EXPERIMENTAL STUDY

Effect of tryptophan on the early tri-iodothyronine uptake in mouse thymocytes

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Abstract

Objective: We have studied the effect of tryptophan on cellular [¹²⁵I]tri-iodothyronine (T³) uptake by mouse thymocytes.

Materials and methods: Mouse thymocytes (20 × 10⁶ cells/ml) were suspended in Krebs–Ringer solution buffered by Tris–HCl and incubation (23 °C at pH 7.45 ± 0.6), in the presence or absence of 1 mM tryptophan, was started by adding 25 pM [¹²⁵I]T³. At the end of incubation, samples were cooled in ice, centrifuged over a 30% sucrose cushion and the cell-associated radioactivity was measured in the pellet.

Results: Tryptophan reduced both the total and the saturable fraction of [¹²⁵I]T³ uptake by 44% (P ≤ 0.0009) and 60% (P ≤ 0.0006) respectively, following 1 min of incubation. This effect was specific and dose-dependent, being maximal at 5 mM concentration (−82%). In contrast, the pre-exposure of cells to tryptophan for up to 2 h had no effect on the subsequent uptake of [¹²⁵I]T³, in the absence of tryptophan. The effect of n-tryptophan on saturable T³ uptake was not different from that obtained using the l-stereoisomer. Tryptophan reduced the Vₘₐₓ of the initial rate of saturable [¹²⁵I]T³ uptake by two-thirds without affecting the apparent Kₘ (2.2 nM) of the process, thus indicating the non-competitive nature of the inhibition. In sodium-free medium the saturable [¹²⁵I]T³ uptake was reduced by 43%. The inhibitory effect of tryptophan on [¹²⁵I]T³ uptake was exerted in both the presence and the absence of sodium. In fact, the inhibitory effect of tryptophan on T³ transport was greater and significantly different (P = 0.0046) from that obtained by sodium depletion alone.

Conclusions: Tryptophan interferes with both the sodium-dependent and -independent components of [¹²⁵I]T³ uptake by a dose-dependent, non-competitive mechanism which operates in cis-modality at the plasma membrane level of mouse thymocytes.

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Introduction

Due to the intracellular localization of thyroid hormone receptors, iodothyronines must enter the cells to exert their biological actions. Several studies have focused on tri-iodothyronine (T³) and thyroxine (T⁴) entry mechanism into different cell types of rodents (1–7) and humans (8, 9); these have shown the existence of a regulatory mechanism at the plasma/cell interface. The mechanism, depending on the tissue studied, appears to operate both for T³ and T⁴ (1, 4, 5, 7–9) or T³ only (2, 3, 6). Despite some biochemical differences, almost all the mechanisms described for thyroid hormone transport showed analogies to some systems which mediate amino acid transport into cells (10–12). In mouse thymocytes, we have previously shown that a saturable, pH- and partly Na⁺-sensitivity mechanism mediates T³ entry (13). This process seemed to share several biochemical characteristics with the amino acid transport system ASC for neutral amino acids (10). In different tissues, the involvement of amino acid transport systems L or T (14–17), based on the effect of tryptophan and/or leucine on the rate of T³ or T⁴ transport, have been reported (14–17). In particular, the existence of a close relationship between tryptophan and T³ transport at the plasma membrane has been described in erythrocytes and cultured astrocytes (14, 15). Moreover, these interactions seem to be modulated by thyroid status in vivo (18). In mouse thymocytes, where over four-fifths of T³ bound to the nucleus derive directly from plasma (19), the role of transport systems at the plasma membrane level may be relevant to the intracellular fate of iodothyronines. The purpose of the present study was therefore to evaluate the effect of tryptophan on early T³ uptake by mouse thymocytes.
Materials and methods

Chemicals

All chemicals were reagent grade and were obtained from Sigma (St Louis, MO, USA). \(^{125}\text{I}\text{T3}\) (specific activity 3300 \(\mu\text{Ci/\text{g}}\)) was obtained from New England Nuclear (Florence, Italy). The purity of labelled T3 was tested by thin-layer chromatography on silica gel plates using a mixture (1:3:16) of formic acid/methanol/chloroform as in Sato & Cahnmann (20). Negligible deiodination occurred in all the conditions assayed.

Cell preparation and cellular uptake measurements

Isolated thymocytes were obtained from BALB-c mice, 3–6 weeks old, housed and fed using standardized procedures. Isolation of thymocytes was performed as previously described (21). Cell viability was tested before and after experiments and, as assessed by trypan blue dye exclusion, was always greater than 95%. Isolated cells were suspended in modified Krebs–Ringer solution buffered by Tris–HCl at physiological pH, as previously described (3). Preincubation, where appropriate, was carried out at 23 °C, in the presence or absence of 1 mM tryptophan, for 2, 1 or 0.5 h. In those experiments in which cells were pre-exposed to tryptophan, the amino acid was removed by washing the cells three times in incubation medium, before starting incubation with the labelled T3.

Incubation was started by adding tracer amounts (25pM) of \(^{125}\text{I}\text{T3}\) to 1ml medium containing 20 × 10^6 cells. Incubation was performed at 23 °C for the times specified in the text. at pH 7.45 ± 0.6, in the presence or absence of 1 mM \(L\)- and/or \(D\)-tryptophan, unless otherwise specified. Experiments were stopped by cooling samples in ice and 3 × 200 \(\mu\text{L}\) aliquots were immediately removed and layered in a plastic microtube over an equal volume of 30% sucrose; samples were then centrifuged, and the radioactivity in the pellet counted to determine the total amount of labelled T3 associated with cells. The non-specific cell-associated radioactivity was measured as the \(^{125}\text{I}\text{T3}\) uptake in the presence of 15 \(\mu\text{M}\) unlabelled T3 and represented the non-saturable fraction of \(^{125}\text{I}\text{T3}\) uptake. The saturable \(^{125}\text{I}\text{T3}\) uptake was obtained by subtracting the non-saturable fraction from the total \(^{125}\text{I}\text{T3}\) uptake and has been used throughout the study. In the experiments carried out in the absence of sodium, an iso-osmotic amount of choline chloride was added to the medium to replace sodium chloride.

All experiments were performed in triplicate and the results of at least three independent experiments were plotted. The INSTAT Graphpad software (1990–92) for DOS was used in the statistical analysis. Statistical analysis was performed by using the unpaired t-test or the Mann–Whitney test (two tailed) where appropriate.

Results

Table 1 Effect of tryptophan stereoisomer on \(^{125}\text{I}\text{T3}\) uptake. The uptake is expressed as % \(^{125}\text{I}\text{T3}/10^6 \text{cells}\) and the data represent the means ± s.d. of four different experiments.

<table>
<thead>
<tr>
<th></th>
<th>Total uptake</th>
<th>Variation</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>(a) Basal</td>
<td>1.62 ± 0.22</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(b) Plus excess T3</td>
<td>0.50 ± 0.07</td>
<td>—99</td>
<td>&lt;0.0001</td>
<td>—</td>
</tr>
<tr>
<td>(c) Plus (L)-tryptophan</td>
<td>0.91 ± 0.13</td>
<td>—44</td>
<td>0.0009</td>
<td>—</td>
</tr>
<tr>
<td>Plus excess T3 + (L)-tryptophan</td>
<td>0.53 ± 0.09</td>
<td>—67</td>
<td>&lt;0.0001 ns(^1)</td>
<td>—</td>
</tr>
<tr>
<td>Plus (\alpha)-tryptophan</td>
<td>1.01 ± 0.12</td>
<td>—38</td>
<td>0.0019</td>
<td>ns(^2)</td>
</tr>
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</table>

Cells were incubated with 25pM \(^{125}\text{I}\text{T3}\) for 1 min at 23 °C, with the variables listed above. 1 mM \(L\)- and \(L\)-tryptophan were used throughout. Excess unlabelled T3 added was 15 \(\mu\text{M}\) and after experiments and, as assessed by trypan blue dye exclusion, was always greater than 95%. Isolated thymocytes were obtained from BALB-c mice, 3–6 weeks old, housed and fed using standardized procedures. Isolation of thymocytes was performed as previously described (21). Cell viability was tested before and after experiments and, as assessed by trypan blue dye exclusion, was always greater than 95%. Isolated cells were suspended in modified Krebs–Ringer solution buffered by Tris–HCl at physiological pH, as previously described (3). Preincubation, where appropriate, was carried out at 23 °C, in the presence or absence of 1 mM tryptophan, for 2, 1 or 0.5 h. In those experiments in which cells were pre-exposed to tryptophan, the amino acid was removed by washing the cells three times in incubation medium, before starting incubation with the labelled T3.

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All experiments were performed in triplicate and the results of at least three independent experiments were plotted. The INSTAT Graphpad software (1990–92) for DOS was used in the statistical analysis. Statistical analysis was performed by using the unpaired t-test or the Mann–Whitney test (two tailed) where appropriate.

Results

Total \(^{125}\text{I}\text{T3}\) uptake following 1 min of incubation was 1.62 ± 0.22%/10^6 cells and the non-saturable fraction of uptake represented 31% of the total (Table 1). The addition of \(L\)-tryptophan (1 mM) during the incubation period significantly reduced total \(^{125}\text{I}\text{T3}\) uptake at that time. The inhibitory effect of \(L\)-tryptophan and that of an excess of unlabelled T3 were not additive, indicating that the effect of that amino acid was exerted only on the saturable fraction of T3 uptake (Table 1). The non-saturable fraction of \(^{125}\text{I}\text{T3}\) uptake (see Materials and methods) was subtracted to obtain the saturable uptake, as previously described (3). The saturable \(^{125}\text{I}\text{T3}\) uptake by thymocytes was linear in the first minute whereas the equilibrium between entry and exit rate was reached within 30 min, similarly to previous studies (3) (not shown). Following 1 min of incubation, the saturable fraction of \(^{125}\text{I}\text{T3}\) uptake was reduced by the simultaneous addition of 1 mM \(L\)-tryptophan (1.13 ± 0.16 vs 0.45 ± 0.15%/10^6 cells; —60%, \(P = 0.0006\)).

To investigate whether the inhibitory effect of tryptophan is exerted in cis, in trans or in both, thymocytes were preloaded up to 2 h in the presence of 1 mM tryptophan, which was then removed before starting the incubation. The pre-exposure to tryptophan

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had no effect on the subsequent uptake of T3 (1.58 ± 0.23 vs 1.53 ± 0.18%/10^6 cells; \( P \) = not significant (ns)). This finding suggests that the effect of tryptophan is restricted to cis-inhibition at the T3 binding site on the outer membrane.

As the uptake of T3 by thymocytes has been proven to be stereospecific (3), the comparative effect of L- and D-tryptophan was investigated. The addition of 1 mM D-tryptophan significantly reduced total [125I]T3 uptake to an extent (−38%) which was similar to that obtained with the L-stereoisomer (Table 1). The inhibitory effect of D-tryptophan on the saturable fraction of [125I]T3 uptake (−50%; \( P = 0.0061 \)) was also similar to that obtained using the L-stereoisomer (0.54 ± 0.11%/10^6 cells; \( P = \) ns). This finding indicated that the effect of tryptophan is exerted on a structure not able to discriminate between amino acid enantiomers.

The described effect of L-tryptophan was dose-dependent as shown in Fig. 1; in fact, the incubation of cells in the presence of increasing concentrations of the L-tryptophan (0.1–5 mM) progressively reduced the amount of [125I]T3 uptake to 18% of the control at the maximal concentration of tryptophan: the half maximal inhibitory concentration (IC50) of tryptophan was about 0.35 mM.

The effect of L-tryptophan on the initial rate of [125I]T3 uptake is shown in Fig. 2. The initial rate of T3 uptake, measured in the presence of increasing amounts of unlabelled T3 was saturable as already described (3) and was measured in the presence or absence of 1 mM tryptophan. The rate of transport was analyzed by plotting the data according to the Eadie–Hofstee plot, in both the experimental conditions. This plot, shown in Fig. 2, revealed that the apparent \( K_m \) of [125I]T3
transport was 2.2 nM and was not affected by the addition of tryptophan whereas the V\textsubscript{max} was 4.5 pM/10\textsuperscript{6} cells per min and was reduced by about two-thirds (1.78 pM/10\textsuperscript{6} cells per min) by the addition of L-tryptophan. These parameters suggest a non-competitive inhibition between these two substrates.

The described carrier which facilitates T3 entry in thymocytes was reported to be partly dependent on extracellular sodium (14). The combined effect of sodium deprivation and tryptophan was therefore tested on cellular T3 uptake. The removal of extracellular sodium from the medium decreased the saturable [\textsuperscript{125}I]T3 uptake by 43\% (P \approx 0.0052) as compared with basal experimental conditions. The inhibition exerted on [\textsuperscript{125}I]T3 uptake, in the presence of sodium, by using 1 mM tryptophan was greater and significantly different (P = 0.0046) from that obtained by sodium depletion alone (Fig. 3). The effect of tryptophan was already maximal as no further inhibition was obtained by combining the effect of the addition of tryptophan and that of the absence of sodium (Fig. 3).

**Discussion**

The results of this study indicate that tryptophan inhibits, in a dose-dependent fashion, the early saturable T3 uptake by mouse thymocytes to a considerably higher extent than reported in rat cardiac myocytes (7) and in choriocarcinoma cells (17).

A close relationship among the T3 transport system and amino acid transport systems L and/or its variant T was reported in different tissues (5, 14–16, 18) and the presence of a counter-transport mechanism shared by tryptophan and T3 has also been suggested in erythrocytes (14). In thymocytes, however, several biochemical characteristics of the effects of tryptophan were different from those previously reported (5, 14, 15, 17) and even the involvement of the T3 carrier described (3, 13) in that process may be questionable. In fact, the following peculiar findings emerged from the present investigation: (a) the effect of tryptophan on T3 uptake was evident only when both substrates (T3 and tryptophan) were present during incubation time; in contrast, in tryptophan-preloaded cells, when the incubation was carried out in the absence of tryptophan, no measurable inhibition was recorded. According to Guidotti et al. (10) and Christensen (12) it can be concluded that no trans-inhibition phenomena occurred and the effect of tryptophan was only exerted at the external T3-recognizing site at the plasma membrane level; (b) the initial rate of T3 influx in thymocytes was modified by tryptophan by changing the V\textsubscript{max} of the process, whereas the apparent K\textsubscript{m} was not modified, indicating a non-competitive nature of the inhibitory process; (c) the inhibitory effect of tryptophan was not stereospecific since the D-form had the same effect as the L-form, whereas the described carrier for T3 in these cells was strongly stereospecific (3); (d) the inhibitory effect of tryptophan on T3 transport was exerted both in the presence and the absence of sodium. A similar effect was previously obtained in this tissue by changing the external pH, whose variation inhibited [\textsuperscript{125}I]T3 uptake without discriminating between the sodium-dependent and sodium-independent component (13). Also, the coexistence of these two components of transport is typical of several transmembrane amino acids trafficking (12). However, tryptophan is usually transported into cells by a sodium-independent mechanism (22) and there is no evidence that it may interact with membrane structures related to ionic movements (10–12, 14, 22), thus suggesting that, in this tissue, its effect is exerted at more than one level on the plasma membrane.

These characteristics of the effect of tryptophan on the early [\textsuperscript{125}I]T3 uptake described above are unlikely to support the existence of a common transport system shared by tryptophan and T3 (10, 12).

A possible mechanism of tryptophan inhibition of T3 uptake by thymocytes may be similar to the one suggested by Kragie et al. (23). These authors showed, in an elegant study based on molecular modelling, that

![Figure 3](https://www.eje.org/)

**Figure 3** Combined effect of sodium depletion and tryptophan addition on [\textsuperscript{125}I]T3 uptake. Cells were incubated in the presence or the absence of sodium, for 1 min at 23 °C with or without tryptophan (trp; 1 mM). In Na\textsuperscript{+}-free experiments sodium chloride was replaced by equal concentrations of choline chloride. The results, expressed as % of specific T3 uptake/10\textsuperscript{6} cells, are the means ± S.D. of four separate experiments.
several substances, including those characterized by an alanine side chain and an outer phenyl ring, may conformationally block the iodothyronine membrane transporter. This interaction seems to be due to the thyromimetic action of these substances (23). Tryptophan is indeed an aromatic amino acid which is structurally similar to and shares the same transport system with tyrosine, the precursor of iodothyronines (12, 22); also, similarly to tryptophan in this study, thyroxine and thyronine inhibited L-T3 binding with the same IC50 in L- and D-conformations (23). Tryptophan may, therefore, induce a conformational change in the T3 membrane transporter that impairs its function (12). The kinetic characteristics of the effect of tryptophan on the saturable 125I-T3 uptake (i.e. the non-competitive inhibition exerted only in cis modality, the lack of stereospecificity) may be consistent with this hypothesis, which does not imply a direct involvement of the plasma membrane T3 carrier described in thymocytes (3).

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