The uptake and efflux of reverse triiodothyronine (rT3) in JAr cells were investigated. Uptake of 125I-rT3 was time dependent and reversible with a saturable component of around 70 per cent of total uptake after 30 min of incubation. Efflux was not saturable. Kinetic analysis of the initial specific uptake rates revealed an uptake process with a Michaelis constant of 3.04 ± 0.53 μM (mean ± SEM, n=15) and a corresponding maximum velocity of 9.65 ± 2.49 pmol/min/mg protein (n=15). Uptake of rT3 was stereospecific, but not specific for rT3, as unlabelled l stereoisomers of thyroid hormone analogues were more effective as inhibitors of 125I-rT3 uptake than rT3. Unlabelled T3 and thyroxine (T4) (10 μM) reduced cellular uptake of 125I-rT3 by around 82 and 74 per cent, respectively. The calculated inhibition constants Ki were 1.23 ± 0.29 μM (n=4) and 0.66 ± 0.19 μM (n=4) for T3 and T4, respectively. Similarly, rT3 reduced cellular uptake of 125I-T3 and 125I-T4 by 34 and 23 per cent, respectively. The calculated inhibition constants Ki were 1.75 ± 0.55 μM (n=8) and 1.08 ± 0.36 μM (n=8) for the inhibition of 125I-T3 and 125I-T4 uptake, respectively. Reverse T3 inhibited efflux of 125I-T3 from the cells by around 20 per cent, but did not inhibit efflux of 125I-T4. These results suggest that uptake of rT3 in JAr cells may occur via a single, saturable membrane carrier, which also interacts with T3 and T4, while efflux of rT3 may occur by passive diffusion.

INTRODUCTION

In the placenta, thyroxine (T4) is inactivated to reverse T3 (rT3) and triiodothyronine (T3) to 3,3'-diiodothyrosine (3,3'-T2) by a type III deiodinase (Salvatore et al., 1995) and large amounts of rT3 appear in the maternal and fetal circulations of the human placental lobule perfused with T4 via the maternal circuit (Mortimer et al., 1996).

Nothing is known of trophoblast cell membrane handling of rT3. We have previously shown that human trophoblast has a cell membrane transport mechanism for T3 (Mitchell, Manley and Mortimer, 1992a) and have extended these studies to demonstrate that in the human choriocarcinoma cell line, JAr, there are carrier-mediated, saturable membrane transport mechanisms for T3 and T4 which interact with certain amino acids (Mitchell, Manley and Mortimer, 1992b, 1994; Mitchell et al., 1995). As structurally similar amino acids can mutually inhibit amino acid transport (Yudilevich and Sweiry, 1985; Christensen, 1990), we postulated that rT3, present at a higher concentration in fetal than in maternal plasma (Yoshida et al., 1987), may regulate trophoblast transport of thyroid hormone.

The aim of the present study, using JAr cells as a model of the human trophoblast, was to examine whether membrane transport mechanisms mediating uptake and efflux of rT3 could be demonstrated and to determine whether rT3 modulates the uptake and efflux of T3 and T4.

MATERIALS AND METHODS

Reagents

Materials were purchased from the following sources: 125I-rT3 (790–1250 μCi/μg), 125I-T3 (3300 μCi/μg) and 125I-T4 (1250 μCi/μg) from Du Pont Company, Wilmington, DE, USA; fetal calf serum from Commonwealth Serum Laboratories, Melbourne, Victoria, Australia, BCA Protein Reagent from Pierce Chemicals, Rockford, IL, USA; six-well tissue culture plates from Costar, Cambridge, MA, USA; and LH-20 Sephadex from Pharmacia LKB Biotechnology, Uppsala, Sweden. All other chemicals and cell culture media were from Sigma Chemicals, St Louis, MO, USA.

Methods

The JAr cell line was purchased from American Type Culture Collection, Rockville, MD, USA. The procedures for maintenance of cultures and preparation of cells for uptake experiments were as described previously (Mitchell, Manley and Mortimer, 1992b). Briefly, cells were maintained in continuous culture at 37°C in a humidified atmosphere of 95 per cent air
and 5 per cent CO₂. Culture medium was RPMI 1640 supplemented with 10 per cent (v/v) fetal calf serum. Cells were subcultured three times a week. For uptake experiments 3 × 10⁴ cells were plated into each well of the six-well tissue culture plates. Medium was changed 24 h after plating. Cells were cultured for 2–3 days. At the end of uptake experiments, viability of the cells was assessed by the trypan blue exclusion method and was always over 90 per cent.

The procedures for uptake and efflux studies and the determination of the kinetic parameters (Michaelis constant \(K_m\) and maximum velocity \(V_{max}\)) of initial cellular uptake of \(^{125}\text{I}-\text{rT}3\) were as previously described for \(\text{T}3\) and \(\text{T}4\) (Mitchell, Manley and Mortimer, 1994; Mitchell et al., 1995), except that \(^{125}\text{I}-\text{rT}3\) (100 pm) and \(\text{rT}3\) (0–10 μm) were used. Briefly, prior to uptake experiments, cells were incubated for 1 h in Hank’s balanced salts solution (HBSS). All incubations were carried out at 37°C. To terminate uptake, incubation medium was aspirated, cells were washed twice with ice-cold HBSS and immediately lysed in 1 m NaOH. Cell-associated radioactivity was determined by counting the radioactivity of the cell lysates in a Packard γ-counter with a counting efficiency of 84 per cent. To study time course of cellular uptake, cells were incubated for 30 min in HBSS containing 100 pm \(^{125}\text{I}-\text{rT}3\) with or without an excess (10 μm) unlabelled \(\text{rT}3\). At intervals of 1, 2, 5, 10, 15, and 30 min, medium was aspirated and cells were lysed. An uptake curve for the saturable process was obtained by subtracting non-saturable uptake in the presence of an excess of unlabelled ligand from the total uptake in the absence of an unlabelled ligand. To study efflux of \(^{125}\text{I}-\text{rT}3\) from the cells, the cells were incubated with \(^{125}\text{I}-\text{rT}3\) for 30 min, washed and then incubated in fresh HBSS with and without 10 μm excess unlabelled \(\text{rT}3\) for 30 min. After 1, 2, 5, 10, 15 and 30 min of incubation medium was aspirated and replaced with fresh HBSS to prevent re-uptake of the hormone released into the medium. The cells were pre-incubated without an excess of unlabelled \(\text{rT}3\) in order to allow the entry of \(^{125}\text{I}-\text{rT}3\) into the cells via the saturable uptake mechanism. If the cells were pre-incubated with a 10 μm excess of unlabelled \(\text{rT}3\), in addition to the trace amount of \(^{125}\text{I}-\text{rT}3\), the saturable uptake mechanism which mediates uptake of \(\text{rT}3\) into the cells, would be saturated. Consequently, any cell-associated radioactivity present at the end of the pre-incubation period under these experimental conditions would reflect \(^{125}\text{I}-\text{rT}3\) bound non-specifically to the cell surface, and not \(\text{rT}3\) taken up into the cells. Under such experimental conditions it would have been, therefore, impossible to study efflux of intracellular hormone from the cells.

To determine the kinetic parameters of uptake, the cells were incubated in the presence of 100 pm \(^{125}\text{I}-\text{rT}3\) and unlabelled \(\text{rT}3\) (0–10 μm) for 2 min. Results from 15 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).

The specificity of \(\text{rT}3\) uptake process was examined by incubating the cells for 30 min in the presence of 100 pm \(^{125}\text{I}-\text{rT}3\) with or without an excess (10 μm) of unlabelled L-\(\text{rT}3\), D-\(\text{rT}3\), L-\(\text{T}3\), D-\(\text{T}3\), L-tryptophan (Trp) or L-phenylalanine (Phe). \(^{125}\text{I}-\text{rT}3\) tracer taken up in the presence of an analogue was expressed as a percentage of that taken up in the absence of an analogue. The procedure to examine the effect of unlabelled \(\text{rT}3\) on the uptake of \(^{125}\text{I}-\text{T}3\) and \(^{125}\text{I}-\text{T}4\) was similar, except that the cells were incubated for 30 min with either 30 pm \(^{125}\text{I}-\text{T}3\), or 50 pm \(^{125}\text{I}-\text{T}4\) with and without an excess (10 μm) unlabelled \(\text{rT}3\). Results were expressed as a percentage of initial uptake rate in the presence of an inhibitor compared with the control value in the absence of an inhibitor. Inhibition constants (\(K_i\)) were calculated by fitting data to the one-site competition model using non-linear regression by GraphPad PRISM software.

Evidence of metabolism of \(^{125}\text{I}-\text{rT}3\) by duplicate cultures of JAr cells during uptake experiments was sought by analysing radioactivity present in the cells and in the medium after incubation with 100 pm \(^{125}\text{I}-\text{rT}3\) for 30 min at 37°C by LH-20 Sephadex chromatography as described by Otten, Mol and Visser (1983). Cells were lysed in ethanol and media were extracted with tertiary amyl alcohol. Free iodine, iodothyronine degradation products and intact \(\text{rT}3\) were eluted from the column with 0.1 mm HCl, 10 per cent ethanol in NaOH and 50 per cent ethanol in NaOH, respectively.

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

Results are expressed as mean ± SEM, and \(n\) is the number of independent determinations. Statistical analysis was performed using Student’s \(t\)-test using the statistical software package Sigma Stat (Jandel Scientific, San Rafael, CA, USA). A probability of <0.05 was regarded as significant.

RESULTS

Uptake of \(^{125}\text{I}-\text{rT}3\) in the human choriocarcinoma cell line JAr was time dependent and increased during 30 min incubation to a value of about 3 per cent of added \(^{125}\text{I}-\text{T}3\) (Figure 1). Uptake was saturable, being reduced in the presence of a 10 μm excess of the unlabelled ligand. After 30 min of incubation, saturable \(^{125}\text{I}-\text{rT}3\) uptake was around 70 per cent of total uptake. The early phase of the uptake curve was approximately linear allowing the use of the measurement at 2 min as an estimate of the initial uptake rate.

Uptake of \(^{125}\text{I}-\text{rT}3\) was reversible, with the label being progressively released from the cells during the incubation in the non-radioactive medium (Figure 2). Efflux of \(^{125}\text{I}-\text{rT}3\) from the cells was, however, not saturable, as it was not reduced in the presence of an excess (10 μm) of extracellular \(\text{rT}3\). Uptake of \(^{125}\text{I}-\text{rT}3\) was significantly \((P<0.05)\) reduced by 68.6 ± 2.4 per cent \((n=9)\) after 30 min of incubation with unlabelled L-\(\text{rT}3\) (10 μm) while unlabeled D-\(\text{rT}3\) (10 μm) inhibited only 20.4 ± 1.0 per cent \((n=9)\) of \(^{125}\text{I}-\text{rT}3\) uptake, indicating that uptake was stereospecific. Unlabelled L-steroisomers of thyroid hormones were more effective inhibitors of \(^{125}\text{I}-\text{rT}3\)
Specific for rT3 uptake process with a Michaelis constant (K) of 10 μM unlabelled rT3 that they are smaller than the symbol.

The initial rate of specific uptake of 125I-rT3 was progressively inhibited by subtracting non-saturable uptake in the presence of 10 μM unlabelled rT3 from the total uptake in the absence of the unlabelled rT3. Values shown are means ± SEM (n=9). Absence of error bars indicates that they are smaller than the symbol.

Unlabelled 1-rT3 (10 μM) significantly (P<0.05) reduced uptake of 125I-T3 and 125I-T4 to 66.0 ± 7.0 per cent (n=4) and 77.2 ± 4.0 per cent (n=7) respectively of the control uptake after 30 min of incubation. Similarly, external rT3 significantly (P<0.05) reduced efflux of 125I-T3 from the cells. The amount of 125I-T3 released after 30 min incubation in the presence of 10 μM external unlabelled rT3 was reduced to 82.9 ± 2.1 per cent (n=9) of control dishes. Efflux of 125I-T4 was, in contrast to efflux of 125I-T3, not reduced in the presence of 10 μM excess of external rT3.

Increasing concentrations of unlabelled T3 and T4 (0–10 μM) progressively inhibited the initial rate of specific uptake of 125I-rT3 (Figure 4). In the presence of maximal concentration of either T3 or T4 (10 μM) the initial rate of specific 125I-rT3 uptake was reduced by around 58 and 51 per cent, respectively. The calculated inhibition constants Ki were 1.23 ± 0.15 μM (n=4) and 0.66 ± 0.10 μM (n=4) for the inhibition of 125I-rT3 uptake by T3 and T4 respectively. Similarly, increasing concentrations of unlabelled rT3 (0–10 μM) progressively inhibited the initial rate of specific uptake of 125I-T3 and 125I-T4 (Figure 4). The calculated inhibition constants Ki were 1.75 ± 0.55 μM (n=8) and 1.08 ± 0.36 μM (n=8) for the inhibition of 125I-T3 and 125I-T4 uptake, respectively, by rT3.

Only minimal evidence of metabolism of 125I-rT3 by the cells was found after uptake for 30 min. Free iodine, degradation products and intact 125I-rT3 accounted for 4.6, 3.5 and 91.9 per cent, respectively, of the radioactivity recovered from the cells, and 3.6, 1.0, and 95.8 per cent, respectively, of the radioactivity recovered from the incubation media. The corresponding figures for the radioactivity recovered from the medium incubated without the cells were 3.1, 1.2 and 95.8 per cent.

Figure 1. Time dependence of 125I-rT3 uptake in the human choriocarcinoma cell line, JAr. Cells were incubated at 37°C with 100 pm 125I-rT3 in the absence (■) or in the presence (▲) of 10 μM unlabelled rT3. Saturable uptake (▲) was obtained by subtracting non-saturable uptake in the presence of 10 μM unlabelled rT3 from the total uptake in the absence of the unlabelled rT3. Values shown are means ± SEM (n=9). Absence of error bars indicates that they are smaller than the symbol.

Unlabelled L-T3 and L-T4 (both 10 μM) inhibited uptake of 125I-rT3 by 82.2 ± 1.3 per cent (n=9) and 73.6 ± 2.7 per cent (n=9) respectively. Ten micromolar phenylalanine also inhibited 37.0 ± 3.7 per cent (n=3) of 125I-rT3 uptake, while 10 μM tryptophan had no significant effect.

Increasing concentrations of unlabelled rT3 (0–10 μM) progressively inhibited the initial rate of specific uptake of 125I-rT3 (Figure 3). The initial rate of specific uptake of 125I-rT3 was reduced by around 60 per cent at the maximal concentration of rT3. Kinetic analysis of the initial specific uptake rates revealed an uptake process with a Michaelis constant (Km) value of 3.04 ± 0.53 μM (n=15) and a corresponding maximum velocity (Vmax) of 9.65 ± 2.49 pmol/min/mg protein (n=15).

Figure 2. Washout of 125I-rT3 from the human choriocarcinoma cell line, JAr. Cells were labelled by incubation for 30 min at 37°C with 100 pm 125I-rT3, washed and incubated in fresh, non-radioactive medium with (■) or without (▲) 10 μM unlabelled rT3 for the indicated times. The values are means ± SEM (n=9). Absence of error bars indicates that they are smaller than the symbol.

Figure 3. Concentration dependence of the initial uptake rate of 125I-rT3 in the human choriocarcinoma cell line, JAr. Cells were incubated for 2 min at 37°C with 100 pm 125I-rT3 in the presence of increasing concentrations of rT3. Values shown are means ± SEM (n=15).
DISCUSSION

Studies of thyroid hormone levels in parallel samples of human arterial and venous cord blood provided evidence that in the placental inner ring deiodination of maternal T4 contributes significantly to the pool of fetal rT3 (Penny et al., 1986). The concentration of total rT3 in umbilical cord serum (1.5–4.5 nM) is much higher than in maternal serum (300–600 pM) and reflects the high concentrations of rT3 in the fetus during the third trimester (Chopra et al., 1975). Reverse T3 circulates almost entirely bound to proteins, with the free fraction (9–18 pM) being about 3 per cent of total rT3 (Chopra, 1974).

Membrane transport of rT3 has been described only in a small number of cells, including rat (Krenning et al., 1982) and human hepatocytes (de Jong et al., 1993), human liver-derived cell line HepG2 (van Stralen et al., 1996), rat anterior pituitary cells (Everts et al., 1995) and human monocytic leukemia cells (Yap and Schussler, 1997). In hepatocytes, rT3 is taken up at least in part by saturable, high-affinity systems (Km value in the nanomolar range in rat hepatocytes) dependent on intracellular energy (Krenning et al., 1982; de Jong et al., 1993), and Na+ gradient (de Jong et al., 1993; van Stralen et al., 1996), and partially inhibited by a monoclonal antibody against the rat hepatocyte membrane (Hennemann et al., 1986). In anterior pituitary cells rT3, T4, and T3 share a common transporter (Everts et al., 1994), while in rat hepatocytes rT3 and T4 share a transporter, which is different from that of T3 (Krenning et al., 1982). Although the interactions between the transport of T3, T4 and rT3 were not examined in human hepatocytes, recent evidence from serum tracer kinetic studies in humans indicates the presence of separate transport processes for T4, T3 and for rT3 from serum to rapidly equilibrating tissues, including the liver (Kaptein, 1997). Reverse T3 uptake in human monocytic leukaemia cells is mediated by transthyretin, a plasma transport protein for T4 and rT3 (Yap and Schussler, 1997).

The aim of the present study was to examine whether the JAr cells possess a membrane transport mechanism mediating uptake and efflux of rT3, and whether transport of rT3, T3 and T4 interact in these cells. We chose JAr cells as a model of human trophoblast. Although these cells are transformed, they do have taurine and T3 transporters that are kinetically identical to those of normal trophoblast that have been previously described in these cells (Kulanthaivel et al., 1991; Mitchell, Manley and Mortimer, 1992a). In the present study uptake of 125I-rT3 was time dependent, reversible and saturable suggesting that a carrier-mediated process was involved. We have previously described carrier mediated transport of T4 and T3 in these cells (Mitchell, Manley and Mortimer, 1992b; Mitchell et al., 1995). The saturation kinetics of 125I-rT3 uptake indicated a mechanism with a single Km value of 3 μM which was similar to the Km value of 1 μM previously found for T3 uptake in these cells, but higher than the Km value of 60 nM previously found for T4 uptake. The Km for rT3 uptake in JAr cells was around 500 times higher than Km for rT3 uptake found in rat hepatocytes (6 nM;
Krenning et al., 1982). As we previously found with T₃ uptake in JAR cells (Mitchell, Manley and Mortimer, 1992b), the saturation data suggested the presence of a second saturable uptake site with a $K_m$ value in the picomolar range, but again the precision of the fit was not improved by use of a two-site model.

We have previously shown that uptake and efflux of T₃ and Trp in JAR cells interact (Mitchell, Manley and Mortimer, 1994). In the present study Trp did not inhibit uptake of rT₃ and Phe inhibited only 37 per cent of rT₃ uptake. These results suggest that in JAR cells rT₃ was not taken up via an amino acid carrier.

We found no significant deiodination of rT₃ by JAR cells under the experimental conditions used in the present study. These are similar results to those of Roti et al. (1982), who found no conversion of rT₃ to other labelled compounds by rat placental homogenates.

We examined the efflux of $^{125}$I-rT₃ from the cells in the presence of an excess of unlabelled rT₃ in the external medium. This was done to determine whether rT₃ was released from the cells via a saturable process, similarly to the saturable release of T₃ from these cells described by us previously, and to determine whether both uptake and efflux of rT₃ in JAR cells were mediated by the same or different transporters. Efflux of rT₃, in contrast to rT₃ uptake and efflux of T₃ (Mitchell, Manley and Mortimer, 1994) in these cells, was not saturable.

We postulated that rT₃, which is present at a higher concentration in fetal than in maternal plasma, may play a role in regulation of uptake and efflux of thyroid hormones in the trophoblast. Although external rT₃ (10 μM) inhibited efflux of T₃ from the cells, it did not inhibit T₄ efflux. While it is not known whether similar interactions occur in the trophoblast in vivo, the results of the present study suggest that rT₃ is unlikely to control the transfer of maternal T₃ to the fetus by inhibiting efflux of thyroid hormone on the fetal side.

T₃ and T₄ both inhibited uptake of rT₃. Inhibition of initial saturable $^{125}$I-rT₃ uptake by unlabelled T₃ and T₄ was dose dependent with calculated inhibition constants ($K_i$) of 1.23 ± 0.15 μM ($n=4$) and 0.66 ± 0.10 μM ($n=4$), respectively. Similarly, rT₃ inhibited uptake of both T₃ and T₄ in a dose dependent way, with $K_i$ values of 1.75 ± 0.55 μM ($n=8$) and 1.08 ± 0.36 μM ($n=8$).

The $K_i$ for the inhibition of T₃ uptake by rT₃ (1.75 ± 0.55 μM, $n=8$) was similar to the $K_m$ for rT₃ uptake (3.04 ± 0.53 μM), and, similarly, the $K_i$ for the inhibition of rT₃ uptake by T₄ was similar to the $K_m$ for T₃ uptake (1.06 ± 0.15 μM, $n=15$, Mitchell, Manley and Mortimer, 1994), suggesting that rT₃ and T₃ shared a common transporter in JAR cells. Similarly, the $K_i$ for the inhibition of T₄ uptake by rT₃ (1.08 ± 0.36 μM, $n=8$) was similar to the $K_m$ for rT₃ uptake (3.04 ± 0.53 μM, $n=15$), however, the $K_i$ for the inhibition of rT₃ uptake by T₄ (0.66 ± 0.10 μM, $n=4$) was significantly ($P<0.001$) higher than the $K_m$ for T₄ uptake (59.4 ± 13.9 nM, $n=12$; Mitchell et al., 1995). These results suggested that while uptake of rT₃ and T₄ in JAR cells interacted, T₄ was probably also taken up by a specific, high-affinity carrier which did not interact with rT₃, in addition to the non-specific carrier which interacted with T₃, rT₃ and T₄. Taken together, these results suggest that rT₃, T₃ and T₄ share a common transporter in JAR cells similarly to rat anterior pituitary cells (Everts et al., 1995). In addition to this non-specific transporter, JAR cells also possess a specific carrier which interacts with T₄, but not with rT₃.

In summary, we demonstrated the presence of a saturable uptake mechanism for rT₃ in the human choriocarcinoma cell line JAR. This uptake process was similar to the uptake of T₃ and T₄ previously described by us in these cells. The kinetic parameters of inhibition of rT₃ uptake by T₃ and T₄ and of T₃ and T₄ uptake by rT₃ suggested that rT₃, T₃ and T₄ may share the same transporter in JAR cells. Efflux of rT₃ from the cells was not saturable indicating that the release of rT₃ from the JAR cells proceeds via a separate mechanism from that mediating uptake of rT₃ and is likely to occur by passive diffusion. Reverse T₃ did not inhibit T₄ efflux and therefore did not provide a mechanism for control of maternofetal T₄ transport.

ACKNOWLEDGEMENTS

This work was supported by the National Health and Medical Research Council of Australia.

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