Different transporters for tri-iodothyronine (T$_3$) and thyroxine (T$_4$) in the human choriocarcinoma cell line, JAR

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Abstract

We investigated transport systems for tri-iodothyronine (T$_3$) and thyroxine (T$_4$) 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 T$_3$ but not saturable T$_4$ 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 T$_4$ uptake from a control value of around 500 nM to around 300 nM ($n=6$). In contrast, the $K_m$ for T$_3$ 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 T$_3$ and T$_4$ suggested that separate uptake systems exist for these two thyroid hormones.

This provides evidence for at least two transporters mediating uptake of T$_3$ and T$_4$ in JAR cells: a specific T$_4$ transporter that does not interact with T$_3$ or structurally similar compounds; and a shared iodothyronine transporter that interacts with T$_3$, T$_4$, nitrendipine and diazepam.

Introduction

Although transfer of thyroxine (T$_4$) from maternal to foetal circulation in the perfused placenta is minimal (Mortimer et al. 1996), T$_4$ 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 (T$_3$), T$_4$ and reverse tri-iodothyronine (rT$_3$) into JAR cells and export of T$_3$ from the cells occurs by saturable processes. Efflux of T$_4$ and rT$_3$, 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 T$_3$ is energy dependent and T$_3$ 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 T$_3$ while T$_4$ is probably taken up by simple diffusion. Separate transport mechanisms for T$_3$, T$_4$ and rT$_3$ have been described in human liver (Kaptein 1997) and separate membrane transporters mediating uptake of T$_3$ and T$_4$ 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 T$_3$ and T$_4$ 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: $^{125}$I-T$_3$ (3300 µCi/µg) and $^{125}$I-T$_4$ (1250 µCi/µg) from

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Du Pont Company, Wilmington, DE, USA; foetal calf serum from Commonwealth Serum Laboratories, Melbourne, Victoria, Australia; bicinchoninic 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 gamma-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/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 nM 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 bicinchoninic 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).
Saturable T₃ but not saturable T₄ 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 T₃ and T₄ transporters was obtained by studies with nitrendipine and diazepam. The kinetic parameters of initial specific uptake of T₃ and T₄ (Michaelis constant, \( K_m \), and maximum velocity, \( V_{max} \)) were determined with and without addition of these drugs. Addition of 100 \( \mu \)M nitrendipine increased \( K_m \) for T₃ uptake from a control value of 294 ± 35.3 nM to 757 ± 152.2 nM \( (n=4) \) (Fig. 3A). The effects of nitrendipine on T₄ uptake were however quite different,
with a reduction in $K_m$ from 509 ± 182 nM to 293 ± 96·8 nM ($n=6$) (Fig. 3B). Similarly, 80 µM 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 µM 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 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 T3 uptake in many cells at much lower concentrations (1–100 µM) (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 T3 uptake without significant effects on saturable T4 uptake, suggesting different mechanisms for uptake of T3 and T4. Nitrendipine and diazepam were the most effective inhibitors of T3 uptake tested in JAR cells. Inhibition of T3 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 T3 and T4, their effects on hormone binding are divergent. Affinity of T3 binding is reduced, consistent with conventional competitive antagonism but these drugs cause a paradoxical increase in T4 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 T4 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 T3, T4, 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 T4. 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).

Irrespective, however, of the interpretation of the increased affinity for T4 in the presence of inhibitors, the data very clearly indicate that T3 and T4 are transported into JAR cells by different carriers.

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Specific thyroxine transporter in JAR cells

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