Different transporters for tri-iodothyronine (T₃) and thyroxine (T₄) in the human choriocarcinoma cell line, JAR

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Abstract

We investigated transport systems for tri-iodothyronine (T₃) and thyroxine (T₄) in the human choriocarcinoma cell line, JAR, using a range of structurally similar compounds to determine whether these thyroid hormones are transported by common or different mechanisms. Saturable T₃ but not saturable T₄ uptake was inhibited by a wide range of aromatic compounds (nitrendipine, nifedipine, verapamil, meclofenamic acid, mefenamic acid, diazepam, phenytoin).

Nitrendipine and diazepam were the most effective inhibitors of saturable thyroid hormone uptake. Nitrendipine decreased the K_m for T₄ uptake from a control value of around 500 nM to around 300 nM ($n=6$).

In contrast, the K_m for T₃ uptake was increased from a control value of around 300 nM to around 750 nM ($n=4$). Diazepam had similar effects. This divergent shift in affinity for the uptake of T₃ and T₄ suggested that separate uptake systems exist for these two thyroid hormones.

This provides evidence for at least two transporters mediating uptake of T₃ and T₄ in JAR cells: a specific T₄ transporter that does not interact with T₃ or structurally similar compounds; and a shared iodothyronine transporter that interacts with T₃, T₄, nitrendipine and diazepam.

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Introduction

Although transfer of thyroxine (T₄) from maternal to foetal circulation in the perfused placenta is minimal (Mortimer *et al.* 1996), T₄ has been found in cord blood of infants unable to synthesise their own hormone (Vulsma *et al.* 1989). While this provides convincing evidence for the passage of maternal thyroid hormone across the placenta, at least to a foetus lacking thyroid hormone, the mechanisms mediating this transfer are not well understood.

We have previously described membrane transporters for thyroid hormones in human trophoblast and the human choriocarcinoma cell line, JAR. Transport of tri-iodothyronine (T₃), T₄ and reverse tri-iodothyronine (rT₃) into JAR cells and export of T₃ from the cells occurs by saturable processes. Efflux of T₄ and rT₃, on the other hand, occurs by passive diffusion (Mitchell *et al.* 1992, 1995, 1999a,b). Membrane transport of thyroid hormone has now been characterised in a wide range of cells, with reported differences in thyroid hormone uptake and efflux kinetics between and within cell types. In hepatocytes, for example, uptake of T₃ is energy dependent and T₃ efflux occurs by passive diffusion (Hennemann *et al.* 1984). Human and rat lymphocytes and erythrocytes, rat

synaptosomes and cardiac myocytes (Kragie 1994 (review), Everts *et al.* 1996) actively transport T₃ while T₄ is probably taken up by simple diffusion. Separate transport mechanisms for T₃, T₄ and rT₃ have been described in human liver (Kaptein 1997) and separate membrane transporters mediating uptake of T₃ and T₄ are also suggested for rat hepatocytes (Krenning *et al.* 1981). These findings taken together suggest that the processes involved in uptake and efflux of thyroid hormones may be relatively specific for individual thyroid hormones and may be different depending on tissue type.

Our previous studies have shown that thyroid hormones are taken up into JAR cells by at least two transporters with differing affinities (Mitchell *et al.* 1999a,b). The aim of this study was to determine if T₃ and T₄ were transported by a distinct or shared mechanism in order to further characterise iodothyronine transport in JAR cells.

Materials and Methods

Reagents

Materials were purchased from the following sources: ¹²⁵I-T₃ (3300 µCi/µg) and ¹²⁵I-T₄ (1250 µCi/µg) from

Du Pont Company, Wilmington, DE, USA; foetal calf serum from Commonwealth Serum Laboratories, Melbourne, Victoria, Australia; bicinechonic acid Protein Reagent from Pierce Chemicals, Rockford, IL, USA; and six-well tissue culture plates from Costar, Cambridge, MA, USA. All other chemicals and cell culture media were from Sigma Chemicals, St Louis, MO, USA. Nitrendipine was a gift from Bayer Pharmaceutical Division, West Haven, CO, USA.

Cell culture

The JAR cell line was purchased from American Type Culture Collection, Rockville, MD, USA. Cells were maintained in continuous culture at 37 °C in humidified atmosphere of 95% air and 5% CO₂. Growth medium was RPMI 1640 supplemented with 10% (v/v) foetal calf serum. Cells were subcultured three times a week. For uptake experiments, 3×10^5 cells were plated into each well of the six-well tissue culture plates. Medium was changed 24 h after plating, with cells cultured for 2–3 days. At the end of the uptake experiments, viability of the cells was assessed by the trypan blue exclusion test and was always over 90%.

Uptake studies

The procedure for uptake studies and the determination of the kinetic parameters (Michaelis constant, K_m , and the maximum velocity, V_{max}) of the initial cellular uptake of ¹²⁵I-T₃ and ¹²⁵I-T₄ were as previously described (Mitchell *et al.* 1992, 1995). In brief, prior to all uptake experiments cells were incubated for 1 h in Hanks' balanced salts solution (HBSS). All incubations were carried out at 37 °C. To terminate uptake, incubation medium was aspirated, cells were washed twice in ice-cold HBSS and immediately lysed in 1 M NaOH. Cell-associated radioactivity was determined by counting the radioactivity in a Packard γ-counter with a counting efficiency of 84%. ¹²⁵I-labelled thyroid hormone taken up was expressed as femtomoles per minute per milligram of cellular protein (fmol/min per mg protein). The specificity of the uptake process was examined by incubating cells for 30 min in the presence of 30 pM ¹²⁵I-T₃ or 50 pM ¹²⁵I-T₄, with or without 10 μM excess of unlabelled thyroid hormones, 10 mM unlabelled amino acids, tryptophan, phenylalanine, leucine, glycine, alanine, glutamine, α-(methylamino)-isobutyric acid (Me-AIB) and 2-amino-2-norbornane-carboxylic acid (BCH). The effect of various compounds on the initial rate of specific uptake of thyroid hormone was determined by incubating cells for 2 min in the presence of 30 pM ¹²⁵I-T₃ or 50 pM ¹²⁵I-T₄ with or without 10 μM unlabelled thyroid hormone and with or without 100 μM nitrendipine, nifedipine, verapamil, meclofenamic acid, mefenamic acid, diazepam, phenytoin, indocyanine green, procion red,

reactive red, reactive blue, acid blue and 50 μM iopanoic acid. Drugs and dyes were dissolved in ethanol and diluted in HBSS. The final concentration of ethanol did not exceed 0.4%, which had no effect on cellular uptake of T₃ or T₄. Results from three to seven determinations were pooled. Further characterisation of the transport system was carried out using inhibitor studies to determine the kinetic parameters of inhibition of thyroid hormone uptake by various compounds. The cells were incubated for 2 min in the presence of 30 pM ¹²⁵I-T₃ or 50 pM ¹²⁵I-T₄ and unlabelled T₃ and T₄ (0–10 μM) with or without 100 μM final concentration of unlabelled nitrendipine and 80–100 μM final concentration of diazepam. Results from 4–8 determinations were pooled and data fitted to the Michaelis–Menten equation using a non-linear curve-fitting program (GraphPad Prism, GraphPad, San Diego, CA, USA).

We sought evidence of metabolism of ¹²⁵I-T₃ and ¹²⁵I-T₄ by duplicate cultures of JAR cells during uptake experiments by analysing radioactivity present in the cells and in the medium after incubation with the tracers for 30 min at 37 °C. As in our previous studies, we found evidence of only minimal metabolism of tracers by the cells.

Determination of the cellular protein content

The protein content of cell lysates was determined with the bicinechonic acid reagent (Pierce Chemicals) which is a modification of the Biuret reaction using BSA as a standard.

Statistical analysis

Statistical analysis was performed by Student's *t*-test and one-way ANOVA followed by multiple comparison of means against a single control (Bonferroni's *t*-test) using the statistical software package SigmaStat (Jandel Scientific, San Rafael, CA, USA). Results were expressed as means and standard errors of the mean, and *n* is the number of independent determinations, each in triplicate. A probability of <0.05 was regarded as significant.

Results

Uptake of ¹²⁵I-T₃ and ¹²⁵I-T₄ in JAR cells was inhibited by the L-system amino acids tryptophan, phenylalanine, leucine and the synthetic amino acid analogue 2-amino-2-norbornane-carboxylic acid (BCH). Tryptophan was the most effective inhibitor, reducing saturable ¹²⁵I-T₃ and ¹²⁵I-T₄ uptake by 85.5% (*n*=3) and 63.6% (*n*=4) respectively. The effects of L-system amino acids on thyroid hormone uptake are shown in Fig. 1. Amino acids transported by other amino acid carriers had no significant effect on the uptake of T₃ and T₄ (results not shown).

Figure 1 Effect of amino acids on the saturable uptake of (A) $^{125}\text{I-T}_3$ ($n=3-4$) and (B) $^{125}\text{I-T}_4$ ($n=4-7$) in JAR cells. Cells were incubated for 30 min at 37°C with 30 pM $^{125}\text{I-T}_3$ or 50 pM $^{125}\text{I-T}_4$ with and without tryptophan, phenylalanine, BCH and leucine. Values are shown as mean $\pm$ S.E.M.. * $P<0.05$.

Saturable T_3 but not saturable T_4 uptake (Fig. 2) was inhibited by a wide range of aromatic compounds, suggesting that there may be different transporters for these two thyroid hormones in JAR cells.

Further evidence of distinct T_3 and T_4 transporters was obtained by studies with nitrendipine and diazepam. The kinetic parameters of initial specific uptake of T_3 and T_4 (Michaelis constant, K_m , and maximum velocity, $V_{\max}$)

Figure 2 Effect of various compounds on the saturable uptake of (A) $^{125}\text{I-T}_3$ ($n=3-5$) and (B) $^{125}\text{I-T}_4$ ($n=3-6$) in JAR cells. Cells were incubated for 2 min at 37°C with 30 pM $^{125}\text{I-T}_3$ or 50 pM $^{125}\text{I-T}_4$ in the absence or presence of verapamil, phenytoin, mefenamic acid, meclofenamic acid, nifedipine, nitrendipine and diazepam. Values are shown as mean $\pm$ S.E.M.. * $P<0.05$.

were determined with and without addition of these drugs. Addition of $100\ \mu\text{M}$ nitrendipine increased K_m for T_3 uptake from a control value of $294 \pm 35.3\ \text{nM}$ to $757 \pm 152.2\ \text{nM}$ ($n=4$) (Fig. 3A). The effects of nitrendipine on T_4 uptake were however quite different,

Figure 3 Effect of 100 μ M nitrendipine on the initial rate of specific uptake of (A) 125 I- T_3 or (B) 125 I- T_4 in the human choriocarcinoma cell line, JAR. Cells were incubated for 2 min at 37 °C with either 30 pM 125 I- T_3 or 50 pM 125 I- T_4 in the presence of increasing concentrations (0–10 μ M) of appropriate unlabelled thyroid hormone, and with $\circ$ (Panel A – T_3 , $n=4$; Panel B – T_4 , $n=6$) or without $\blacksquare$ (Panel A – T_3 , $n=5$; Panel B – T_4 , $n=6$) 100 μ M nitrendipine. Values shown as means $\pm$ S.E.M.

with a reduction in K_m from 509 ± 182 nM to 293 ± 96.8 nM ($n=6$) (Fig. 3B). Similarly, 80 μ M diazepam increased the K_m for T_3 uptake from 192 ± 20.7 nM to 936 ± 176.3 nM ($n=5$), whereas the K_m for T_4 uptake in the presence of 100 μ M diazepam decreased from 660 ± 228.7 nM to 483 ± 97.8 nM ($n=8$) (Fig. 4).

Discussion

Studies of cell membrane transport of thyroid hormones and their analogues have shown differences in mechanisms

Figure 4 Effect of 80 μ M diazepam on the initial rate of specific uptake of 125 I- T_3 (A) or 100 μ M diazepam on the initial rate of specific uptake of 125 I- T_4 (B) in the human choriocarcinoma cell line, JAR. Cells were incubated for 2 min at 37 °C, with either 30 pM 125 I- T_3 or 50 pM 125 I- T_4 in the presence of increasing concentrations (0–10 μ M) of appropriate unlabelled thyroid hormone, with $\circ$ ($n=5$) or without $\blacksquare$ ($n=5$) 80 μ M diazepam (Panel A – T_3), or with $\circ$ ($n=8$) or without $\blacksquare$ ($n=8$) 100 μ M diazepam (Panel B – T_4). Values are shown as means $\pm$ S.E.M.

of uptake and efflux for T_3 and T_4 in some cell types but not others. The present study confirms our previous findings of at least two types of transporters for thyroid hormones in JAR cells (Mitchell *et al.* 1999b). Uptake of T_3 and T_4 in JAR cells was inhibited by the L-system amino acids tryptophan, phenylalanine, leucine and the synthetic amino acid analogue BCH. The magnitude of the inhibition of T_3 uptake by these agents compared with their effects on T_4 uptake suggests however a subtle difference in the uptake mechanisms for these two iodothyronines.

Thyroid hormone uptake in many cells, including JAR cells (Mitchell *et al.* 1994, 1995, Prasad *et al.* 1994), is inhibited by high (10 mM) concentrations of aromatic and other L-system amino acids. The spectrum of inhibition appears to relate to amino acid structure rather than biological considerations (Christensen 1989). A range of small molecules with multiple aromatic rings inhibits T_3 uptake in many cells at much lower concentrations (1–100 μ M) (Krenning *et al.* 1981, Topliss *et al.* 1989, 1993). It is possible that aromatic rings or similar planar functional groups in these inhibitory compounds are required to inhibit thyroid hormone uptake (Chalmers *et al.* 1993).

We had previously reported a preliminary account of the effects of nitrendipine on thyroid hormone uptake in JAR cells (Mitchell *et al.* 1999b). A larger range of structurally similar but pharmacologically diverse compounds (verapamil, phenytoin, mefenamic acid, meclofenamic acid, nifedipine, iopanoic acid and diazepam) were tested for effects on thyroid hormone uptake in the present study. All significantly inhibited saturable T_3 uptake without significant effects on saturable T_4 uptake, suggesting different mechanisms for uptake of T_3 and T_4 . Nitrendipine and diazepam were the most effective inhibitors of T_3 uptake tested in JAR cells. Inhibition of T_3 uptake by diazepam, reported in human hepatoblastoma cells, neuroblastoma cells and, with less efficiency, rat pituitary cells (Kragie & Doyle 1992), was thought to be due to the structural similarity of thyroid hormones and diazepam.

Although nitrendipine and diazepam both inhibit uptake of T_3 and T_4 , their effects on hormone binding are divergent. Affinity of T_3 binding is reduced, consistent with conventional competitive antagonism but these drugs cause a paradoxical increase in T_4 binding affinity.

It is difficult to explain how a competing ligand can increase the affinity for transport of a substrate. We postulate that two transporters of different affinity take up T_4 and that inhibition of the lower affinity component results in a shift to the left of the saturation curves, which is interpreted as an increase in affinity. In a mathematical model (Fig. 5) we show how a saturation curve (B) is shifted to the right by the addition of a lower affinity process (A). The precision of the data in the present experiments is insufficient to clearly resolve the saturation data into two components, so that we see only the affinity shift in the total uptake data.

The present data are consistent therefore with a low affinity transporter (A in Fig. 5), which interacts with T_3 , T_4 , L-system amino acids and several other structurally similar but functionally unrelated compounds, plus a high affinity transporter (B in Fig. 5) which is specific for T_4 . Inhibition of the low affinity transporter results in an apparent affinity increase as the uptake data moves from curve A+B to curve B (Fig. 5).

Figure 5 Model of two T_4 transporters with differing affinities. A ($\blacktriangle$) is the uptake of 125 I- T_4 through a low-affinity, broad specificity transporter inhibited by nitrendipine and diazepam. B ($\bullet$) is the uptake of 125 I- T_4 through the high-affinity specific T_4 transporter unaffected by nitrendipine or diazepam. We postulate A+B ($\blacksquare$) is the observed T_4 uptake in JAR cells, which is a summation of the uptake by the shared iodothyronine transporter (A) and T_4 uptake through the specific T_4 transporter (B).

Irrespective, however, of the interpretation of the increased affinity for T_4 in the presence of inhibitors, the data very clearly indicate that T_3 and T_4 are transported into JAR cells by different carriers.

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