Rapid stimulation of hepatic oxygen consumption by 3,5-di-iodo-L-thyronine

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INTRODUCTION

Mitochondria are a target of thyroid-hormone action. In vivo, application of thyroid hormones for several days provokes an increase in their number and respiratory capacity, alterations in their size and inner surface area (for reviews see [1,2]) as well as in a set of proteins [3]. Whether thyroid hormones exert an additional rapid action on increasing mitochondrial activity is a matter of debate.

$T_3$ (1 $\mu$m) significantly increased $O_2$ consumption ($Q_{O_2}$) in the isolated perfused liver of hypothyroid and euthyroid rats [4]. $Q_{O_2}$ of mitochondria isolated 20 or 30 min after an intravenous injection of $T_3$ was likewise increased [5,6]. When mitochondria were isolated 15 min after the injection, no effect on $Q_{O_2}$ was detectable, whereas other parameters (ADP/O, H+/O, pH gradient, adenine nucleotide translocation) were significantly altered [7-9]. The alterations of ADP/O and H+/O ratios, however, were not reproduced by others [5,6] and were recently suggested to be an experimental artefact [10]. The enhanced adenine nucleotide translocation, on the other hand, was also detected in the isolated perfused liver [11].

With respect to the mechanism(s) involved, opinions are also controversial. Sterling [12] claimed to have evidence that these effects are due to $T_3$ binding at the adenine nucleotide translocase. Several observations argue against this hypothesis, however (for a review see [13]). A direct stimulation of oxidative phosphorylation in submitochondrial particles by $T_3$ was reported [14], whereas others failed to obtain any $T_3$ effect on intact mitochondria [9].

The present study was carried out to investigate the rapid effect of iodothyronines on $Q_{O_2}$ in the isolated perfused liver, to verify its specificity by use of thyroid-hormone analogues, and to approach the underlying mechanism(s).

MATERIALS AND METHODS

Chemicals

3'-Acetyl-$T_3$, Dibit, L-94901 and the ether-bridge analogues were from Smith Kline and French Research (Welwyn, Herts., U.K.), and 3,3',5-tri-iodopropionic acid was from Sigma Chemie G.m.b.H. (Munich, Germany). All other iodothyronines and thyroid-hormone analogues were from Henning Berlin G.m.b.H. All tested compounds were > 99 % pure. For application, they were dissolved in a minimum of 1 m NaOH before dilution with water, and tested at 1 pm except where otherwise stated.

6-n-Propyl-2-thiouracil was from Sigma (Munich, Germany), and enzymes were from Boehringer Mannheim (Mannheim, Germany). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany) or Boehringer Mannheim.

Animals

Male Wistar rats (specific-pathogen-free) from Wiga (Charles River, Sulzfeld, Germany) were used throughout this study. Over the entire experimental period, veterinary supervision was provided. Hypothyroidism was provoked by an intraperitoneal injection of 0.25 mCi of Na$^{31}$I 28 days before the experiments. The hypothyroid state was monitored by measurement of serum $T_4$ (< 10 ng/ml) and hepatic malic enzyme activity (< 2 units/mg of protein). Experiments were performed in rats fasted overnight.

Abbreviations used: $T_3$, 3,5-di-iodo-L-thyronine; $T_2$, 3,3',5-tri-iodo-L-thyronine; $T_4$, thyroxine; Dimit, 3,5-dimethyl-3'-isopropyl-L-thyronine; Dibit, 3,5-dibromo-3'-isopropyl-L-thyronine; L-94901, 3,5-dibromo-3'-pyridazinone-L-thyronine; $Q_{O_2}$, $O_2$ consumption.

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Perfusion system

Rats were anaesthetized with pentobarbital, and isolated livers were perfused in a recirculating system with Krebs/Henseleit bicarbonate buffer saturated with O₂/CO₂ (19:1), pH 7.4, at 37°C, with 10 mM-alanine as substrate. Q₀ was continuously monitored by a Clark-type electrode. Q₀ of controls (n = 7) increased linearly during perfusion, from 1.90 ± 0.20 μmol/min per g after 30 min of preperfusion to 2.16 ± 0.09 after 30 min perfusion time, 2.35 ± 0.12 after 60 min, and 2.48 ± 0.13 after 90 min. Hepatic function was monitored by alanine uptake (210 ± 30 μmol/2 h per g), glucose production (30 ± 10 μmol/h per g), urea production (60 ± 15 μmol/2 h per g) and the release rate of glutamate-oxaloacetate transaminase (< 1.5 units/2 h per g).

Spectrophotometric analysis

Substrate concentrations and enzyme activities were determined as described in Bergmeyer [15], except for malic enzyme [16]. A unit of enzyme activity is defined as turnover of 1 μmol of substrate/min. Protein was measured by the Bio-Rad method.

Statistical analysis

Statistical studies were performed with a computer program using Student’s t test; P < 0.01 was considered significant.

RESULTS

Effect of iodothyronines in the isolated perfused liver

In order to examine the rapid effect of iodothyronines on hepatic Q₀, a series of compounds was tested in the isolated perfused liver of hypothyroid rats. T₃, T₂ and T₄ stimulated Q₀, at a concentration as low as 1 pm (Fig. 1). Stimulation by T₂ was statistically significant only after 30 min, whereas stimulation by T₃ and T₄ was slower, being significant after 60 min. Q₀ was roughly similar in all three cases 90 min after addition of iodothyronines to the perfusate. Other iodothyronines tested (3-monooiodothyronine, 3,3'-T₂, 3',5'-T₂, rT₃; nos. 2, 4, 5, 10) as well as 3,5-di-iodothyroline (no. 1) had no effect (Table 1).

In dose/response experiments, T₃ and T₄ (Fig. 2) showed a maximal response at 1 pm. No further effects were detectable at higher concentrations, whereas they were diminished at lower concentrations.

T₃ is produced from T₄ in the liver and kidney by type I iodothyronine monodeiodinase [17]. The enzyme also rapidly degrades iodothyronines to thyronines including the production of T₂ from T₃. The observed different time courses of the Q₀ stimulation by T₃, T₂ and T₄ led to the suggestion that T₄ and T₃ are deiodinated to T₂ to become active for this rapid stimulation. Therefore, perfusion experiments were carried out in the presence of 6-n-propyl-2-thiouracil, a potent inhibitor of deiodinase [17]. Propylthiouracil was applied at 1 μM, in accordance
3,5-Di-iodothyronine stimulates hepatic O₂ consumption

Fig. 1. Effect of iodothyronines (T₁; 1 µM) on the O₂ consumption of isolated perfused livers of hypothyroid rats in the absence or presence of propylthiouracil (PTU; 1 µM).

Data given are ΔO₂ above controls (n = 7); O₂ consumption at 0 min was 1.9 ± 0.2 µmol/min per g (n = 3, mean ± s.e.m.) ○, T₁; □, T₂; □, T₃, *P < 0.01 versus T₁, T₂, and controls; **P < 0.01 versus perfusion without PTU.

with [18]. Control perfusions with propylthiouracil alone revealed no influence of the inhibitor on Q₀₂; T₁ and T₂ lost all their stimulative activity, whereas T₃ was as potent as in the absence of propylthiouracil (Fig. 1). Thus T₃ can be perceived to be the sole thyroid hormone to stimulate hepatic Q₀₂ rapidly and significantly.

Effect of thyroid-hormone analogues in the isolated perfused liver

A series of perfusion experiments with thyroid-hormone analogues was performed to confirm the specific T₂ effect (Table 1).

Those of the 3',5'-substituted 3,5-di-iodothyronine analogues with smaller substituents (CH₃, Br; nos. 6–8) gave rise to a stimulation of Q₀₂ comparable with that by T₂, whereas larger substituents (I, acetyl, isopropyl; nos, 9, 12, 13) diminished the effect. Among the 3,3',5'-substituted analogues, Dibit (no. 14) and Dimit (no. 15) stimulated Q₀₂ to a considerable extent; L-94901 (no. 16), with its large pyridazinone substituent in the 3' position, was ineffective.

When rotation of the phenolic ring was prevented by 2'-methylation [19], the T₂ analogue (no. 17) was as active as T₃ itself, whereas the T₃ analogue (no. 18) hardly stimulated Q₀₂ at all. 4'-Methylation (no. 19) also led to a remarkable loss of effectiveness. Replacement of the ether oxygen atom by a sulphur bridge (no. 20) slightly decreased the stimulative activity. The DL-methylene bridge analogue (no. 21) was ineffective.

No analogue with alterations in the side chain was as potent as the iodothyronines with an intact l-alanine side chain (Table 2). Among the acetic acid and ethylamine side-chain analogues, only di-iodothyroacetic acid (no.

Table 2. Effect of side-chain analogues (1 µM) of thyroid hormones on the O₂ consumption of isolated perfused livers of hypothyroid rats in comparison with 'anti-goitre activity' and induction of hepatic mitochondrial glycerol-3-phosphate dehydrogenase activity (from refs. [19,23,44])

Data given are ΔO₂ above controls (n = 7) at 90 min perfusion time; O₂ consumption at 0 min was 1.9 ± 0.2 µmol/min per g or % of T₂ action (T₂ = 100 %); n = 2 (single values given). Nos. 29 and 30 are l-isomers. Key: tTriam; tDL; 'full agonist (refs. [23,44]); Ac, -COCH₃; AG, anti-goitre test (ref. [19]); GPDH, induction of hepatic mitochondrial glycerol-3-phosphate dehydrogenase activity (refs. [23,44]).

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>R₁</th>
<th>R₃</th>
<th>ΔO₂ (µmol/min per g)</th>
<th>ΔO₂ (% T₂)</th>
<th>AG (%) T₂</th>
<th>GPDH (%) T₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>D-T₂</td>
<td>H</td>
<td>H</td>
<td>0.14, 0.17</td>
<td>23</td>
<td>7.5</td>
<td>44</td>
</tr>
<tr>
<td>23</td>
<td>D-T₃</td>
<td>H</td>
<td>H</td>
<td>0</td>
<td>0</td>
<td>0.12</td>
<td>0.5</td>
</tr>
<tr>
<td>24</td>
<td>Triprop</td>
<td>CH₂CH₂CO₂H</td>
<td>H</td>
<td>0.40, 0.46</td>
<td>54</td>
<td>4.5</td>
<td>1.9</td>
</tr>
<tr>
<td>25</td>
<td>Diac</td>
<td>CH₂CO₂H</td>
<td>H</td>
<td>0.17, 0.25</td>
<td>23</td>
<td>&lt; 0.5</td>
<td>1.9</td>
</tr>
<tr>
<td>26</td>
<td>Triac</td>
<td>CH₂CO₂H</td>
<td>I</td>
<td>0.14, 0.24</td>
<td>23</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Tetra</td>
<td>CH₂CO₂H</td>
<td>I</td>
<td>0.23, 0.32</td>
<td>32</td>
<td>4.3³</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Diam</td>
<td>CH₂CH₂NH₂</td>
<td>H</td>
<td>0.06, 0.12</td>
<td>6</td>
<td>2²</td>
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<tr>
<td>29</td>
<td>N-AcT₂</td>
<td>CH₂CH(NHAc)CO₂H</td>
<td>H</td>
<td>0.28, 0.24</td>
<td>30</td>
<td>4.3³</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>N-AcT₃</td>
<td>CH₂CH(NHAc)CO₂OH</td>
<td>I</td>
<td>0.24, 0.32</td>
<td>32</td>
<td>4.3³</td>
<td></td>
</tr>
</tbody>
</table>
25) showed a noteworthy effect. Alterations at the amino acid group (nos. 24, 29, 30) led to a loss, but not to abolition, of stimulation. The isomeric D-T₃ (no. 22) and D-T₄ (no. 23) were barely active or inactive.

**Effect of cycloheximide in the isolated perfused liver**

It is generally accepted that iodothyronines exert their action on intermediary metabolism by increasing the synthesis of specific proteins via the nuclear pathway [20]. In order to show that ongoing protein synthesis is not involved in the rapid T₃ effect on hepatic Qₒₒ, protein synthesis was inhibited by the addition of cycloheximide [21,22]. The effect of T₃ was still apparent (Fig. 3).

**Effect of T₃ on malic enzyme induction in vivo**

Only a little information is available on T₃ effects on increasing the synthesis of specific enzymes by the nuclear pathway [19,23]. Therefore, the T₃ effect on the induction of hepatic malic enzyme activity as a very sensitive parameter of thyroid-hormone action was measured. T₃ was injected intraperitoneally into hypothyroid rats fed on a carbohydrate-rich diet, at a daily dose of 50 mg/100 g body wt. for 3 days. This led to a minimal increase in malic enzyme activity (0.29 ± 0.04 munit/g of liver above control values of 0.32 ± 0.05 munit/g). In comparison, a similar dose of T₄ increased malic enzyme activity by 2.6 ± 0.2 munits/g above controls, as could be expected from data in the literature [24].

**DISCUSSION**

The essential finding of the present study is the demonstration that T₃ is an additional metabolically active thyroid hormone that rapidly increases mitochondrial Qₒₒ at a concentration as low as 1 μM. T₂ appears to act on mitochondria via a direct pathway.

Besides T₃, application of T₄ and T₂ resulted in an increased Qₒₒ. The different time courses and the propylthiouracil experiments (Fig. 1) demonstrate that T₃ and T₂ must be considered as ‘preprohormone’ and ‘prohormone’ for the rapidly acting hormone T₄. This conclusion is ascertained by the experimental series with thyroid-hormone analogues (Tables 1 and 2). The volumes [23] of the phenolic-ring substituents of T₃ analogues can easily be correlated with the rapid mitochondrial and long-term nuclear effects: H (0.03 cm³/mol; no. 3), CH₃ (0.14; no. 6) and Br (0.15; nos. 7, 8) rapidly stimulate hepatic Qₒₒ, but are less effective or ineffective on nuclear-mediated effects. Conversely, the larger substituents I (0.20; no. 9), acetyl (0.25; no. 12), and isopropyl (0.34; no. 13) are less effective at the mitochondrial, but quite effective at the nuclear pathway. The charges of the substituents will also play a considerable role in the effectiveness, as they lead to alterations in the conformation of the molecules. The effect of Dimit (no. 15: 66 %) in comparison to 3'-isopropyl-T₃ (no. 13: 18 %) and Dibit (no. 14: 24 %) might result from this phenomenon.

2'-Methylation leads to a fixed proximal 5' position [19]. As 2'-CH₃T₃ (no. 18) is a poor inhibitor of T₄ 5'-deiodination [25], it might be suggested that it is hardly deiodinated. In fact, this compound failed to stimulate hepatic Qₒₒ as did T₃ in the presence of propylthiouracil (Fig. 1).

The sulphur-bridge analogue (no. 20) had little effect, and the methylene-bridge analogue (no. 21) was found to be a full agonist, on the nuclear pathway, whereas the contrary was discovered for the rapid stimulation of hepatic Qₒₒ. The inhibitory concentration for T₄ 5'-deiodination of methylene-bridged T₃ is very high, but that of sulphur-bridged T₃ is comparable with that of T₄ itself [25]. It can be concluded from these data that in the present study the sulphur-bridged T₃ was deiodinated and thus able to stimulate Qₒₒ, whereas the methylene-bridged T₃ was not deiodinated, and consequently was without effect.

The results of the experiments with the side-chain analogues (Table 2) also demonstrate potential differences in their mechanism of action: whereas D-T₃ (no. 23) and tri-iodothyroacetic acid (no. 26) stimulate mitochondrial glycerol-3-phosphate dehydrogenase activity, they are inactive in increasing Oₒ consumption. Hepatic Qₒₒ is, on the other hand, rapidly stimulated by tri-iodopropionate (no. 24), di-iodothyroacetic acid (no. 25) and the N-acetyl derivatives (nos. 29 and 30), compounds with very low nuclear activity.

In summary, the experiments with thyroid-hormone analogues confirm the specificity of the T₃ effect on hepatic Qₒₒ. The data also suggest that the mechanism of the T₃ effect may be distinct from that mediating enzyme induction in response to T₄.

**Target cell organelle**

Thyroid hormones exert an effect not only on nuclei and mitochondria, but also at the cell membrane: they rapidly (< 1 min) stimulate the uptake of sugar and Ca²⁺ into rat thymocytes [26,27] and of amino acids and Ca²⁺ into the liver [4,28]. In isolated erythrocyte membranes, iodothyronines and thyroid-hormone analogues (0.1 nm) stimulated Ca²⁺-ATPase activity within 60 min [29]. This stimulation seems to be rather unspecific, because not only T₃, T₄ and T₂, but also 3,5-diiodothyrosine, was effective. An increase in cytosolic Ca²⁺ should in turn increase the activities of some mitochondrial enzymes.

**Fig. 3. Effect of T₃ (1 μM) on the O₂ consumption of isolated perfused livers of hypothyroid rats in the presence of cycloheximide (20 μg/ml)**

Data give are ΔO₂ during perfusion; O₂ consumption at 0 min was 1.9 ± 0.2 μmol/min per g. Single values of four experiments are given. ○, Control; ●, T₃.
3,5-Di-iodothyronine stimulates hepatic $O_2$ consumption

(e.g. pyruvate dehydrogenase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase) [30] and thus lead to an enhanced $Q_o$. Ca$^{2+}$-mediated effects on mitochondria, however, were found to be associated with an elevation of the intramitochondrial ATP/ADP ratio [31–33], whereas a significant decrease was observed after application of T$_2$ [11]. There is also a considerable lag period between the onset of Ca$^{2+}$ influx (approx. 2 min) [26–28] and $Q_o$ stimulation (approx. 20 min) [28], and cf. Fig. 1). Ca$^{2+}$ can thus be considered unlikely to be a messenger for the rapid T$_2$ effect on hepatic $Q_o$.

In order to exclude the participation of the nuclear pathway in the rapid $Q_o$ effect, the following experiments were carried out. (i) T$_3$ was added to the perfusion system at 1 nM, a concentration known to induce, for example, phosphoenolpyruvate carboxykinase in isolated liver systems [34]. A rapid action on $Q_o$ could still be detected in the presence of the inhibitor of protein biosynthesis, cycloheximide [21,22] (Fig. 3). (ii) Application of T$_3$ in vivo hardly induced hepatic malic enzyme activity (cf. the Results section). Furthermore, the fastest known nuclear effect described in the literature is the induction of the spot 14 mRNA, coding for a protein with so-far unknown function [20]. The induction of this mRNA, however, is not apparent 30 min after application of T$_3$ [20], when the stimulation of $Q_o$ in the isolated perfused liver is already significant (Fig. 1). Thus a participation of the nuclear pathway in the rapid stimulation of hepatic $Q_o$ seems rather unlikely.

Preliminary findings with isolated liver mitochondria suggest that T$_3$ at 1 pm can produce a rapid stimulation of $Q_o$. It is noteworthy that this stimulation is very rapid, as there was no preincubation. It was reported previously that T$_3$ directly enhanced oxidative phosphorylation in submitochondrial particles [14] and increased ADP/O ratios in intact mitochondria [35]. These T$_3$ effects are presumably due to microsomal contaminations containing deiodinase and/or to T$_3$ impurities in the T$_3$ used. Palacios-Romero & Mowbray [9] found a direct T$_3$ effect on mitochondria only after incubation with liver homogenate, indicating that deiodination of T$_3$ to T$_2$ might be involved.

It can be assumed from the literature cited above and the data presented here that T$_3$ stimulates hepatic $Q_o$ directly and specifically at the mitochondrial level. Uptake and transport of T$_3$ in the liver as well as deiodination of T$_3$ and T$_2$ to T$_3$ seem to take time, as can be deduced from the time differences between the effects in isolated mitochondria and the isolated perfused liver on one hand, and between the three effective iodothyronines on the other. It should be investigated whether other cell organelles (e.g. plasma membrane, cytosol [36]) contribute to the effect observed in the isolated perfused liver.

Conclusions

T$_3$ has so far been considered as an intermediate product in the degradative pathways of T$_3$, 5'-Deiodination of T$_3$ to T$_2$ contributes to only about 5% of T$_3$ disposal [17]. Consequently serum T$_3$ concentrations in humans are low (0.4–5.3 ng/dl) compared with T$_3$ (116–147 ng/dl) [17,37]. Serum T$_3$ concentrations were inversely correlated with age [38] and were higher in men than in women, whose metabolic clearance and production rates of T$_3$, however, are higher than those of men [37]. In hyperthyroidism, a 4-fold increase in serum T$_3$ was observed [17,37].

In the present paper, iodothyronines at a concentration of 1 pm stimulated only $Q_o$ of isolated perfused livers. At higher concentrations (10 nM–10 μM), T$_3$ was found to stimulate rapidly ATP-consuming processes such as lipogenesis [39], gluconeogenesis [4,28,40], and urea synthesis [4], as well as adenine nucleotide translocation from mitochondria to cytosol [11]. When the T$_3$ concentration was gradually decreased from 1 μM to 10 pm, a concomitant decrease in gluconeogenesis was detectable [40], indicating the metabolic significance of our finding.

If the T$_3$ effect is present not only in the liver, but also in other organs (e.g. heart, muscle), T$_3$ might play a significant role in thermogenesis. Energy intake, for example, influences T$_3$ levels in humans: after eating a high-energy diet for several days, serum T$_3$ concentrations were 36% higher than after a low-energy diet, and the T$_3$ production rate was even doubled [41]. Thus T$_3$ might take part in the high-energy-induced thermogenesis observed in humans [42]. This phenomenon might also contribute to obesity. Deiodinase activity and serum free T$_3$ concentration were decreased in Zucker rats [43]; consequently, T$_3$ and T$_2$-influenced mitochondrial activity should be lowered, thereby probably limiting energy consumption.

Taken together, a new scheme of regulation of mitochondrial activity can be concluded: the novel metabolically active thyroid hormone, T$_3$, acts directly via the mitochondrial pathway in a short-term fashion, whereas T$_3$ exhibits its long-term effects via the nuclear pathway by the induction of certain mitochondrial proteins. It should be investigated whether this scheme of regulation is limited to the liver, or is also apparent in other organs.

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