Thyroid Hormone Export Regulates Cellular Hormone Content and Response*

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Thyroid hormones (THs)1 regulate growth, differentiation, and other critical functions in a variety of cell types by acting through nuclear receptors (TRs). TRs belong to a large superfamily of structurally related transcription factors that include receptors for steroids, vitamin D, and retinoids (1–3). TRs bind to specific DNA elements and regulate transcription by binding to TH, commonly 3,5,3'–triiodo-L-thyronine (T3). The intracellular concentration of free T3 determines the rate and degree of TR occupancy and, consequently, the extent of TH-regulated transcriptional responses (1–3).

The role of cellular transport in modulating intracellular hormone concentration is poorly understood. Several studies of cellular TH accumulation have demonstrated saturable, stereospecific, energy-requiring TH uptake (4–16); however, these findings have been inconsistent (17, 18). Moreover, few studies have addressed outward TH transport (19–22) and there is no evidence linking active TH transport to TH action in mammalian cells.

There are suggestions that the ATP-binding cassette (ABC)/multidrug-resistance (mdr) family of proteins can actively transport steroid hormones. Various cells overexpressing mdr-related proteins have been shown to exhibit accelerated export of dexamethasone, corticosterone, and aldosterone, as well as attenuation of glucocorticoid-induced apoptosis (23–27). In addition, selective outward transport of dexamethasone by an ABC protein was shown to modulate the biological potency of this steroid in yeast (28). However, there is no prior evidence that mdr-related proteins transport TH and no direct evidence that hormone transport regulates the actions of nuclear-acting hormones in mammalian cells.

We have investigated whether hormone transport can regulate hormone action in mammalian cells using poorly differentiated HTC rat hepatoma cells specially adapted for resistance to a permeable bile acid ester (HTC-R cells) (29). HTC-R cells exhibit enhanced capability to export bile acids and overexpress several plasma membrane proteins representing apparently novel members of the ABC/mdr family (29). Here we report that HTC-R cells show resistance to TH that can be explained by reduced cellular TH concentration due to enhanced TH efflux. Because verapamil, a known inhibitor of mdr/P-glycoprotein function (30), inhibits TH efflux and reverses TH resistance, we suggest that mdr/P-glycoproteins mediate TH resistance in these cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—HTC and HTC-R cells were grown in 10-cm dishes in RPMI 1640 with 10% newborn bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Rat hepatocytes in primary culture were prepared as described (31). Ventricular cardiocytes, atrioocytes, and fibroblasts from newborn rat hearts in primary culture were prepared as described (32, 33). For transfections, cells were trypsinized, resuspended in buffer (PBS, 0.1% glucose), and mixed with the appropriate reporter genes and receptor expression vectors, human thyroid hormone receptor β1 (hTRβ1) a gift from R. M. Evans, and human estrogen receptor a gift from P. Chambon. The reporter genes consisted of either a synthetic TH response element (DR-4) containing two copies of a direct repeat spaced by four nucleotides (AGGT-CAAGAGGTTCA) or a estrogen responsive element (vitA2) from the frog vitellogenin A2 gene (TCAGGTCAcagTGACCTGA) cloned in the HindIII site of the pUC19 polylinker immediately upstream of a minimal (−32/+45) thymidine kinase promoter linked to chloramphenicol acetyltransferase coding sequences (tkCAT). Cells in 0.5 ml of buffer (15–5 million cells) were electroporated using a Bio-Rad gene pulser at 0.33 kV/m and 960 microfarads. After electroporation, cells were pooled in growth medium (RPMI with 10% charcoal-treated, hormone stripped, newborn bovine serum), plated in 6-well dishes, and treated with either vehicle (ethanol) or hormone. CAT activity was determined 24–36 h later as described (34). The concentration of hormone required for half-maximal induction of CAT activity (EC50) was calculated using a curve-fitting program and is expressed as mean ± S.E.

Cell Uptake—Measurements of TH uptake were done in cells grown in monolayer to 80–100% confluency in 6-well plates and incubated at 37 °C. Cells were washed twice with PBS prewarmed to 37 °C and incubated in RPMI medium containing 0.01% newborn bovine serum and 1 mM 125I-T3 (2200 Ci/mmol, Du Pont NEN). Uptake was stopped at various times by aspirating the medium and washing the cells six times with 1 ml each of ice-cold PBS (35). Cells were then detached with 1 ml

1 The abbreviations used are: TH, thyroid hormone; TR, thyroid hormone receptor; T3, 3,5,3'-triiodo-L-thyronine; ABC, ATP-binding cassette; mdr, multidrug-resistant; HTC, rat hepatoma cells; tk, thymidine kinase; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline.
Thyroid Hormone Export Regulates Response

Fig. 1. Response of resistant (HTC-R) and non-resistant parental (HTC) cells to T₃ and estradiol. A, both cell types were transfectioned by electroporation (15) with the hTRβ1 expression vector (1 μg) and the DR-4 tkCAT reporter gene (10 μg). After transfection, cells were pooled and plated in 6-well dishes and treated with vehicle (ethanol) or with T₃ at increasing concentrations (10⁻¹⁵ to 10⁻⁶ M) for 24-36 h. Data are expressed as percent of maximal activity of fold activation (ratio of CAT activity of T₃-treated cells to that of ethanol-treated controls). These results are representative of at least five independent experiments and show the mean ± S.D. of triplicate plates. B, both cell types were transfected by electroporation with the human estrogen receptor expression vector (1 μg) and the vitA2tkCAT reporter gene (10 μg). After transfection, cells were pooled and plated in 6-well dishes and treated with vehicle (ethanol) or with estradiol at increasing concentrations (10⁻¹¹ to 10⁻⁶ M) for 24-36 h. Data are expressed as percent of maximal activity of fold activation (ratio of CAT activity of estradiol-treated cells to that of ethanol-treated controls). These results are representative of three independent experiments and show the mean ± range of duplicate plates.

Efflux Studies—Cellular TH efflux was measured as described (37). Following 3 h preincubation of cells at 37 °C in RPMI medium containing 0.01% newborn calf serum and 125I-T3 (1 nM). Uptake was measured at 37 °C in RPMI medium containing 0.01% newborn bovine serum and 125I-T3 (1 nM). Uptake is depicted for the first 5 min (inset) as well as over 4 h (mean ± S.D. of triplicates of a representative experiment).

RESULTS AND DISCUSSION

We first assessed responsiveness to TH by co-transfecting both HTC parental (HTC cells) and HTC-R cells with cDNAs encoding the human TRβ1 (hTRβ1) as well as a reporter gene encoding a TH responsive element upstream of CAT coding sequences. In the absence of co-transfected TRs, CAT production in HTC parental cells was not stimulated by T₃ (10⁻⁷ M) indicating the absence or very low content of endogenous TRs in these cells. HTC-R cells required approximately 10-fold higher T₃ concentration than HTC parental cells for half-maximal induction of relative CAT activity (EC₅₀ 2.2 ± 0.4), range 0.5-3.8 nM, n = 7 for HTC cells compared to 17.9 ± 4.6, range 8.0-40.7 nM, n = 8 for HTC-R cells (p < 0.03) (Fig. 1A). The absolute CAT activity, however, did not differ between HTC and HTC-R cells. Moreover, in contrast to TH, HTC-R and HTC parental cells exhibited equal sensitivity to estradiol (Fig. 1B), indicating selectivity in the HTC-R cells' hormone resistance.

We then studied TH uptake to test whether the TH resistance of HTC-R cells is due to altered TH transport. Although initial uptake (to 5 min) of T₃ by HTC-R cells was at least as rapid as for HTC cells (Fig. 2, inset), steady state T₃ accumulation by HTC-R cells was significantly less (Fig. 2). The decreased T₃ accumulation in conjunction with similar initial entry rates suggest enhanced TH efflux from the HTC-R cells.

Efflux was therefore studied directly in cells pre-loaded with 125I-T₃. T₃ efflux curves were bi-exponential, showing a rapid initial component and a slower terminal component (Fig. 3A). T₃ efflux could be described by a two-compartment model; a small rapidly exchanging compartment (postulated to represent plasma membrane binding) and a larger, more slowly exchanging compartment (postulated to be intracellular). In both cell types the slow compartment comprised at least 95% of total cellular T₃. The difference in steady state T₃ content between HTC and HTC-R cells was accounted for by the difference in fractional efflux rates from the larger, slow compartment (Table I). As shown in Fig. 3A and Table I, the fractional rate of T₃ efflux was much greater from the HTC-R cells than from HTC cells at 37 °C. In contrast to their differential handling of T₃, no difference was observed between the cell types in
the rate of efflux of $^{125}$I-iodide (Fig. 3B), a finding in agreement with the similar sensitivity of both cell types to estradiol (Fig. 1B).

Analysis by chromatography of cells and media from 1 to 24-h incubations revealed progressive accumulation of metabolites (mainly iodide) in the incubation media. However, T$_3$ comprised more than 93% of radiolabel associated with radioactive products (mainly iodide) in the incubation media. How-

ever, T$_3$ comprised more than 93% of radiolabel associated with both HTC and HTC-R cells as well as the radiolabel in the efflux media from these cells. Therefore, the difference in T$_3$ content and in efflux cannot be ascribed to altered T$_3$ metabolism.

To characterize further the mechanism of enhanced T$_3$ efflux by HTC-R cells, we studied the effects of excess TH, cold incubation, and verapamil. Preincubation with excess T$_3$ or T$_4$ in concentrations (10$^{-4}$ M) sufficient to produce a 30,000–70,000-fold increase in cell-associated hormone inhibited efflux of radiolabeled T$_3$ in HTC-R cells, suggesting that the efflux mechanism in these cells is saturable. Excess T$_4$ was at least as effective an inhibitor of T$_3$ efflux as was excess T$_3$, suggesting that both hormones share the same transporter (Table I). T$_3$ efflux was also markedly slowed at 4°C (Table I, Fig. 3A). Verapamil had no significant effect on T$_3$ efflux from HTC cells, but produced concentration-dependent inhibition of T$_3$ efflux from the HTC-R cells (Table I). Fig. 3A shows that verapamil (10$^{-4}$ M) slowed TH extrusion in HTC-R cells, such that the percent T$_3$ remaining in these cells at 60 min increased from 11.2 ± 2 to 34.2 ± 10% (p < 0.01), approaching values found in HTC cells (50.9 ± 11 and 56.6 ± 6% in the absence and in the presence of verapamil, respectively). These findings are also consistent with the presence in both cell types of a basal hormone efflux which is nonsaturable and verapamil-insensitive. Moreover, the presence of verapamil during a 3-h incubation with $^{125}$I-T$_3$ (1 nM) increased total T$_3$ content of HTC-R cells by a factor of 1.9 (p < 0.01; one-way analysis of variance and the Student-Neuman-Keul test) over that in HTC-R cells incubated without verapamil. In contrast, the drug had no significant effect on T$_3$ content of HTC parental cells. Chromatographic analysis of cells and efflux media showed that verapamil, present during preincubation and efflux periods, did not affect T$_3$ metabolism in either HTC or HTC-R cells. These data are consistent with enhanced T$_3$ efflux as the major determinant of the lower T$_3$ content in HTC-R cells.

To test whether a verapamil-sensitive TH extrusion process plays a role in the observed TH resistance of HTC-R cells, we measured the functional response to T$_3$ in both cell lines in the presence and absence of verapamil (10$^{-4}$ M). As shown in Fig. 4, there was a significant verapamil-induced shift in CAT activity toward greater T$_3$ sensitivity in HTC-R cells (EC$_{50}$ 12.7 ± 4.4

**TABLE I**

| Experimental condition | T$_3$ efflux rate constant (min$^{-1}$) |
|------------------------|----------------------------------------|
|                        | HTC cells | HTC-R cells |
| 37°C control           | 0.0010 ± 0.0027 | 0.034 ± 0.0053$^a$ |
| 4°C                    | 0.0017 ± 0.0010$^b$ | 0.0034 ± 0.0006$^a$ |
| 37°C + verapamil (100 μM)$^d$ | 0.0080 ± 0.0001 | 0.014 ± 0.0055$^a$ |
| 37°C + verapamil (10 μM)$^d$ | Not tested | 0.027 ± 0.0055$^a$ |
| 37°C + T$_3$ (100 μM)$^d$ | 0.011 ± 0.0012 | 0.012 ± 0.0059$^a$ |
| 37°C + T$_4$ (100 μM)$^d$ | 0.0059 ± 0.0032 | 0.0034 ± 0.0027$^a$ |

$^a$p < 0.005 compared to parental HTC cell controls.

$^b$Incubation at 4°C during efflux period only.

$^c$p < 0.005 compared to controls of the same cell type.

$^d$Verapamil, excess T$_3$ or excess T$_4$ present in the indicated concentrations during preincubation and efflux periods.

$^e$p < 0.01 compared to controls of the same cell type.
The accompanying paper (43) reports that the immunosuppressant FK 506, also a P-glycoprotein inhibitor (39), selectively decreases dexamethasone content and responsiveness in mammalian L929 cells but not in HeLa cells. These findings complement our own and indicate that hormone transport may be a general regulatory step for controlling tissue specific actions of nuclear-acting hormones.

Collectively, these findings represent the first demonstration in mammalian cells of a saturable, temperature-sensitive, verapamil-inhibitable TH efflux mechanism that controls cellular TH content and TH responsiveness, presumably by modulating access of TH to TRs. Inhibition of TH efflux by verapamil, as well as the previously reported overexpression of ABC/mdr-related genes in HT-C-R cells (29), implicate an ABC/mdr-related protein(s) as the mediator(s) of TH transport. Clarification of whether the TH transporter is a known or novel mdr-related protein must await its cloning and functional characterization. Altered hormone transport represents one potential explanation for hormone resistance syndromes that occur in the absence of receptor mutations (40), and for heterogeneity of response in different tissues in patients with subclinical hypothyroidism and TH resistance (41, 42). Finally, our findings, in conjunction with those in the accompanying manuscript (43), suggest that active hormone efflux constitutes a novel mechanism for physiologic regulation of the action of TH and potentially other hormones that act through the nuclear hormone receptor gene superfamily.

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