Type 2 Iodothyronine Deiodinase Is Highly Expressed in Human Thyroid

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Abstract

Type 2 iodothyronine deiodinase (D2) is a recently cloned selenodeiodinase thought to provide intracellular 3,5,3′ triiodothyronine (T3) to a restricted group of tissues. We report here the presence of D2 mRNA in human thyroid at levels 50–150-fold higher than in placenta. Surprisingly, while type 1 deiodinase (D1) is known to be present in human thyroid, D2 has not been evaluated previously. D2 mRNA was especially high in thyroids from Graves' patients and in follicular adenomas. Stimulated thyroids had higher D2 to D1 mRNA ratios than normal or multinodular glands suggesting differential regulation of D1 and D2 expression. Microsomes from normal, Graves', and TSH-stimulated thyroids contained low K_m D2 activity resistant to propylthiouracil (1 mM) or to inactivation by *N*-bromoacetyl T3, agents which block or inactivate D1. At 2 nM thyroxine (T4), 100 times the physiological-free T4 levels, 60–80% of T4 to T3 conversion in stimulated, but only 27% of that in normal thyroids, is catalyzed by D2. We conclude that intrathyroidal T4 to T3 conversion by D2 may contribute significantly to the relative increase in thyroidal T3 production in patients with Graves' disease, toxic adenomas, and, perhaps, iodine deficiency. (*J. Clin. Invest.* 1996. 98:962–968.)
Key words: thyroid • iodide peroxidase • selenocysteine • selenium • deiodinase

Introduction

The monodeiodination of thyroxine (3,5,3′,5′ tetraiodothyronine [T4])[1] to 3,5,3′-triiodothyronine (T3) is the first step in thyroid hormone action. Both types 1 and 2 iodothyronine deiodinases (D1 and D2) catalyze this reaction in most vertebrates. D1 has been cloned and is a selenocysteine-containing enzyme which is expressed in rat liver, kidney, thyroid and pituitary glands (1). It is thought to catalyze the production of much of the circulating T3 [reviewed in reference 2]. D1 is extremely sensitive to inhibition by the thiourea drug 6-*n*-propylthiouracil (PTU) (K_i [rT3] 200 nM) by an uncompetitive mechanism (3, 4).

D2 was initially identified because of the presence of a PTU-insensitive pathway for T4 to T3 conversion in rat pituitary and cerebral cortex (5–8). Further studies identified D2 activity in human brown adipose tissue (BAT), placenta, pituitary tumors, and human keratinocytes (9, 10). We have sequenced and transiently expressed the human D2 cDNA which was identified in Genbank by virtue of its sequence similarity to the *Rana catesbeiana* D2 (11, 12). We showed that the protein encoded by the hD2 cDNA has the kinetic characteristics of D2 including a low K_m and limited inhibition by 1 mM PTU. Surprisingly, we also found that the ~ 7.5 kb mRNA encoding D2 was expressed in human cardiac and skeletal muscle in addition to cerebral cortex and placenta (12). The D2 activity in human skeletal muscle was comparable to that in BAT from chronically cold exposed rats (13), suggesting it contributes to the PTU-insensitive plasma T3 production in humans (14).

These surprising results led us to reevaluate our concepts of the physiological role of D2. In the course of comparing D2 and D1 expression in various tissues, we found D2 mRNA and enzyme activity in the thyroid of a patient with Graves' disease at levels much higher than those in human placenta or keratinocytes (15). D2 mRNA was present in normal thyroid, in thyroid tissue from a patient with hyperthyroidism due to a thyroid stimulating hormone (TSH)-producing pituitary tumor, in thyroid adenomas, multinodular goiters all at levels similar to those in two human pituitary tumors. Kinetic studies of T4 and rT3 metabolism by human thyroid microsomes showed that both D2 and D1 are present but that most of the T3 generated from T4 in microsomes from stimulated human thyroid is catalyzed by D2, but by D1 in normal thyroid microsomes. D2 mRNA is not present in rat FRTL-5 cells. Thus, it appears that the human thyroid can produce T3 via D2-catalyzed monodeiodination of T4 as well as from the direct hydrolysis of thyroglobulin (Tg). Both of these sources may contribute to the disproportionate increase in T3 production which is characteristic of the hyperthyroid state (16).

Methods

Sources of mRNA. Samples of thyroid tissue or mRNA were obtained in Boston or Naples from patients with the following diagnoses: Graves' disease (n = 5), a thyroid from a patient with hyperthyroidism secondary to a TSH-producing pituitary tumor, follicular adenoma (one toxic and one nonfunctioning composed of Hurthle cells), multinodular goiter (MNG) (n = 3), and normal tissue surrounding a benign follicular adenoma (n = 2). Two snap frozen postmortem samples of normal thyroid with 4–5 h of death were obtained from Zoion (New York, NY) for use in kinetic studies. All of the hyperthyroid patients were receiving Tapazole and iodide at the time of surgery. Graves' patients 1 and 2 had suppressed serum TSH concentrations although serum thyroid hormone levels were in the high–normal range. The mRNA samples in Fig. 1 *B* were obtained from pa-

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Received for publication 30 April 1996 and accepted in revised form 14 June 1996.

1. *Abbreviations used in this paper:* BAT, brown adipose tissue; BrAct3, *N*-bromoacetyl-3-5-3′-triiodothyronine; D1, type 1 iodothyronine deiodinase; D2, type 2 iodothyronine deiodinase; D3, type 3 iodothyronine deiodinase; MNG, multinodular goiter; PTU, 6-*n*-propylthiouracil; T_3, 3,5,3′ triidothyronine; T_4, 3,5,3′,5′-tetraiodothyronine; rT3, reverse T3; Tg, thyroglobulin.

J. Clin. Invest.

0021-9738/96/08/0962/07 $2.00
Volume 98, Number 4, August 1996, 962–968

tients treated in Naples, at II Medical School (courtesy of Professor A. Fusco and G.F. Fenzi). Total mRNA was also obtained from two nonsecreting anterior pituitary tumors which stained for TSH, LH, and FSH (Pit-1) and LH (Pit-2) courtesy of Drs. Peter Black, Rona Carroll, and Ms. Kathleen Dashner. Total mRNA from FRTL-5 (rat thyroid) cells and from BAT taken from rats exposed to 4°C overnight were included for comparison.

All human and animal tissues were obtained under protocols approved by the Institutional Review Board at the Brigham and Women's Hospital, Boston, MA.

RNA preparation, Northern blotting and densitometric analyses. Total RNA was prepared from different tissues by guanidine thiocyanate standard techniques as previously described (17). Poly(A)$^+$ RNA from full-term human placenta was isolated by chromatography on oligo-(dT) cellulose type 7 (Pharmacia fine Chemicals, Piscataway, NJ). For Northern analysis, extracted RNA was electrophoresed in 1.5% agarose gel and blotted as described (17). After prehybridization for 6 h at 42°C in a solution containing 40% formamide, 0.64 M NaCl, 40 mM PO_4 buffer (pH 5.6), 0.8 mg/ml denatured thymus DNA, 1.6% SDS, 70% dextran sulfate, and 2× Denhardt's solution (1 × = 0.2% Ficoll, 0.2% BSA and 0.2% polyvinylpyrrolidone), hybridization was performed for 12 h at 42°C in the same solution in the presence of ^{32}P-labeled cDNA probe (random primed). Filters were washed three times for 20 min at room temperature in 2 × SSC-0.1% SDS and then two more times for 20 min in 0.5 × SSC-0.1% SDS at 42°C. Filters were then exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY) for various times (2 h to 7 d) using a fluorescence-intensifying screen. Probes used for all three blots were as follows: D1, rat D1 coding region derived from G21-plasmid digested with XhoI and HindIII (∼ nt 1–778) (1); D2, PCR-generated human D2 coding region (nt 40–862) (12); and D3, human D3 coding region derived from BS-hD3 plasmid digested with BamHI and HindIII (nt 221–1061) (18). Multiple exposures of each blot were made to allow a comparison of the densities of the thyroid D2 mRNA signal to that in human placenta to that of β-actin. Densitometric quantitation of the autoradiographs with appropriate exposure times was performed by a Molecular Dynamics computing densitometer (Sunnyvale, CA).

Microsome preparation. Human thyroid tissues were homogenized in ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM DTT, 0.25 M Sucrose and 1 mM EDTA (homogenization buffer). Homogenates were centrifuged at 20,000 *g* for 20 min at 4°C. The 20,000 *g* supernatant from homogenates of thyroid were centrifuged at 200,000 *g* for 90 min at 4°C to prepare microsomes. Microsomal pellets were resuspended in the homogenization buffer and stored at −70°C.

DNA transfections. Human D2 and rat D1 cDNA subcloned into D10 expression vector (19) were transfected into HEK-293 cells by calcium phosphate precipitation as described previously (20). The deiodination kinetics of rat D1 are not different from that of human D1 (21–23). 2 d after transfection, cells were harvested and sonicated in 0.1 M potassium phosphate, 1 mM EDTA, pH 6.9 (PE buffer), containing 25 mM DTT. Transfection efficiencies were monitored by assay of human growth hormone (GH) in the media derived by cotransfecting a thymidine kinase (TK) promoter-directed human growth hormone-expressing plasmid, pTKGH (20).

5′ Deiodinase assays. Reactions contained 5–300 μg of protein, 0.1 nM 3,3′,5, ^{125}I-5′ tetraiodothyronine (T4) purified by LH-20 chromatography, 2 nM cold T4, 20 mM DTT, and 1 mM PTU in PE buffer in a final volume of 300 μl (D2 conditions) or 0.2 nM 3,3′,^{125}I-5′ triiodothyronine (reverse T3 or rT3) purified by LH-20 chromatography, varying concentrations of unlabeled reverse T3, 10 mM DTT, or other reagents added as indicated (D1 conditions). Incubations were for 60 or 120 min at 37°C. ^{125}I was separated from unreacted substrate or iodothyronine products by trichloroacetic acid precipitation after addition of 200 μl of horse serum as described (24). Deiodination was linear with both protein concentration and time and the quantity of enzyme assayed was adjusted to consume < 30% of substrate. Deiodination of T4 and reverse T3 produced equimolar concentrations of labeled I- and T3 (from T4) or 3,3′ diiodothyronine (from reverse T3) as assessed by paper chromatographic separation of the reaction products. All reactions were in duplicate, and all experiments were repeated at least three times, with similar results.

N-Bromoacetyl[^{125}I]T_3 ($BrAcT_3$) was synthesized from bromoacetylchloride and T_3 as described previously (25). The product was purified on LH-20 Sephadex by elution with ethanol and purity verified by thin layer chromatography in ethyl acetate/glacial acetic acid (9:1) and the concentration of the product determined from the specific activity of the T_3 used in the starting material as described previously (18). In all the experiments, labeled $BrAcT_3$ was diluted with 0.01–3.0 pmol of unlabeled $BrAcT_3$ synthesized in the same manner.

Results

D2 mRNA is highly expressed in human thyroid. To characterize D2 expression in human tissues we analyzed three Northern blots containing 30 μg total mRNA derived from normal and pathological human thyroid tissues, two human anterior pituitary adenomas, and 2 μg poly (A)$^+$ from human placenta. After hybridization with the hD2 probe, a prominent band of 7–8 kb appears in all the thyroid samples (Fig. 1, *A–C*). It is particularly high in most of the Graves' thyroids (patients 2, 4, 5), in the Hurthle cell and toxic adenomas and in the TSH-toxic thyroid. High levels of D2 mRNA are also present in pituitary adenoma 1 (staining for TSH, LH, and FSH) and much lower levels in Pit-2 (weak LH staining). D2 is present in BAT from cold-exposed rats, but no D2 mRNA is present in FRTL-5 cells, even after long exposure of this blot. The levels of D2 mRNA in 30 μg of total mRNA from human thyroids are so much higher than that found in 2 μg of human placental poly A$^+$ that overexposure of the D2 band occurs when the blot is exposed sufficiently long (18 h for Fig. 1 *B* and 16 h for Fig. 1 *C*) to allow direct comparisons of D2 mRNA content between thyroid and placenta on the same blot. In addition to the major 7.5-kb band, there are a number of shorter transcripts in thyroid mRNA, the most prominent of which are ∼ 4.0 and ∼ 1.8 kb in size. By exposing the blots for various intervals to obtain densitometric analyses in the linear range for D2 and actin mRNA (see Methods), we estimate that the expression of D2 mRNA in normal thyroid is ∼ 150 times that in placenta and ∼ 45 times that in the BAT mRNA and is similar to that in pituitary adenoma 1. D2 expression in the Graves' -2 and 3 samples (corrected for actin) and in the two adenomas is severalfold higher than in normal thyroid (Fig. 1 and data not shown). Expression in MNG-1 was similar to normal thyroid but it was much lower, similar to placenta, in MNG-2 and 3. Although the number of samples from euthyroid patients is small, it is clear that thyroid expresses the highest levels of D2 mRNA of any normal human tissue yet examined (12). The presence of high levels of D2 mRNA in a human pituitary adenoma relative to placenta is not unexpected given the presence of D2 activity in pituitary tumors (10), but this has not been reported previously.

Comparison of thyroidal D2 mRNA expression with that of D1 and D3. Previous studies have shown D1 activity in normal and pathological human thyroids (26), but there is only one report documenting the level of D1 mRNA. (21). Despite the differences in D2 mRNA expression among the various samples, particularly in the 5 Graves' thyroids, expression of D1 mRNA was remarkably similar from sample to sample (Fig. 1). In particular, compare the much higher D2/D1

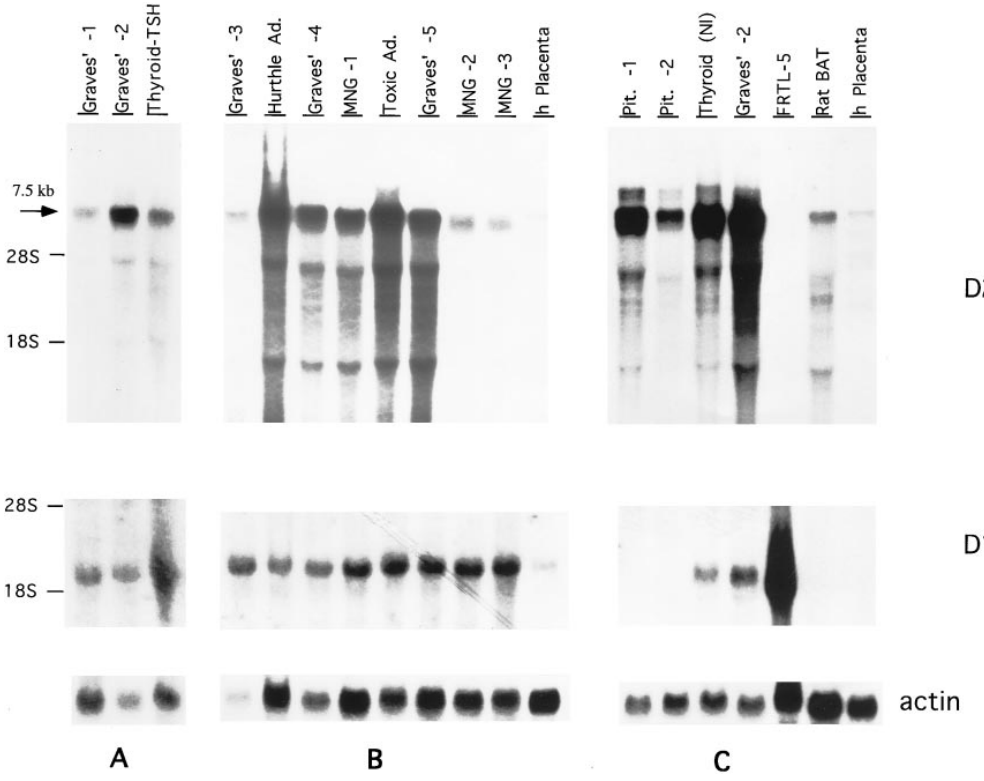

Figure 1. D2 and D1 mRNA expression in three Northern blots of human thyroid mRNA (*A*), human thyroid and placental mRNA (*B*) human thyroid, placenta, and pituitary tumor mRNA together with mRNA from cold-exposed rats BAT and FRTL-5 cells (*C*). With the exception of the human placenta, (2 μg Poly A+) ~ 30 μg of total RNA was loaded in each lane. Each membrane was probed with the appropriate ^{32}P-labeled cDNA corresponding to either the D2 or D1 coding region, (see Methods) stripped after each autoradiograph and subsequently probed with β-actin cDNA in a single hybridization bag as a control for uniform loading and quality of the RNA samples.

mRNA ratios in Graves'-2 and 5 thyroids and in the thyroid adenomas to those in the TSH-toxic, multinodular goiter, and normal thyroid samples. In general, it would appear that D1 mRNA is relatively constitutively expressed whereas D2 mRNA may vary more closely with the degree of thyroid stimulation. Interestingly, FRTL-5 cells express high levels of D1 mRNA despite the complete absence of D2 expression (Fig. 1 *C*). Also, there is no D1 expression in the pituitary tumors despite the presence of D1 in total rat pituitary mRNA (1).

We reprobed the same blots with a human D3 cDNA probe and found no signal in the thyroidal mRNA (data not shown).

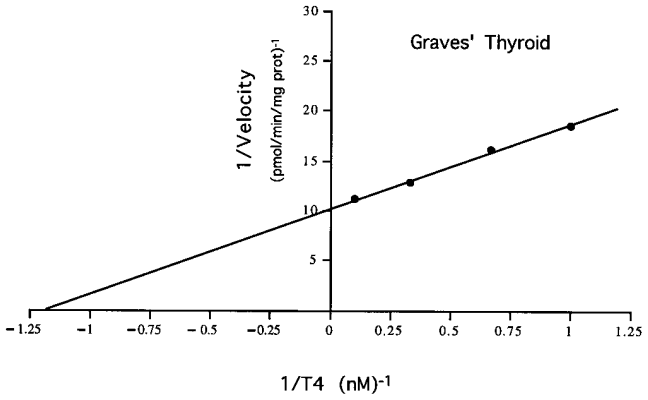

Figure 2. Lineweaver-Burk plot showing 5′-deiodination by microsomes from Graves'-2 thyroid. Microsomal protein (16 μg) was incubated for 1 h with 1, 1.5, 3, and 10 nM T4 , in the presence of 20 mM DTT and 1 mM PTU. The reaction rate was measured by the accumulation of ^{125}I-, as indicated in Methods.

Characterization of D2 and D1 enzyme activities in human thyroid microsomes. As expected from the Northern blots, human thyroid microsomes express both D2 and D1 activities (Table I). Both the Graves-2 and thyroid-TSH samples catalyze T4 deiodination by a low K_m PTU-insensitive mechanism and reverseT3 (rT3) 5′ deiodination by a high K_m process (Table I and Fig. 2). Kinetics of in vitro expressed D1 and D2 analyzed at the same time are shown for comparison (Table I). The difference in the V_{max} values for D2 between the Graves'-2 and TSH-stimulated thyroid was ~ 10-fold, similar to the dif-

Table I. Quantitation of the D2 and D1 Activities of Human Thyroid Microsomes

Assay conditions	Sample	K_m	V_{max}
			pmol/min mg prot
Type 2 deiodinase	Graves'-2	0.8 nM	0.11
T4 (1–10 nM),	Thyroid-TSH	11 nM	0.013
1 mM PTU,	D2* (in vitro)	7.1 nM	1.4
20mM DTT			
Type 1 deiodinase	Graves'-2	0.28 μM	166
rT3 (0.1–1 μM),	Thyroid-TSH	0.31 μM	29
10 mM DTT	D1* (in vitro)	0.24 μM	105

*D2 and D1 are sonicates from HEK-293 cells transfected with D10 expression vector containing cDNAs encoding human D2 or rat D1. HEK-293 cells transfected with vector alone expressed neither D1 nor D2 activity. The V_{max} for each preparation was determined by Lineweaver-Burk analysis using varying concentrations of either T4 (for D2) or reverse T3 (for D1) at the stated DTT concentrations.

Table II. Effects of PTU and BrAcT3 on the 5′ Deiodinase Activities of Human Thyroid Microsomes in Comparison with In Vitro–Expressed D2 and D1

Assay Conditions	Sample	Control	+PTU (1 mM)	+BrAcT3 (0.7 pmol)
		pmol/deiodinated per mg protein	*% control*	*% control*
Type 2 Deiodinase	Graves'-2	1.8	79	88
(2 nM T4,	Thyroid-TSH	0.15	75	71
20 mM DTT)	D2*	35	80	55
	Mean (SE)		78 (1.5)	71 (9.5)
Type 1 deiodinase	Graves'-2	7300	0.3	6.6
(1 μM rT3,	Thyroid-TSH	240	2.6	21
10 mM DTT)	D1*	5000	1.4	2.6
	Mean (SE)		1.4 (0.6)	10 (4.5)

*D2 and D1 are sonicates from HEK-293 cells transfected with D10 expression vector containing cDNAs encoding human D2 or rat D1. HEK-293 cells transfected with vector alone expressed neither D1 nor D2 activity. Incubations were for 1 h except for the D2 assay of Thyroid-TSH microsomes which was 2 h.

ference in D2 mRNA expression (Fig. 1 *A*). The V_{max} for the Graves-2 thyroid of 0.11 pmol/mg protein per min is ~ 20-fold that of highly stimulated rat BAT (27) and that in the TSH-stimulated tissue is comparable to that previously found in BAT from chronically cold-stimulated rats or in human placenta, keratinocytes, or skeletal muscle (12, 13, 15).

Methodological approaches to differentiate T4 to T3 conversion by D1 and D2 in human thyroids. Previous studies have demonstrated that T4 to T3 conversion in thyroid from humans or other species, or in FRTL-5 rat thyroid cells, is inhibited by PTU arguing that this is catalyzed by D1 (28). However, all studies of T4 to T3 conversion in human thyroid reported to date have used T4 substrate concentrations of 0.3–10 μM, appropriate for hD1 (K_m T4 ~ 2.0 μM) (22), but 100–1,000-fold higher than for D2 (K_m T4 < 10 nM) (12). Nonetheless, even though the K_m of D1 for T4 is 200–1,000-fold higher than for D2, the D1 content of thyroid tissue could be sufficiently high that it could catalyze some T4 to T3 conversion even at low T4 concentrations. A critical factor determining which pathway catalyzes T3 production from T4 in vivo is the concentration of intracellular-free T4 in the thyroid cell. Free T4 in human plasma is ~ 20 pmol/liter and can rise 5–10-fold during extreme hyperthyroidism (29). There are no data regarding the intracellular T4 concentrations in thyroid cells. This is because it is not known whether the T4 released during thyroglobulin (Tg) hydrolysis remains compartmentalized in the phagolysosome until it is released from the cell or enters the cytosolic compartment.

To assess the potential physiological role of human thyroidal D2 in T4 to T3 conversion, we explored various approaches for differentiating between D1 and D2 catalyzed pathways. While it would seem that PTU could be used to resolve this issue directly, the conditions required for demonstration of D2-catalyzed T4 to T3 conversion (low substrate, high DTT) make D1 catalyzed T4 to T3 conversion less sensitive to PTU than is the case when high rT3 concentrations are used (30, 31). We, therefore, wished to complement the studies using PTU with independent methods to differentiate between T4 5′ deiodination by D2 and D1.

D1 and D2 activities are differentially susceptible to inactivation by BrAcT3. In contrast to results with overexpressed D1 and D3 (18, 32), we are unable to specifically label the transiently expressed hD2 enzyme with BrAc[^{125}I] T3 or T4 (12). Therefore, we hypothesized that BrAcT3 might be used to differentially inactivate D1(33), but not D2, activity in thyroid microsomes. To test this hypothesis, we incubated increasing concentrations of BrAcT3 for 10 min with sonicates of HEK-293 cells transiently expressing D1 or D2 before assay of deiodinase activity. The susceptibility of D2 to inactivation by BrAcT3 is ~ 1,000-fold less than that of D1 (Fig. 3). D1 activity is inhibited > 80% by 0.07 pmol BrAcT3, whereas > 30 pmol are required to obtain similar inhibition of D2. Thus the two selenoenzymes differ markedly in their sensitivity to this reagent suggesting that, consistent with the failure of BrAcT3 to interact specifically with D2 (12), this enzyme is relatively insensitive to inactivation by BrAcT3. Thus, exposure to appropriate amounts of BrAcT3 is an independent technique for selectively inactivating D1.

Effects of PTU and BrAcT3 on 5′ deiodinase activities in microsomes from human thyroid. Thyroid microsomes or sonicates of HEK-293 cells were incubated with either 2 nM T4 or 1 μM rT3 under either D2 or D1 conditions in the presence or absence of PTU or after 10 min of exposure to 0.7 pmol BrAcT3. PTU inhibited low K_m T4 deiodination by only 20–25% in both thyroid and D2 preparations while blocking high K_m rT3 deiodination by > 97% in all three specimens, consistent with its expected effects. Furthermore, while BrAcT3 inactivated D1 by 79–97% in both thyroid and D1-expressing HEK-293 cell sonicates, it caused a maximum inhibition of 29% of low K_m T4 to T3 conversion by the D2 pathway in thyroid microsomes. Thus, these two independent approaches indicate that even at T4 concentrations 100-fold higher than the free T4 concentration in euthyroid plasma, the bulk of T4 to T3 conversion by stimulated human thyroid microsomes is catalyzed by D2.

Use of differential saturation of D2 by T4 to compare 5′ deiodinase activities in normal and stimulated human thyroid. Since the K_m of hD2 is ~ 1,000-fold lower than hD1 (2 nM vs 2 μM), an alternate method for analyzing the relative roles of

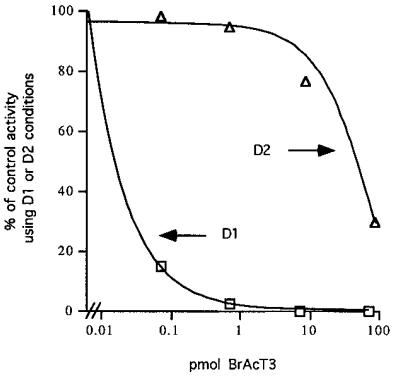

Figure 3. Effect of exposure to BrAcT3 on 5′ deiodination of rT3 by D1 and D2. Sonicates (5 μg protein) from HEK-293 cells transfected with either rat D1 or human D2 cDNA in D10 expression vector were incubated for 10 min at room temperature with 0, 0.07, 0.7, 7, and 70 pmol BrAcT3. After the incubation, deiodination assays were performed using rT3 under D1 (1 μM rT3, 10mM DTT) or D2 (0.3 pmol rT3, 1 mM PTU, 20 mM DTT) conditions. Results are the percentage of the activity found in the absence of BrAcT3 (*control*). The scale of the abscissa is logarithmic. This plot is representative of three similar experiments.

Table III. Comparison of 5′ Deiodinase Activity in Microsomes from Normal and Stimulated Human Thyroids

Thyroid sample	Fraction of 5′ deiodination by D2	D2 activity at 10 nM T4
	%	*fmol/min per mg protein*
1 Normal	23	0.48
2 Normal	35	0.43
3 Normal	22	0.63
4 Thyroid-TSH	58	3.5
5 Graves-2	80	80

All assays were performed using 20 mM DTT with 30–300 μg of microsomal protein for 2 h. Normal thyroids were obtained at surgery (1) or after death (2–3).

D2 and D1 in T4 5′ deiodination is to compare tracer $^{125}I^-$ production by thyroid microsomes at low (2 nM) and high (100 nM) T4 concentrations in the presence of the identical concentrations of tracer T4. We tested this approach with transiently expressed D2 and D1 enzymes. Tracer I^- released from 2 nM T4 during D2 catalyzed reactions was suppressed to background levels at 100 nM T4 but $^{125}I^-$ release by D1 was unaffected. Therefore, the fraction of I^- production by D2 at 2 nM T4 is the difference between tracer iodide production at 2 and at 100 nM T4 and that by D1 is the difference between tracer iodide released at 100 nM and the assay blank. Using this approach, 27% (22–35) of 2 nM T4 was metabolized by D2 in normal thyroid, whereas, D2 is the source of 60–80% of T3 in the stimulated thyroids (Table III).

Quantitation of D2 activity in thyroid microsomes can also be obtained using this approach. An approximation of the V_{max} of D2 can be made by measuring $^{125}I^-$ production at 10 nM T4 reflecting contributions of both D2 and D1 and then subtracting from this $^{125}I^-$ production at 100 nM T4, a measure of D1 activity. D2 activity in normal human thyroid was 0.48 to 0.63 fmol /mg of protein whereas that in the stimulated thyroids was ~ 5 and 50 times higher. While this approach underestimates the V_{max} of the stimulated thyroids by approximately twofold (compare results for the stimulated thyroids in Tables I and III), the underestimation is roughly proportional since D2 activity Graves-2 thyroid is 10–20-fold that with thyroid-TSH by both methods. Interestingly, the D2 mRNA levels in these three normal thyroids were only 1/4 those of the Graves-2, similar to results shown in Fig. 1 *C* (data not shown). Thus there is a large discrepancy between the ~ 100-fold higher D2 activity and the only ~ 4 higher mRNA level in the Graves'-2 versus normal thyroid.

Discussion

The present results are the first demonstration of D2 mRNA and activity in human thyroid tissue. Not only is the D2 mRNA expressed in human thyroid, but, even in normal tissue it is 50–100-fold higher than present in any other normal human tissue examined (12). Since the human D2 mRNA has only recently been identified, studies of D2 mRNA expression were not possible previously. However, it is surprising that D2 activity in the stimulated thyroid has been overlooked. In fact, there are many reports demonstrating T4 to T3 conversion in thyroids of various species, but, in general, this has been evaluated using high T4 or rT3 concentrations which obscure contributions of a low K_m pathway to T3 production. In one report using FRTL-5 rat thyroid cells, no evidence of PTU-insensitive T4 to T3 conversion was found using 1 nM T4 as substrate even though D1 activity was present (34). This is consistent with the absence of D2 in FRTL-5 cell mRNA (Fig. 1 *C*). In an extensive series of studies, Laurberg and colleagues identified PTU-sensitive T4 to T3 conversion in perfused dog thyroid (reviewed in reference 28). Since the perfusate was not supplemented with T4, this presumably reflects endogenous D1-catalyzed T4 to T3 conversion. Interestingly however, when dog thyroids were perfused with TSH, there was a transient increase in the T3 to T4 ratio in the thyroid effluent that was not blocked by PTU pretreatment (28). The authors speculated that this was due to a more rapid release of T3 than T4 during the Tg hydrolysis induced by TSH but an acute TSH-induced increase in D2 activity could also explain this phenomenon.

The presence of high levels of D2 mRNA and activity in stimulated human thyroid tissue is unexpected for several reasons. First, D2 activity is reduced by substrate in pituitary and cerebral cortex by an as yet poorly defined posttranslational mechanism (35). This process has been thought to provide a mechanism by which certain critical tissues (brain, BAT, and somatotrophs) can maintain intracellular T3 levels constant when plasma T4 concentrations fall, as in iodine deficiency (2). One would have assumed that, especially in the hyperactive thyroid, D2 activity would be low even if D2 mRNA were highly expressed if the D2 enzyme were exposed to the high levels of T4 transiting the thyroid cell. The results in Table I and Fig. 2 suggest that this does not occur or that the antithyroid drugs given before surgery reduced the intrathyroidal T4 to levels ineffective in the down-regulation of D2.

Second, if T4 down-regulates the D2 enzyme at the posttranslational level, it would be logical to expect negative regulation of the *dio* 2 gene by T3 at the transcriptional level. We have observed such negative regulation of D2 mRNA in vivo in the rat central nervous system (data not shown). To the extent that circulating or intrathyroidal T3 has access to nuclear T3 receptors in the thyroid cell, one would have expected D2 mRNA to be reduced in those very conditions in which the D2 mRNA is high, Graves' disease and a toxic adenoma. This suggests potent intracellular messengers are stimulating D2 transcription or prolonging D2 mRNA half-life overcoming the negative effect of high circulating T3.

What could such messengers be? Previous studies in rat brown adipocytes have shown that D2 mRNA transcription is induced by a combination of α1 and β adrenergic agonists (36–38). Both protein kinase A and C pathways are stimulated by TSH and under certain circumstances by thyroid stimulating antibodies in Graves' disease (39). While this can explain the high D2 mRNA in the Graves' patients (and in the toxic adenoma which presumably contains an activating mutation in the TSH receptor) it does not explain why the D2 mRNA levels are 10-fold lower in the patient with TSH-stimulated hyperthyroidism. Furthermore, since both T3 and TSH stimulate D1 transcription (reviewed in reference 40), it is surprising that there is only modest parallelism between the expression of D1 and D2 mRNAs in the various thyroids (Fig. 1). Clearly human thyroidal D2 mRNA expression is strongly influenced by as yet unidentified factors.

What is the physiological or pathophysiological significance of the high levels of D2 in the human thyroid? It seems likely that D2 contributes to the relative hypersecretion of T3 in the Graves' patient. This has been documented in many studies and has been attributed to both the increased T3/T4 ratio in Graves' Tg (16) and to high D1 levels in the toxic gland (28, 41, 42). However, the results in Tables II and III indicate that at T4 concentrations up to 2 nM, at least 10-fold higher than the circulating-free T4 concentrations in the most thyrotoxic patients, $>$ 60% of T4 to T3 conversion is catalyzed by D2 in stimulated thyroids, but only $\sim$ 30% in normal glands. Thyroidal T3 production from T4 is minimally inhibited by PTU and BrAcT3 (Table I), agents which are very effective at blocking D1 activity. These results indicate an increasingly important role for D2 in thyroidal T3 production as the human thyroid cell becomes more stimulated. However, the relative role of D2 will be greater the lower the intracellular thyroidal T4 concentration. The absence of knowledge in this area makes a precise estimate difficult.

D2 mRNA has not been demonstrated previously in human pituitary tumors though D2 activity is present (10). This indicates that D2 is expressed in the glycoprotein-producing pituitary cells especially in those expressing TSH.

The availability of the D2 mRNA has thus revealed a number of unexpected results which must lead to a reassessment of our current views of human thyroid physiology. The presence of D2 in human thyroid and its potentially important role as a source of circulating T3, especially in hyperactive glands, as well as the presence of significant quantities of D2 in skeletal and cardiac muscle (12) all point to the need for an open mind as we reassess the nature of the enzymatic pathways by which human tissues activate the prohormone T4 both within and outside of the thyroid gland.

Acknowledgments

We thank Professor G.F. Fenzi and A. Fusco and Drs. Peter Black, Rona Carroll, Francis Moore, Jr., and Ms. Kathleen Dashner for providing us with human tissues or mRNA samples.

This work was supported by grants from the National Institutes of Health (DK 36256 and DK 07529).

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