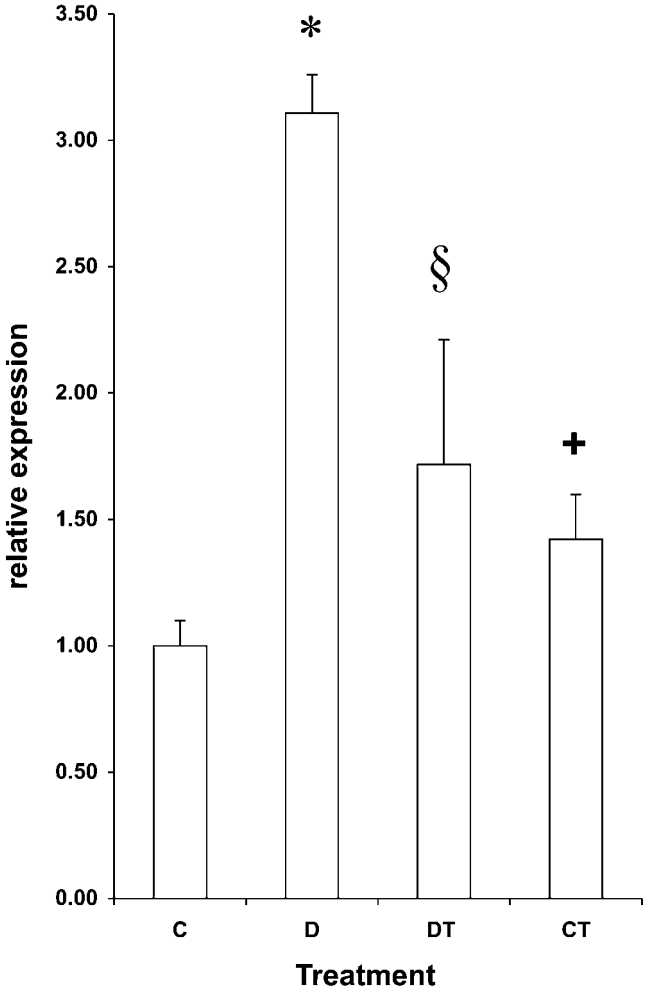


Figure 2. Effects of HFD and T_2 on hepatic transcription of PPARα. Relative expression of PPARα with respect to standard diet-fed rats (C), HFD (D), HFD + T_2 (DT), and C + T_2 (CT). Data (mean ± SD) are reported as fold induction with respect to controls after normalization for GAPDH mRNA. Significant differences are reported: * C vs. D, $P < 0.01$; § D vs. DT, $P < 0.05$; + D vs. CT, $P < 0.001$.

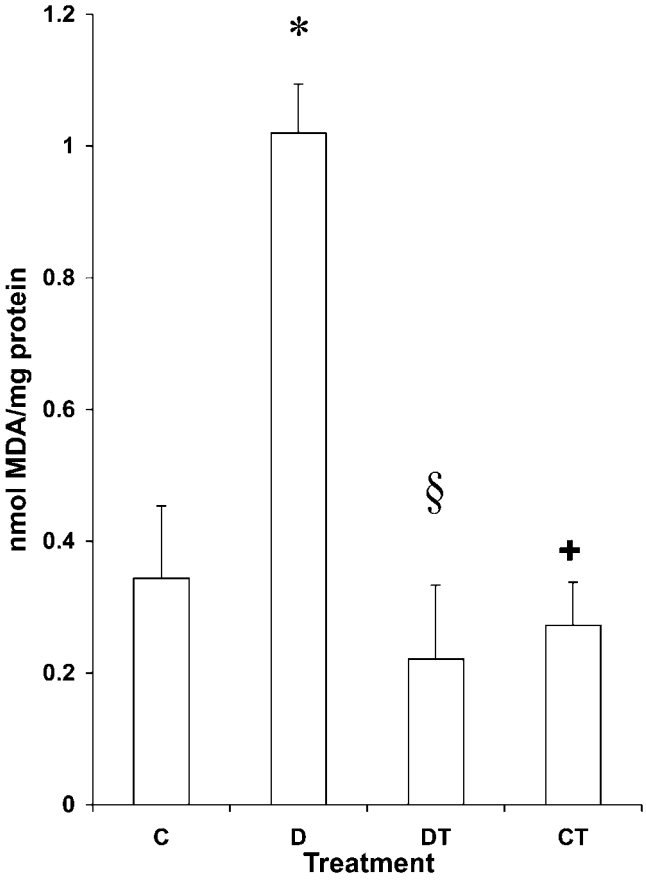


Figure 3. Effects of HFD and T_2 on hepatic lipid peroxidation. Liver lipid peroxidation evaluated by the TBARs assay in standard diet-fed rats (C), HFD (D), HFD + T_2 (DT), and C + T_2 (CT). Data (mean ± SD) are expressed as nmol MDA/mg protein. Significant differences are reported: * C vs. D, $P < 0.001$; § D vs. DT, $P < 0.001$; + D vs. CT, $P < 0.001$.

C rats (2.5-fold induction with respect to C; $P < 0.001$). Conversely, expression of MT-1 in DT rats was similar to that observed in D rats. T_2 did not affect the transcription of either MT isoforms in CT rats (Figs. 5B and C).

Discussion

Despite the plethora of studies, the molecular mechanisms leading to fatty liver development are still not completely understood. An excess of dietary fat intake leads to increased FA oxidation in mitochondria and peroxisomes (31). An elevated rate of FA oxidation results in increased release of reactive oxygen species (ROS) and H_2O_2 from mitochondria and peroxisomes, respectively (32).

The ligand-activated transcription factor PPARα controls fat metabolism at multiple levels, altering the transcription of numerous genes, many of which are involved in FA catabolism (4, 33). A high-fat diet presumably provides more long-chain FAs entering the hepatocytes with consequent activation of PPARα and upregulation of those PPARα-target genes involved in FA catabolism. It has been widely documented that PPARα expression is also upregu-

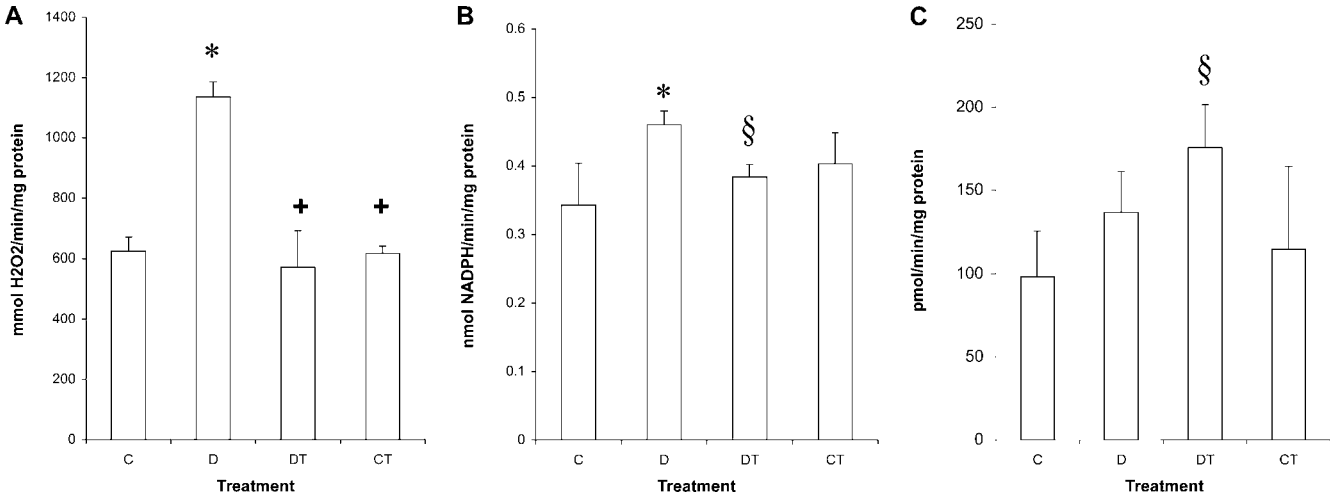
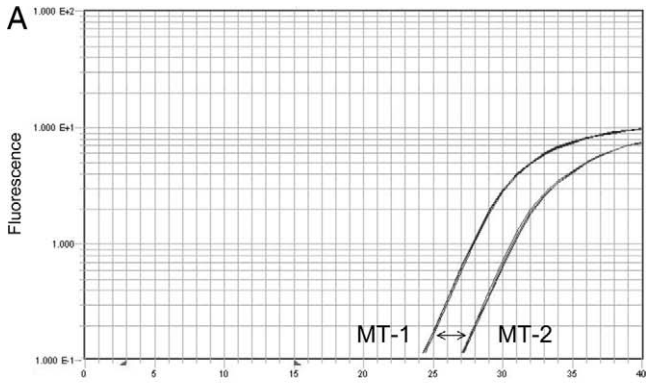
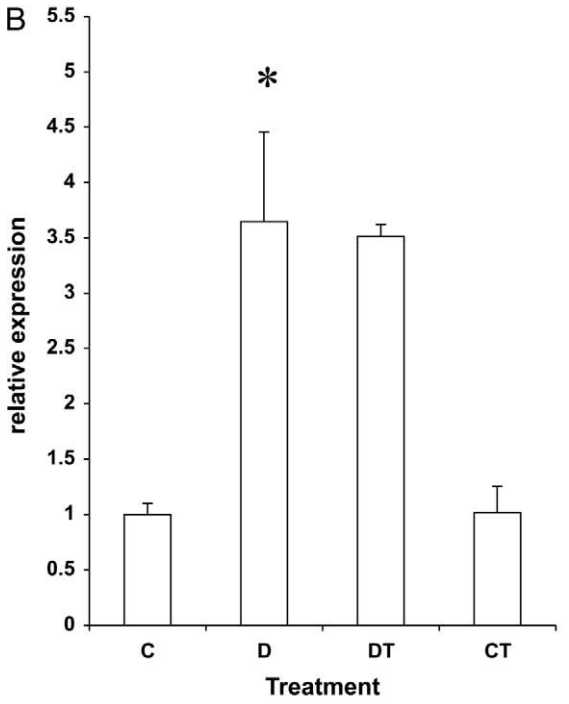


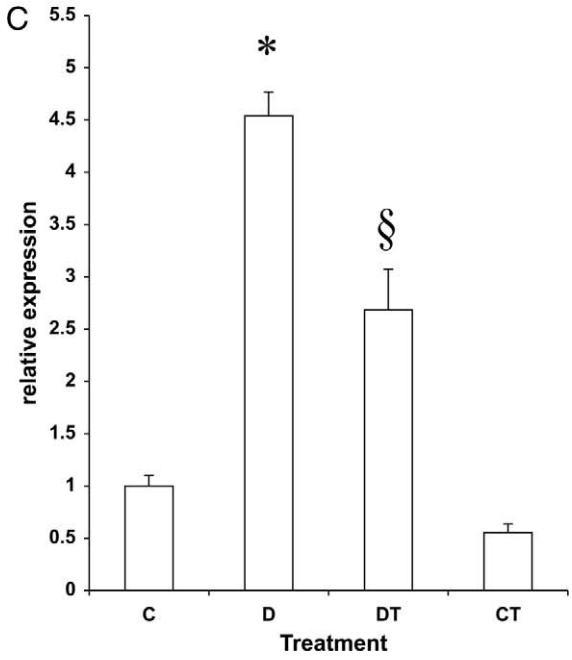
Figure 4. Effects of HFD and T_2 on catalase and Se-dependent glutathione peroxidase activity, and mitochondrial H_2O_2 production. (A) Catalase specific activity determined in standard diet-fed rats (C), HFD (D), HFD + T_2 (DT), and C + T_2 (CT). Data (mean ± SD) are expressed as μmol H_2O_2/min/mg protein. Significant differences are reported: * C vs. D, $P < 0.01$; § D vs. DT, $P < 0.01$; + D vs. CT, $P < 0.001$. (B) Se-dependent glutathione peroxidase specific activity determined in standard diet-fed rats (C), HFD (D), HFD + T_2 (DT), and C + T_2 (CT). Data (mean ± SD) are expressed as nmol NADPH/min/mg protein. Significant differences are reported: * C vs. D, $P < 0.01$ and § D vs. DT, $P < 0.05$. (C) Mitochondrial H_2O_2 production determined in standard diet-fed rats (C), HFD (D), HFD + T_2 (DT), and C + T_2 (CT). Data (mean ± SD) are expressed as pmol/min/mg of protein. Significant differences are reported: § C vs. DT, $P < 0.05$.

lated in the liver of HFD-fed rodents and in obese Wistar rats (31, 34–36), as well as in human NAFLD biopsies (37). In line with these reports, we observed that HFD induces PPARα expression in rat liver. Our data also indicate that, after 30 days of treatment, T_2 reduces the HFD-induced upregulation of PPARα, and this effect parallels the reduced lipid droplet accumulation observed in liver sections of DT rats with respect to D rats. However, the possibility that at shorter times after T_2 administration, PPARα expression may be upregulated can not be excluded. Increased FA oxidation usually leads to higher oxyradical production and consequent oxidative stress (3, 4). We indeed observed an increased lipid peroxidation in the liver of D rats, as well as a stimulation of catalase and Se-dependent GPx activities. All these effects were partially counteracted by T_2 administration to HFD-fed rats. In contrast, an increase in mitochondrial H_2O_2 production was observed in the liver of DT rats, in line with previous data showing an increase in mitochondrial FA oxidation (19). Peroxisomes are the main site for oxidation of long- and very long-chain FAs, the main component of dietary lipids (4, 31). On the other hand, to enter mitochondria, long-chain FAs require the activity of carnitine palmitoyl-transferase (CPT; 38). A previous report showed that activity of mitochondrial CPT was stimulated by HFD, and it was further stimulated by T_2, pointing to CPT activation as a key step in mediating the stimulation of mitochondrial FA catabolism by T_2 (19). In DT rats, the T2-induced CPT stimulation and increased influx of FAs in mitochondria may partly explain our observations that although T_2 increases H_2O_2 production in mitochondria, it prevents both the stimulation of H_2O_2 metabolism at the peroxisomal and microsomal level, as well as the tissue lipid peroxidation induced by HFD alone, this indicating that the mitochondrial H_2O_2 production contributes little to the overall oxidative stress conditions of the liver (19). Overall, the results confirm previous data showing that T_2 leads to reduced hepatic fat accumulation by stimulating FA metabolism at the mitochondrial level (19). MTs represent a main component of the nonenzymatic antioxidant system of the cell (12). MTs play a key role in liver damage and regeneration (39, 40), as well as in energy metabolism (16, 41). When the transcription profiles of MT-1 and MT-2, the two isoforms constitutively expressed in the liver, were evaluated, a marked upregulation of both isoforms was found in D rats, thereby supporting the protective role of MTs in liver (42). Concomitant administration of T_2 to D rats partially counteracts the upregulation of MT-2 but not that of MT-1. The observation that T_2 alone does not alter the transcription profile of MTs in liver from standard diet-fed rats indicates that the T_2 effects on hepatic MT expression are secondary to the effects of the hormone at the level of molecules and/or pathways associated with fat intake.

The HFD-induced upregulation of MTs could be due to an imbalance in the level of essential metals and/or oxidative stress conditions. However, no significant changes in the hepatic levels of total zinc and copper were observed by Coupled Plasma-Atomic Emission Spectroscopy as a function of HFD and/or T_2 treatment. This excludes a primary role for these essential metals in the HFD-induced upregulation of MTs, even if changes in availability of free (rapid exchangeable) zinc ions induced by HFD cannot be ruled out. Therefore, oxidative stress appears to be the most probable candidate for the observed upregulation of MT transcription, in particular of MT-2, whereas MT-1 expression remains upregulated in D rats, regardless of the







reduction in lipid accumulation and oxidative stress associated with T_2 administration. A previous report showed that the level of MT-2 mRNA in adipose tissue was increased in obese subjects (43). On the other hand, the antioxidant N-acetylcysteine reduced lipid droplet formation and downregulated MT-2 in 3T3L1 adipocytes (44). Overall, these data and our results point to a specific role of the MT-2 isoform in the defense mechanism against oxidative stress.

However, regulation of MT gene expression is rather complex, involving several transcription factors, signaling pathways, and responsive elements. ROS can induce MT expression both through antioxidant response elements (ARE) and metal responsive elements (MRE; Ref. 45), as well as through several redox-sensitive transcription factors (46). At present, the molecular mechanisms responsible for the differential regulation of MT-1 and MT-2 transcription, as well as their functional implications, are still unclear. The relationship between MT-2 transcription, fat oxidation, and oxidative stress might be related to the different scavenging activities exerted by the two MT isoforms *in vitro,* with MT-1 representing a more selective scavenger of $O_2{}^{*-}$ and MT-2 of *OH (47).

Interestingly, Ye *et al.* (2001) demonstrated that MT localizes in the intermembrane space of liver mitochondria, suggesting a role for this protein in the fine-tuning of mitochondrial energy metabolism by modulating reversible zinc binding (15). Moreover, a recent study shows that MT synthesis is induced by an increase in oxidative stress at the mitochondrial level (48). The possible role of MT on the mitochondrial function in our experimental conditions deserves further investigation.

Overall, the results of this study demonstrate that in rat liver, HFD-induced lipid accumulation is associated with increased levels of PPARα and MT expression and with a condition of oxidative stress. On the other hand, the concomitant administration of T_2 is able to prevent the HFD-induced fat accumulation and partially counteracts PPARα and MT upregulation, as well as oxidative stress. These effects may be explained by a selective stimulation exerted by T_2 on FA catabolism in mitochondria at the expense of peroxisomal and microsomal oxidation. However, there is a possibility that the effects of T_2 on the

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Figure 5. Effects of HFD and T_2 on hepatic transcription of metallothioneins. (A) Transcription profile of the two MT-1 and MT-2 metallothionein liver isoforms. Ct values for C rats are represented in triplicate (ΔCt of about 3 cycles). Relative expressions of MT-1 (B) and MT-2 (C) isoforms with respect to standard diet-fed rats (C), evaluated by quantitative RT-PCR in liver of HFD (D), HFD + T_2 (DT), and C + T_2 (CT) rats. Data (mean ± SD) are reported as fold induction with respect to controls after normalization for GAPDH mRNA. Significant differences are reported on the graph: * C vs. D, $P < 0.001$ for both isoforms and § D vs. DT, $P < 0.001$) for MT-2. Other pair wise comparison not indicated on the chart are: for MT-1, C vs. DT ($P < 0.001$) and D vs. CT ($P < 0.001$); for MT-2, C vs. DT ($P < 0.001$) and D vs. CT ($P < 0.001$).

liver are indirect and may depend on a reduced fat influx into hepatocytes caused by a primary action of T_2 on other tissues (such as adipose tissue). *In vitro* experiments are planned to address the direct effects of T_2 on primary hepatocytes.

In conclusion, our study indicates that T_2 is able to prevent fat accumulation in the liver of HFD-fed rats, at the same time affording protection from the oxidative stress conditions associated with an excess of dietary fats, and further addresses to T_2 as a promising drug for weight loss treatment.

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