Thyroid hormone export from cells: contribution of P-glycoprotein

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Abstract

Verapamil inhibits tri-iodothyronine (T\textsubscript{3}) efflux from several cell types, suggesting the involvement of multidrug resistance-associated (MDR) proteins in T\textsubscript{3} transport. The direct involvement of P-glycoprotein (P-gp) has not, however, been investigated. We compared the transport of \textsuperscript{125}I-T\textsubscript{3} in MDCKII cells that had been transfected however, been investigated. We compared the transport of \textsuperscript{125}I-T\textsubscript{3} in MDCKII cells (MDCKII) and examined the effect of mdr1 cDNA (MDCKII-MDR) versus wild-type MDR inhibitors (VX 853 and VX 710) on \textsuperscript{125}I-T\textsubscript{3} efflux. We confirmed by Western blotting the enhanced expression of P-gp in MDCKII-MDR cells. The calculated rate of \textsuperscript{125}I-T\textsubscript{3} efflux from MDCKII-MDR cells (around 0.15/min) was increased twofold compared with MDCKII cells (around 0.15/min). Overall, cellular accumulation of \textsuperscript{125}I-T\textsubscript{3} was reduced by 26% in MDCKII-MDR cells compared with MDCKII cells, probably reflecting enhanced export of T\textsubscript{3} from MDCKII-MDR cells rather than reduced cellular uptake, as P-gp typically exports substances from cells. Verapamil lowered the rate of \textsuperscript{125}I-T\textsubscript{3} efflux from both MDCKII and MDCKII-MDR cells by 42% and 66% respectively, while nitrendipine reduced \textsuperscript{125}I-T\textsubscript{3} efflux rate by 36% and 48% respectively, suggesting that both substances inhibited other cellular T\textsubscript{3} transporters in addition to P-gp. The specific MDR inhibitors VX 853 and VX 710 had no effect of \textsuperscript{125}I-T\textsubscript{3} efflux rate from wild-type MDCKII cells but reduced \textsuperscript{125}I-T\textsubscript{3} 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 (T\textsubscript{3}) efflux from cells that did not express MDR1 (Cavalieri et al. 1999). Photoaffinity labelling of these latter cells with \textsuperscript{125}I-T\textsubscript{3} revealed a 90–100 kDa protein that interacted with \textsuperscript{125}I-T\textsubscript{3} 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 T\textsubscript{3} export in these cells. Although the available evidence of verapamil-sensitive T\textsubscript{3} 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 T\textsubscript{3} 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, nitrendipine) 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: 125I-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., Dedham, MA, USA; BCA protein reagent from Pierce Chemicals, Rockford, IL, 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 mdrl 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 125I-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 125I-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 125I-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 125I-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 nitrendipine (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 125I-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. 125I-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 125I-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 Cavaliere 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; benzo-nase) 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 protease 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
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-T$_3$ 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-T$_3$ 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 T$_3$ from MDCKII-MDR cells rather than reduced cellular uptake. Efflux of $^{125}$I-T$_3$ was significantly enhanced in MDCKII-MDR cells compared with wild-type MDCKII cells (Fig. 2). The calculated rate of $^{125}$I-T$_3$ efflux from the intracellular compartment of MDCKII-MDR cells (0.276 ± 0.026/min, n=5) was increased twofold compared with MDCKII cells (0.134 ± 0.008/min, n=5).

**Effect of verapamil, nitrendipine, VX 710 and VX 853 on cellular efflux of T$_3$**

Verapamil lowered the rate of $^{125}$I-T$_3$ efflux from both MDCKII and MDCKII-MDR cells by around 42% and 66% respectively, while nitrendipine reduced $^{125}$I-T$_3$ efflux rate by 36% and 48% respectively (Fig. 4). VX 710 and VX 853 had no significant effect of T$_3$ efflux from MDCKII cells, but reduced T$_3$ export in MDCKII-MDR cells by around 50% (Fig. 4).
Metabolism of $^{125}$I-T₃ 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-T₃ 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-T₃.

Discussion

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

Figure 2

Export of $^{125}$I-T₃ from MDCKII cells (○) and MDCKII cells transfected with human mdr1 cDNA (●). Cells were labelled by incubation for 30 min with 30 pM $^{125}$I-T₃, washed, and then incubated again in non-radioactive medium for the indicated times as described in Materials and Methods. Values shown are means ± 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 ± s.e.m. (n=3–5). *P<0·01.

Figure 3

Time dependence of $^{125}$I-T₃ 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-T₃ in the absence (●) or the presence (○) of 10 μM unlabelled T₃. Values shown are means ± s.e.m. (n=3).

Figure 4

Effect of inhibitors on export of $^{125}$I-T₃ 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-T₃ or 50 pM $^{125}$I-T₃, 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 ± s.e.m. (n=4).
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 T₃ from placental cells occurs via a saturable membrane transporter, which is trans-inhibited by T₃ and tryptophan (Mitchell et al. 1994). The present study, 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. T₃ 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 ¹²⁵I-T₃ revealed a 90–100 kDa protein that interacts with ¹-T₃ 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 T₃ 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 T₃ by calcium channel blockers nitrédipine 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 nitrédipine 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 T₃ 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 T₃ release from MDCKII-MDR cells in the presence of the calcium channel blockers, verapamil and nitrédipine, and the specific MDR inhibitors, VX 710 and VX 853, directly confirming the involvement of P-gp in the export of T₃ from the cell. Verapamil and nitrédipine inhibited the rate of efflux of T₃ from both the wild-type MDCKII and the MDCKII-MDR cells, confirming the non-specific inhibition of cellular T₃ transporters by these compounds. In contrast, neither VX 710 nor VX 853 had any significant effect on T₃ efflux from wild-type MDCKII cells, but reduced the rate of T₃ 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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