3,5-diiodo-L-thyronine, by modulating mitochondrial functions, reverses hepatic fat accumulation in rats fed a high-fat diet

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Background/Aims: Mitochondrial dysfunction is central to the physiopathology of steatosis and/or non-alcoholic fatty liver disease. In this study on rats we investigated whether 3,5-diiodo-L-thyronine (T2), a biologically active iodothyronine, acting at mitochondrial level is able to reverse hepatic steatosis after its induction through a high-fat diet.

Methods: Hepatic steatosis was induced by long-term high-fat feeding of rats for six weeks which were then fed the same high-fat diet for the next 4 weeks and were simultaneously treated or not treated with T2. Histological analyses were performed on liver sections (by staining with Sudan black B). In liver mitochondria fatty acid oxidation rate, mitochondrial efficiency (by measuring proton conductance) and mitochondrial oxidative stress (by measuring H₂O₂ release, aconitase and SOD activity) were detected.

Results: Stained sections showed that T2 treatment reduced hepatic fatty accumulation induced by a high-fat diet. At the mitochondrial level, the fatty acid oxidation rate and carnitine palmitoyl transferase activity were enhanced by T2 treatment. Moreover, by stimulating mitochondrial uncoupling, T2 caused less efficient utilization of fatty acid substrates and ameliorated mitochondrial oxidative stress.

Conclusion: These data demonstrate that T2, by activating mitochondrial processes, markedly reverses hepatic steatosis in vivo.

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1. Introduction

Fatty liver or steatosis refers to a histopathological condition in which an excess accumulation of lipids, primarily triglycerides within hepatocytes occurs [1]. The clinical significance of fatty liver is generally thought to be one of the leading causes of hepatic dysfunction worldwide. Indeed, fatty liver can develop into steatohepatitis which then progresses to fibrosis, cirrhosis, and liver failure [2,3]. Lipotoxicity, oxidative stress, cytokines, and other proinflammatory mediators may...
each play a role in the transition from steatosis to steatohepatitis [4].

Accumulating evidence indicates that impaired mitochondrial function plays a central role in the fatty liver, and indeed that non-alcoholic fatty liver disease (NAFLD) may be considered a mitochondrial disease [5]. Mitochondria are involved in both fatty acid β-oxidation and oxidative phosphorylation, and at the same time they are an important cellular source of reactive oxygen species (ROS) which are considered an important factor in producing the hepatocyte injury associated with NAFLD [5]. Although modulating caloric intake and increasing physical activity is the mainstay of treatment of metabolic disorders, in the past few years, data from several experimental and clinical investigations suggested that different drugs could be useful to prevent and/or treat steatosis. Since mitochondrial dysfunction is central to the physiopathology of steatosis and steatohepatitis, drugs or other factors improving directly or indirectly mitochondrial function may prove useful in the prevention and/or treatment of these liver diseases.

Our group demonstrated in a previous study on rats that 3,5-diiodo-L-thyronine (T2), a naturally occurring iodothyronine, when administered simultaneously with a high-fat diet (HFD), prevented the development of liver steatosis and excessive body weight gain without inducing thyrotoxicity [6]. In fact, T2 stimulated fatty acid oxidation and mitochondrial uncoupling in the liver, with mitochondria being their major target. In that study, T2 was administered simultaneously with the high-fat diet, and our attention was focused on the prevention of both over-weight and lipid accumulation in the liver. However, the results made us wonder whether T2 administration might be able to reduce a pre-existing hepatic fat accumulation that had already been induced by feeding a high-fat diet to fully address its therapeutic potential for preventing or treating steatosis. To test this hypothesis we evaluated both the steatosis and mitochondrial parameters in rats fed high-fat feed long-term and then administered T2 for 4 weeks simultaneously with the same high-fat diet.

2. Experimental procedures

2.1. Materials and methods

All chemicals used were of analytical grade and were purchased from Sigma (St. Louis, MO, USA).

2.2. Animals and diets

Male Wistar rats aged 5 months (Charles River Italia, Calco, Como, Italy) were kept one per cage in a temperature-controlled room (at a thermoneutral temperature for rats) of 28 °C with a 12 h light–dark cycle. At the start of the study, after 4–7 days of acclimatization, the rats were divided into two groups with a similar mean body weight (about 400 g) and with the body weights normally distributed within each group. The first group (comprising eight rats and referred to as N) received a standard diet [7] for 10 weeks (10.6% fat/J); the second group (comprising 16 rats received a high-fat diet (referred to as D0) [7] for 6 weeks (50% fat/J). After 6 weeks of high-fat feeding (time 0), the D0 group was subdivided into two groups: one (D group) continued to receive the high-fat diet for the next 4 weeks, while for the same 4-week period the other group (DT group) received the same high-fat diet together with a simultaneous daily i.p. injection of T2 (25 μg/100 g b.w.). N and D rats were i.p. injected daily with the same volume of saline throughout the last 4 weeks of treatment (“sham-injection”). The pharmacological dose of 25 μg T2/100 g body wt, was chosen after consideration of some data from studies in which T3 and T2 were used. In fact, 1) in ob/ob mice T3 treatment sufficiently decreased the percentage of lipid in the body, only at 25 μg/100 g body wt. [8]; 2) chronic treatment of rats with doses of 25 μg T2/100 g body wt. did not result in any thyrotoxic effect that might be of clinical relevance [9]; and 3) data present in literature so far, show that T2 serum levels are ~40–100 times lower than those of T3 [10].

The treatment, housing, and sacrificing of animals met the guidelines set by the Italian Health Ministry. At the end of the treatment, the animals were anesthetized by injection of chloral hydrate (40 mg/100 g body weight, i.p.) and blood was drawn via the inferior cava vein. Immediately after blood collection, the liver was removed for further processing. Serum levels of cholesterol, triglycerides and ALT were determined using standard procedures. Thyroid stimulating hormone (TSH) was measured using materials and protocols supplied by Amersham Bioscience, with rat TSH as standard (Biotrak TM rat (rTSH) [125I] assay system).

2.3. Liver lipid droplet and triglycerides content

Sections of livers were fixed in formol-calcium, and 10 μm frozen sections were subsequently stained with Sudan Black B for the detection of fat [11].

Lipid was extracted from frozen tissues in chloroform: methanol by the methods of Folch et al. [12]. The triglyceride concentration was determined using the Infinity triglyceride kit (Sigma).

2.4. Measurement of mitochondrial respiration parameters, fatty acid oxidation rate and carnitine palmitoyl-transferase system (CPT) activity assay

Mitochondria were isolated as previously reported [13]. Mitochondrial oxygen consumption was measured polarographically using a Clark-type electrode, as previously reported [13] and the rate of mitochondrial fatty acid oxidation was assessed in the presence of palmitoyl-L-carnitine (40 μM). CPT system (CPT1 plus CPT2) activity was measured spectrophotometrically (at 412 nm) [14].

2.5. Basal and palmitate-induced proton leak

Basal and induced proton leak kinetics were obtained by parallel measurements of mitochondrial oxygen consumption (using a Clark-type electrode) and membrane potential (using safranin O and a JASCO dual-wavelength spectrophotometer (511–533 nm)) in presence or in absence of 75 μM palmitate as previously described [13].

2.6. Mitochondrial aconitase and superoxide dismutase (SOD) activity assays and H₂O₂ release

Aconitase and SOD specific activity was measured spectrophotometrically as previously described [7]. The rate of mitochondrial H₂O₂ release was assayed by following the linear increase in fluorescence (ex 312 nm and em 420 nm) due to the oxidation of homovanillic acid by H₂O₂ in the presence of horseradish peroxidase (using a fluorometer) [15].

2.7. Western blot analysis

Uncoupling protein (UCP3 and UCP2) protein levels in mitochondrial lysates (30 μg of protein) were determined using 13% SDS–polyacrylamide gel as described by de Lange et al. [16].
2.8. Data analysis

All data are expressed as the means ± SE. Respiration rates at the highest membrane potential common to all the curves were used to test for differences in proton leak. Differences between groups were compared by ANOVA followed by the Newman–Keuls test to correct for multiple comparisons. Differences were considered statistically significant at \( P < 0.05 \). All analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

3. Results

3.1. T2 decreases body weight gain and metabolic efficiency without suppressing TSH

At time 0 (i.e., after 6 weeks feeding), D\(_0\) rats showed significantly greater increases in body weight gain (about +60%), in energy intake (+20%) and in energy efficiency (+35%) than N rats (data not shown). Following the last 4 weeks of treatment, the body weight gain and energy efficiency was still significantly greater in D rats than in N rats. Moreover, although in DT animals there was a 17% increase in energy intake (even if not significant), DT rats (i.e., D\(_0\) rats treated with T2 for the last 4 weeks) exhibited a significantly decrease both in body weight gain (−37%) and energy efficiency (−48%) than D rats (Table 1).

At this time point (10 weeks), the livers of D rats were lighter in colour than those of the DT group (Fig. 1). Stained sections showed that D livers contained more abundant fat droplets than N livers, whereas fat droplets were markedly fewer in DT sections than in D ones (Fig. 1). Moreover, hepatic triglycerides content was significantly elevated in D rats, while administration of T2 reduced it to a value not significantly different from that of N animals (Table 2).

Table 1
Body weight gain, energy intake and energy efficiency in N, D and DT rats.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>D</th>
<th>DT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g)</td>
<td>15 ± 1</td>
<td>19 ± 1*</td>
<td>12 ± 1#</td>
</tr>
<tr>
<td>Energy intake (kJ)</td>
<td>9348 ± 455</td>
<td>8178 ± 386</td>
<td>9614 ± 402</td>
</tr>
<tr>
<td>Energy efficiency (%)</td>
<td>0.16 ± 0.01</td>
<td>0.23 ± 0.02*</td>
<td>0.12 ± 0.01#</td>
</tr>
</tbody>
</table>

N = rats that had received the standard diet for 10 weeks; D = rats that had received a high-fat diet for 10 weeks and were sham-injected throughout the last 4 weeks; DT = rats that had received the high-fat diet for 10 weeks, with T2 being administered throughout the last 4 weeks.

Data are means ± SE for five different rats in each experimental group.

Energy efficiency = body weight gain/energy intake.

* \( P < 0.05 \) compared to N rats.

# \( P < 0.05 \) compared to D rats.

Table 2
Serum levels of cholesterol, triglycerides, ALT and TSH and hepatic triglycerides content in N, D and DT rats.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>D</th>
<th>DT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>45.5 ± 0.6</td>
<td>78.5 ± 0.6*</td>
<td>62.5 ± 0.5*#</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>105 ± 8.0</td>
<td>250 ± 22.5*</td>
<td>140 ± 18*#</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>23 ± 1</td>
<td>35 ± 2*</td>
<td>20 ± 2#</td>
</tr>
<tr>
<td>TSH (ng/ml)</td>
<td>0.64 ± 0.07</td>
<td>0.73 ± 0.05</td>
<td>0.76 ± 0.06</td>
</tr>
<tr>
<td>Hepatic triglyceride content (mg/ml)</td>
<td>2.8 ± 0.6</td>
<td>5.7 ± 0.2*</td>
<td>3.2 ± 0.4*#</td>
</tr>
</tbody>
</table>

N = rats that had received the standard diet for 10 weeks; D = rats that had received a high-fat diet for 10 weeks and were sham-injected throughout the last 4 weeks; DT = rats that had received the high-fat diet for 10 weeks, with T2 being administered throughout the last 4 weeks.

Data are means ± SE for five different rats in each experimental group.

ALT = alanine aminotransferase.

TSH = thyroid stimulating hormone.

* \( P < 0.05 \) compared to N rats.

# \( P < 0.05 \) compared to D rats.

Fig. 1. Histological analyses of livers obtained from N, D, and DT rats. (A) Livers and (B) representative photomicrographs of livers from N, D, and DT rats. Degree of fat accumulation in liver was evaluated using Sudan black. N = rats that had received the standard diet for 10 weeks; D = rats that had received a high-fat diet for 10 weeks and were sham-injected throughout the last 4 weeks; DT = rats that had received the high-fat diet for 10 weeks, with T2 being administered throughout the last 4 weeks. [This figure appears in colour on the web.]
no substantial differences from the normal euthyroid values (Table 2).

3.2. T2 decreases serum levels of cholesterol, triglycerides and ALT

Serum levels of cholesterol and triglycerides were higher in D rats than in N rats, whereas in DT animals they were significantly reduced (Table 2). In agreement with reports that in NAFLD, a) the increased serum ALT concentration is a consequence of hepatocyte damage [17] and b) the degree of liver steatosis is positively correlated with the liver function parameter ALT [18], the serum ALT level was significantly elevated in D rats, while administration of T2 reduced it to a value not significantly different from that of N animals (Table 2).

3.3. T2 increases hepatic mitochondrial oxygen consumption and fatty acid oxidation

Mitochondrial State 3 (Fig. 2A) and State 4 respiration (Fig. 2B) obtained using succinate as substrate were decreased significantly in D rats (vs. N). Administration of T2 increased them to values not significantly different from those of N animals (Fig. 2). The fatty acid oxidation rate (measured in the presence of palmitoyl-carnitine as substrate) and CPT system activity were increased in D rats vs. N rats and further increased in DT rats (Fig. 3 A and B).

Collectively, these results indicate that T2 increases the ability of mitochondria to import and oxidise fatty acids.

3.4. T2 activates mitochondrial proton leak in liver

Fig. 4A shows that under basal conditions (i.e., in the absence of free fatty acids [19,20], mitochondria from DT rats had to consume more oxygen than N and D rats to maintain a given membrane potential. This indicates that DT rats had an increased basal proton leak (compared to both N and D rats). Indeed, respiration rates measured at the highest membrane potential common to all the curves (180 mV) were statistically significant ($P < 0.05$) when comparing DT rats with N and D rats (Fig. 4B). On the other hand, basal proton leak from N rats was indistinguishable from D rats since the respective kinetic curves were superimposable (Fig. 4A).

As for fatty acid-induced proton leak (i.e., in the presence of physiological amounts of palmitate) among the three groups analysed, D rats had the lowest proton leak and DT rats the highest one (Fig. 4C). Indeed, respira-
tion rates measured at the highest membrane potential common to all the curves (165 mV) were statistically significant ($P < 0.05$) between the groups (Fig. 4D).

At mitochondrial level, neither UCP2 nor UCP3 protein was found to be expressed, thus excluding their possible involvement in the uncoupling effect of T2.

3.5. T2 decreases mitochondrial oxidative stress

When compared to N rats, D rats showed a higher mitochondrial H$_2$O$_2$ release (an indirect index of mitochondrial superoxide production in vitro) (Fig. 5A), an increase in mitochondrial ROS production (as confirmed by the basal/total aconitase activity ratio, a sensitive marker of oxidative stress) (Fig. 5B) and a reduced capacity to eliminate superoxides at the mitochondrial level, since superoxide dismutase activity was significantly inhibited (Fig. 5C).

Administration of T2 to rats fed a high-fat diet reduced mitochondrial oxidative stress (Fig. 5). When compared to D rats, DT rats showed a decrease in H$_2$O$_2$ release and a reduced degree of ROS inhibited aconitase activity (as indicated by an increased basal aconitase/total aconitase ratio). Moreover, increased superoxide dismutase activity was observed in DT rats (vs. D rats), although it was not restored to the control value (N).

4. Discussion

To study the effect of T2 on the fatty liver, we chose rats fed a high-fat diet since in this model, fat accumulation is usually not accompanied by liver injury. Indeed, in another experimental models developed to investigate the biochemical alterations occurring in the fatty liver (namely, rodents fed a choline-methionine deficient diet) hepatocyte injury, fibrosis and cirrhosis occurred [21–23]. In contrast, in the present model of steatosis no signs of inflammation or fibrosis can be detected until week twelve of treatment [24]. Our data show that although the serum ALT level was significantly higher (+51% vs. N) in D rats (as expected with hepatic steatosis), the increase was less marked than that found in rodents with steatohepatitis [18], a result confirming the absence of liver injury in our experimental model.

The present results show that the development of ectopic fat storage in the liver induced by a high-fat diet is associated with alterations in the mitochondrial compartment. Indeed, D rats exhibited reduced respiratory
capacity and increased oxidative stress in their liver mitochondria even if the ability to utilise fat as a metabolic fuel was elevated. The increased mitochondrial fatty acid oxidation observed in the fatty liver would be a compensatory mechanism for the increased hepatic uptake and synthesis of free fatty acids (FFAs) that occur during high-fat feeding. Apparently, this also occurs in patients with steatohepatitis via an increased mitochondrial β-oxidation of fatty acids and ketogenesis [25,26] and enhanced mitochondrial β-oxidation is also found in the liver of the genetically obese (ob/ob) mouse, which exhibits massive steatosis [27]. The mechanisms responsible for the increased mitochondrial β-oxidation observed in the fatty liver are poorly understood but several hypotheses may be put forward.

One possible mechanism is substrate pressure, due to an increased hepatic uptake and synthesis of FFAs in the liver. A second mechanism could be an activation of hepatic peroxisome proliferator-activated receptor alfa (PPAR-α) by the augmented pool of FFAs, while a third mechanism could be enhanced CPT-I activity, which would further increase the entry of long-chain fatty acids into the mitochondria. Indeed, CPT-I expression and/or activity in the liver are enhanced in rodent models of steatosis [27–29]. Actually, the present data and those reported by others indicate that all three mechanisms may be operative. In fact, in mitochondria from the liver of D rats, both CPT system activity and β-oxidation were increased (vs. control rats). Moreover, a very recent study has shown that PPAR-α expression is enhanced in rats fed a high-fat diet [30].

Succinate oxidation shares with lipid oxidation the pathways underlying FADH2 linked respiration. In contrast to the significant increase in oxygen consumption occurring when palmitoyl-carnitine was employed as substrate, decreased respiratory rates were evident when respiration was supported by succinate, thus suggesting that FADH2 linked respiratory pathways are inhibited by high-fat diet feeding. Mitochondrial State 4 respiration, controlled by the activity of the respiratory chain and by the proton leak [31], was decreased in the fatty liver, although no change in the basal proton leak occurred. This seems to support an inhibition of electron chain activity and a substantial role for substrate pressure in enhancing fatty acid oxidation in the liver in rats fed a high-fat diet. However, this increase in lipid oxidation is apparently not sufficient to handle the increased load of hepatic FFAs, the result being that the remaining FFAs are converted into triglycerides that are partly stored in the cytoplasm, causing steatosis. Interestingly, our data suggest that an increase in mitochondrial energy efficiency, as shown by the decrease in the induced proton leak in D rats, also contributes to the fat accumulation observed in D rats.

Under normal conditions, from 0.15% to a few percent of the total amount of oxygen consumed yields superoxide [32] but this may increase significantly in certain conditions. For instance, ROS production is increased when metabolic turnover increases due to an increase in the respiratory chain enzyme levels. Interestingly, fatty acid β-oxidation per se [32,33] can lead to significant mitochondrial ROS generation, probably by increasing both the amount of reducing equivalents and electron transfer within the respiratory chain. Large amount of ROS are also likely to be produced via the concomitant increase in β-oxidation rate (which enhances NADH and FADH2 generation and thus electron delivery to the respiratory chain) and respiratory

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Fig. 5. Effect of T2 on mitochondrial H2O2 release (A), basal aconitase/total aconitase ratio (B) and superoxide dismutase activity (SOD) (C) in liver mitochondria from N, D, and DT rats. N = rats that had received the standard diet for 10 weeks; D = rats that had received a high-fat diet for 10 weeks and were sham-injected throughout the last 4 weeks; DT = rats that had received the high-fat diet for 10 weeks, with T2 being administered throughout the last 4 weeks. Data are means ± SE for five different rats in each experimental group. *P < 0.05 compared to N rats. #P < 0.05 compared to D rats.
chain impairment (as indicated by the decrease in succinate State 3 oxygen consumption, which would partially block electron flow within the respiratory chain). Indeed, D rats exhibited, in addition to hepatic steatosis, an increase in mitochondrial oxidative stress parameters such as an increase in H$_2$O$_2$ production and inhibitions of aconitase and SOD activity.

The most interesting results relate to changes in the efficiency of substrate utilization. Indeed, our data showing a decreased proton leak at the mitochondrial level, and an increase in body weight gain/energy intake ratio clearly indicate a more efficient energy utilization in D animal vs. N. Following T2 treatment, on the other hand, energy utilization efficiency was markedly decreased. In addition, in line with the decreased fat droplets and triglycerides content in the livers, both the serum cholesterol and triglycerides levels and also the ALT level were significantly reduced. The effect of T2 on liver mitochondrial efficiency could not be ascribed to an induction of the expressions of UCP2 and UCP3, which have been proposed by some authors as mediators of an uncoupling effect [19,20] since neither UCP2 nor UCP3 protein were found to be expressed. Thus, the mechanism underlying the uncoupling effect of T2 remains to be elucidated even though previous studies [34,35] had already shown that the addition of T2 to a reconstituted cytochrome-c oxidase (COX) complex led to a decrease in the respiratory control ratio of the complex. The authors concluded that the effect of T2 on COX results in partial uncoupling of oxidative phosphorylation and decrease H+/e-stoichiometry, which explain, at least in part, our data.

At the mitochondrial level, 4 weeks administration of T2 induced further increases in $\beta$-oxidation and CPT system activity. These increases, by allowing better compensation of hepatic load of FFAs, could be one of the mechanisms whereby T2 is able to improve steatosis. In addition, the increase in respiration rates would increase the re-oxidation of NADH, a coenzyme required for both $\beta$-oxidation and the tricarboxylic acid cycle [36]. This, coupled with a concomitant less efficient utilization of substrates, through a stimulation of both the basal and fatty acid-induced proton leak would lead to a greater burning of fat. Further, an increased proton leak would prevent the over-reduction of respiratory complexes and excessive ROS formation [37]. Indeed, our data reveal that T2 administration induced a reduction in hepatic mitochondrial oxidative stress, as indicated by the significant decrease in H$_2$O$_2$ release that was coupled to increases both in the basal aconitase/totai aconitase ratio and SOD activity.

Taken together, the present results highlight the ability of T2 to reduce a pre-existing hepatic fat accumulation that had already been induced by feeding a high-fat diet. These data, together with those previously published [6], support a therapeutic potential role of T2 for preventing or treating steatosis. Preliminary data [38] on the effects of T2 in humans showed that T2 may increase resting metabolic rate, decrease adiposity and body weight, without side effects. Although modulating fat intake and increasing physical activity is the mainstay of treatment of metabolic disorders, such as type 2 diabetes, overweight and NAFLD, if preliminary data on humans are confirmed in larger studies, then pharmacological use of T2 could be considered in clinical trials for the treatment of this liver disease.

Acknowledgement

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References