Thyroid hormone export from cells: contribution of P-glycoprotein

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Abstract

Verapamil inhibits tri-iodothyronine (T₃) efflux from several cell types, suggesting the involvement of multidrug resistance-associated (MDR) proteins in T₃ transport. The direct involvement of P-glycoprotein (P-gp) has not, however, been investigated. We compared the transport of ¹²⁵I-T₃ in MDCKII cells that had been transfected with *mdr1* cDNA (MDCKII-MDR) versus wild-type MDCKII cells (MDCKII), and examined the effect of conventional (verapamil and nitrendipine) and specific MDR inhibitors (VX 853 and VX 710) on ¹²⁵I-T₃ efflux. We confirmed by Western blotting the enhanced expression of P-gp in MDCKII-MDR cells. The calculated rate of ¹²⁵I-T₃ efflux from MDCKII-MDR cells (around 0.30/min) was increased twofold compared with MDCKII cells (around 0.15/min). Overall, cellular accumulation of ¹²⁵I-T₃ was reduced by 26% in MDCKII-MDR cells

compared with MDCKII cells, probably reflecting enhanced export of T₃ from MDCKII-MDR cells rather than reduced cellular uptake, as P-gp typically exports substances from cells. Verapamil lowered the rate of ¹²⁵I-T₃ efflux from both MDCKII and MDCKII-MDR cells by 42% and 66% respectively, while nitrendipine reduced ¹²⁵I-T₃ efflux rate by 36% and 48% respectively, suggesting that both substances inhibited other cellular T₃ transporters in addition to P-gp. The specific MDR inhibitors VX 853 and VX 710 had no effect on ¹²⁵I-T₃ efflux rate from wild-type MDCKII cells but reduced ¹²⁵I-T₃ export in MDCKII-MDR cells by 50% and 53% respectively. These results have provided the first direct evidence that P-gp exports thyroid hormone from cells.

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Introduction

Thyroid hormone plays an essential role in mammalian cellular metabolism. While much is known about the physiology of trans-membrane iodothyronine transport (Kragie 1994, 1996, Abe *et al.* 2002) little is known about the molecular mechanisms of these processes. In addition to cellular uptake, export of thyroid hormone from cells may be an important factor in the regulation of thyroid hormone action at the level of its nuclear receptor (Ribeiro *et al.* 1996).

P-glycoprotein (P-gp; multidrug resistance-associated (MDR)1) is a 170 kDa surface glycoprotein found in a number of normal tissues and interacts with a broad range of substrates and inhibitors, including steroid hormones (Barnes *et al.* 1996). P-gp pumps substrates in a single direction, out of cytoplasm, and is present in high levels in many multidrug-resistant cell lines and tumors. Verapamil-sensitive thyroid hormone efflux from cells has been reported in several cell types (Ribeiro *et al.* 1996, Neves *et al.* 2002) and, as verapamil is a potent inhibitor of

P-gp, this suggested involvement of the MDR transporters in the efflux of thyroid hormone. Verapamil is not, however, a specific inhibitor of P-gp and it was also found to inhibit tri-iodothyronine (T₃) efflux from cells that did not express MDR1 (Cavalieri *et al.* 1999). Photoaffinity labelling of these latter cells with ¹²⁵I-T₃ revealed a 90–100 kDa protein that interacted with ¹²⁵I-T₃ and verapamil. The small size of the protein excluded the possibility that it was P-gp and Cavalieri *et al.* (1999) concluded that a novel, verapamil-sensitive protein was responsible for T₃ export in these cells. Although the available evidence of verapamil-sensitive T₃ efflux from cells suggested that MDR transporters may be involved in exporting thyroid hormone out of cells, we identified a need for further investigations to clarify whether P-gp is indeed a mediator of cellular thyroid hormone efflux.

We investigated this by using a polarized MDCKII cell line that has been stably transfected with MDR1 cDNA (MDCKII-MDR). This is an established model for testing the transport functions of P-gp. We compared uptake and efflux of T₃ in wild-type MDCKII cells (MDCKII) and

transfected MDCKII cells (MDCKII-MDR). We confirmed interaction between thyroid hormone and P-gp by examining the effect of conventional (verapamil, nifedipine) as well as novel (VX 853 and VX 710) inhibitors of P-gp-mediated transport on uptake and efflux of thyroid hormone in these cells. The results provide the first direct evidence that P-gp is involved in the cellular export of thyroid hormone.

Materials and Methods

Materials were purchased from the following sources: ^{125}I -T₃ (114 MBq/μg) from Amersham Biosciences, Amersham, Bucks, UK; foetal calf serum (FCS) from JRH Biosciences, Lexena, KS, USA; six-well tissue culture plates from Costar, Cambridge, MA, USA; mouse anti-P-gp antibody C219 from Signet Laboratories Inc., Dedham, MA, USA; BCA protein reagent from Pierce Chemicals, Rockford, IL, USA. MDR inhibitors VX 853 and VX 710 were a gift from Vertex Pharmaceuticals Inc., Cambridge, MA, USA. All other chemicals and cell culture media were from Sigma Chemical Co., St Louis, MO, USA.

Cell culture

The MDCKII cells (canine kidney cell line) and the MDCKII-derived cells, MDCKII-MDR, (stably transfected with *mdr1* cDNA) were a gift from Professor P Borst, The Netherlands Cancer Institute, Amsterdam, The Netherlands, and have been described previously (Bakos *et al.* 1998). Cell lines were maintained in continuous culture at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Culture medium was Dulbecco's modified Eagles' medium supplemented with 10% (v/v) FCS. Cells were subcultured twice a week. For uptake and efflux experiments, cells were plated into six-well tissue culture plates and grown for 2–3 days until confluent. Medium was changed daily.

Uptake and efflux studies

Uptake studies were carried out as previously described (Mitchell *et al.* 1992, 1994, 1995). Briefly, prior to all experiments cells were incubated for 1 h in Hanks' balanced salt solution (HBSS). All incubations were carried out at 25 °C. To study the time-course of cellular uptake, cells were incubated for 45 min in HBSS containing 30 pM ^{125}I -T₃ with or without 10 μM excess unlabelled T₃. Unlabelled thyroid hormone was dissolved in ethanol and diluted in HBSS. The final concentration of ethanol did not exceed 0.4% and this had no effect on cellular uptake and efflux of ^{125}I -T₃. To terminate uptake, incubation medium was aspirated, cells were washed twice in ice-cold HBSS and immediately lysed in 1 M NaOH.

Cell-associated activity was determined by counting the radioactivity in a Packard γ-counter with a counting efficiency of 84%.

Washout studies were performed to determine whether the exit rate of ^{125}I -T₃ from the cells was enhanced in MDCKII-MDR cells compared with wild-type MDCKII cells and whether it was affected by the presence of either unlabelled T₃ or inhibitors in the external medium. The procedure used to study efflux was as described (Mitchell *et al.* 1994) with some modifications. Briefly, the cells were incubated for 45 min with 30 pM ^{125}I -T₃, washed in non-radioactive HBSS and then incubated for 45 min at 37 °C in non-radioactive medium with or without 10 μM unlabelled T₃, 100 μM verapamil or nifedipine (Sigma Chemical Co.) or 5 μM VX 853 or VX 710. After 1, 2, 5, 10, 15 and 30 min incubation medium was aspirated and replaced with fresh HBSS to prevent re-uptake of the hormone released into the medium. The amount of ^{125}I -T₃ released from the cells in the presence of unlabelled T₃ or an inhibitor during the 45-min incubation in non-radioactive medium and the cell-associated hormone were determined by counting the radioactivity in the aspirated media and the cell lysates respectively. ^{125}I -labelled thyroid hormone tracer released in the presence of an inhibitor during the 45-min incubation in non-radioactive medium was compared with that released in the absence of an inhibitor. The exit rate constants were calculated by linear regression of the efflux data.

We sought evidence of metabolism of ^{125}I -T₃ by duplicate cultures of MDCKII cells during uptake and efflux experiments by analysing radioactivity present in the cells and in the medium after incubation with the tracer for 45 min at 25 °C by LH-20 Sephadex chromatography (Otten *et al.* 1983).

Plasma membrane isolation

Plasma membrane proteins were isolated as described by Cavalieri *et al.* (1999). Cells were grown to confluence, washed and scraped in phosphate-buffered saline, harvested by centrifugation at 180 g for 2 min at 4 °C and resuspended in hypotonic buffer (1 mM Tris, pH 7). Recombinant *Serratia marcescens* nuclease (800 U; benzonase) was added, and the mixture was stirred on ice for 2 h. The cell lysate was centrifuged at 100 000 g for 30 min at 4 °C, and the resulting crude membrane pellet was resuspended in buffer (50 mM Tris and 50 mM mannitol, pH 7.0). All solutions were supplemented with the proteinase inhibitors leupeptin (4 μM), EGTA (2 mM) and phenylmethylsulfonylfluoride (0.5 mM). Aliquots were removed for determination of protein concentration.

Electrophoresis and immunoblotting

Plasma membrane proteins (20 μg) isolated from MDCKII and MDCKII-MDR cells were dissolved in sample

Figure 1 Western blot analysis of cell membrane proteins using the C219 mdr1/P-gp monoclonal antibody. Aliquots of 20 μg plasma membrane protein isolated from MDCKII cells and MDCKII cells transfected with mdr1 cDNA were fractionated on 7.5% SDS-PAGE and transferred to nitrocellulose. The blot was incubated with a 1:1000 dilution of a mouse monoclonal antibody, C219. The immune complexes were detected with the ECL Western blotting analysis system. Line 1, protein standards; line 2, MDCKII; line 3, MDCKII-MDR.

electrophoresis buffer (2% (v/v) SDS, 5% (v/v) mercaptoethanol, 10% (w/v) glycerol and 60 mM Tris, pH 6.8), boiled for 5 min as described by Samson *et al.* (1993) and fractionated by electrophoresis on 7.5% SDS-PAGE gels (1.5 mm thick) (Laemmli 1970). The molecular weight of the bands was estimated using pre-stained SDS-PAGE standards (BioRad, Hercules, CA, USA). The gel was transferred to nitrocellulose membrane by electroblotting (Towbin *et al.* 1979). The nitrocellulose membrane was incubated with a 1:1000 dilution of a mouse monoclonal antibody (C219) against the nucleotide-binding domain of human P-gp (Georges *et al.* 1990). Immune complexes were detected using the ECL Western blotting analysis system (Amersham).

Determination of the cellular protein content

The protein content of cell lysates and the the membrane protein preparation were determined with the BCA reagent, which is a modification of the Biuret reaction, using bovine serum albumin as a standard.

Statistical analysis

Statistical analysis was performed by Student's *t*-test. Results are expressed as means and standard errors of the mean; *n* is the number of independent experiments, each in triplicate. A probability of <0.05 was regarded as significant.

Results

Confirmation of expression of P-gp in MDCKII and MDCKII-MDR cells

Immunoblots of plasma membrane proteins using a specific antibody against P-gp, C219, confirmed strong

expression of P-gp in MDCKII cells transfected with MDR1 cDNA (MDCKII-MDR) and weaker expression in the wild-type MDCKII cells (Fig. 1). This pattern of expression correlated with the enhanced rate of T₃ efflux from MDCKII-MDR cells compared with MDCKII cells (Fig. 2).

Cellular uptake and export of thyroid hormones

Uptake of ^{125}I -T₃ into both MDCKII cells and MDCKII-MDR cells was time-dependent and saturable, being reduced in the presence of an excess of unlabelled ligand (Fig. 3). The overall cellular accumulation of ^{125}I -T₃ was reduced from 6.77 ± 0.52 fmol/mg protein ($n=5$) in MDCKII cells to 5.02 ± 0.21 fmol/mg protein ($n=5$) in MDCKII-MDR cells after 30 min of incubation ($P=0.003$), probably reflecting enhanced export of T₃ from MDCKII-MDR cells rather than reduced cellular uptake. Efflux of ^{125}I -T₃ was significantly enhanced in MDCKII-MDR cells compared with wild-type MDCKII cells (Fig. 2). The calculated rate of ^{125}I -T₃ efflux from the intracellular compartment of MDCKII-MDR cells ($0.276 \pm 0.026/\text{min}$, $n=5$) was increased twofold compared with MDCKII cells ($0.134 \pm 0.008/\text{min}$, $n=5$).

Effect of verapamil, nitrendipine, VX 710 and VX 853 on cellular efflux of T₃

Verapamil lowered the rate of ^{125}I -T₃ efflux from both MDCKII and MDCKII-MDR cells by around 42% and 66% respectively, while nitrendipine reduced ^{125}I -T₃ efflux rate by 36% and 48% respectively (Fig. 4). VX 710 and VX 853 had no significant effect of T₃ efflux from MDCKII cells, but reduced T₃ export in MDCKII-MDR cells by around 50% (Fig. 4).

Figure 2 Export of ^{125}I - T_3 from MDCKII cells (○) and MDCKII cells transfected with human mdr1 cDNA (●). Cells were labelled by incubation for 30 min with 30 pM ^{125}I - T_3 , washed, and then incubated again in non-radioactive medium for the indicated times as described in Materials and Methods. Values shown are means $\pm$ S.E.M. ($n=3-5$). Inset: % cell-associated thyroid hormone remaining in MDCK II cells (open bar) and MDCKII-MDR cells (solid bar) after a 45-min washout in non-radioactive medium. Values are means $\pm$ S.E.M. ($n=3-5$). * $P<0.01$.

Metabolism of ^{125}I - T_3 by MDCKII cells

Since MDCKII cells originate from dog renal epithelium, and kidney expresses type I deiodinase (Leonard & Rosenberg 1978), we sought evidence of metabolism of ^{125}I - T_3 in duplicate cultures of MDCK cells during uptake and efflux experiments by analysing the radioactivity present in the cells and the medium after incubation with the tracer for 45 min at room temperature. Intact hormone and metabolites were resolved by LH-20 Sephadex chromatography. We found only minimal evidence of

metabolism of the tracer by the cells under the experimental conditions employed in our studies. The elution profiles indicated that over 90% of activity present in the medium and cells at the end of incubation was intact ^{125}I - T_3 .

Discussion

Thyroid hormones exert widespread influences on most mammalian tissues by genomic (via nuclear thyroid

Figure 3 Time dependence of ^{125}I - T_3 uptake in the (A) MDCKII cells and (B) MDCKII cells transfected with human mdr1 cDNA. Cells were incubated at 25 °C with 30 pM ^{125}I - T_3 in the absence (●) or the presence (○) of 10 μM unlabelled T_3 . Values shown are means $\pm$ S.E.M. ($n=3$).

Figure 4 Effect of inhibitors on export of ^{125}I - T_3 from MDCKII cells (open bars) and MDCKII cells transfected with human mdr1 cDNA (solid bars). Cells were labelled by incubation for 30 min with either 30 pM ^{125}I - T_3 or 50 pM ^{125}I - T_3 , washed, and then incubated again in non-radioactive medium in the absence (control) or the presence of verapamil (100 μM), nitrendipine (100 μM), or P-gp inhibitors VX 710 and VX 853 (both 5 μM) for the indicated times. Values shown are means $\pm$ S.E.M. ($n=4$).

hormone receptors) and non-genomic mechanisms. Thyroid hormone must enter the cell to bind to its nuclear receptor and there is extensive evidence that cellular transporters specific for thyroid hormone mediate hormone entry. Export of thyroid hormone may be an important factor regulating the intracellular content of hormone. Thyroid hormone transport across cell membranes has been studied extensively and much is known about the physiology of trans-membrane iodothyronine transport (Kragie 1994, 1996, Abe *et al.* 2002). There is, however, much less information about the molecular mechanisms mediating export of thyroid hormones from cells. We have shown previously that efflux of T_3 from placental cells occurs via a saturable membrane transporter, which is trans-inhibited by T_3 and tryptophan (Mitchell *et al.* 1994), but our previous studies did not provide information about the nature of this transporter. In the present study, we investigated whether thyroid hormones are exported from the cell by the MDR protein, P-gp.

P-gp is a 170 kDa surface glycoprotein found in a number of normal tissues and in many multidrug-resistant cell lines and tumors. It interacts with a broad range of substrates, including steroid hormones (Barnes *et al.* 1996), and inhibitors, including calcium channel antagonists (Mickisch *et al.* 1990). Its greatest affinity is for large, hydrophobic cations. It pumps substrates in one direction, typically out of the cytoplasm. Several studies have attempted to examine the interaction of thyroid hormones with MDR proteins (Nelson & Hinkle 1992, Ribeiro *et al.* 1996, Cavalieri *et al.* 1999, Neves *et al.* 2002). Inhibition of thyroid hormone efflux by verapamil has been reported in several cell types (Ribeiro *et al.* 1996, Neves *et al.* 2002). Since verapamil is a potent inhibitor of P-gp (Mickisch *et al.* 1990) these results suggested the involvement of P-gp in the efflux of thyroid hormone. T_3 efflux from cell lines that do not express P-gp is, however, also inhibited by verapamil (Cavalieri *et al.* 1999). Photoaffinity labelling of these latter cells with ^{125}I - T_3 revealed a 90–100 kDa protein that interacts with L- T_3 and verapamil. The small size excluded the possibility that it was P-gp, indicating that a novel, as yet unidentified, verapamil-inhibitable protein was responsible for T_3 export in these cells.

While previous studies provided some evidence suggesting interaction between MDR proteins and thyroid hormone export from cells, they either used drug-selected cell lines (Ribeiro *et al.* 1996) or primary cells (Neves *et al.* 2002) that expressed multiple MDR transporters in addition to P-gp. In addition, the calcium channel blocker verapamil, which is not selective for MDR, was used as an inhibitor of MDR transport. We have previously shown in the human choriocarcinoma cell line, JAR, significant inhibition of both uptake and efflux of T_3 by calcium channel blockers nitrendipine and verapamil (Mitchell *et al.* 1999). JAR cells express minimal levels of P-gp (Mylona *et al.* 1996, Atkinson *et al.* 2003). In contrast, in the present study we used a polarized cell line,

MDCKII-MDR derived from an MDCKII cell line, stably transfected with *mdr1* (P-gp) cDNA (Evers *et al.* 1997, 2000), and over-expressing P-gp (Borst *et al.* 1999). We also used two novel and specific inhibitors of MDR transport, VX 853 and VX 710 as well as the less specific calcium channel blockers verapamil and nitrendipine which are conventionally used as inhibitors of P-gp. A similar approach has been successfully employed to examine interactions of P-gp and cytotoxics used clinically, in which an earlier generation P-gp reversal agent, V-104 (Vertex Pharmaceuticals), effectively inhibited daunorubicin and vinblastine transport in MDCKII-MDR cells (Evers *et al.* 2000).

Using Western blot analysis of the plasma membrane proteins with an anti-P-gp monoclonal antibody C219, we confirmed residual P-gp expression previously reported in the wild-type MDCKII cells (Goh *et al.* 2002) as well as the expected higher expression of P-gp in the MDCKII-MDR cells (Fig. 1). Cellular accumulation of T_3 by MDCKII-MDR was markedly reduced when compared with the wild-type MDCKII cells (Fig. 3). This was most likely due to enhanced export of thyroid hormone from transfected cells rather than reduced uptake, since P-gp mediates unidirectional outward transport of substances from cells. Significantly enhanced efflux of thyroid hormones from MDCKII-MDR cells compared with wild-type MDCKII cells (Fig. 2) further suggested involvement of P-gp in the export of thyroid hormones. We have also shown a significant reduction in the rate of T_3 release from MDCKII-MDR cells in the presence of the calcium channel blockers, verapamil and nitrendipine, and the specific MDR inhibitors, VX 710 and VX 853, directly confirming the involvement of P-gp in the export of T_3 from the cell. Verapamil and nitrendipine inhibited the rate of efflux of T_3 from both the wild-type MDCKII and the MDCKII-MDR cells, confirming the non-specific inhibition of cellular T_3 transporters by these compounds. In contrast, neither VX 710 nor VX 853 had any significant effect on T_3 efflux from wild-type MDCKII cells, but reduced the rate of T_3 efflux from MDCKII-MDR cells by around 50% (Fig. 4).

In addition to inhibiting the activity of P-gp, both VX 710 and VX 853 also interact with another MDR protein, MRP1 (personal communication, C Sorensen, Vertex Pharmaceuticals). Wild-type MDCKII cells have relatively low levels of endogenous expression of functional MRP1 (Goh *et al.* 2002). Transfected and wild-type cells would be expected to have similar expression of MRP1 so the failure of VX 710 and VX 853 to inhibit thyroid hormone efflux in wild-type cells (Fig. 4) excludes MRP1 as a significant thyroid hormone export molecule in transfected cells.

Collectively, the results of the present study provide the first direct evidence of the involvement of P-gp in thyroid hormone export from cells. MDR transporters may participate in the cellular transport of thyroid hormone in

tissues that express them and provide a tissue-specific mechanism for the regulation of thyroid hormone action at the nuclear receptor level.

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