

Type 2 Iodothyronine Deiodinase in Rat Pituitary Tumor Cells Is Inactivated in Proteasomes

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Abstract

The goal of these studies was to define the rate-limiting steps in the inactivation of type 2 iodothyronine deiodinase (D2). We examined the effects of ATP depletion, a lysosomal protease inhibitor, and an inhibitor of actin polymerization on D2 activity in the presence or absence of cycloheximide or 3,3',5'-triiodothyronine (reverse T3, rT3) in rat pituitary tumor cells (GH4C1). We also analyzed the effects of the proteasomal proteolysis inhibitor carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132). The half-life of D2 activity in hypothyroid cells was 47 min after cycloheximide and 60 min with rT3 (3 nM). rT3 and cycloheximide were additive, reducing D2 half-life to 20 min. D2 degradation was partially inhibited by ATP depletion, but not by cytochalasin B or chloroquine. Incubation with MG132 alone increased D2 activity by 30–40% for several hours, and completely blocked the cycloheximide- or rT3-induced decrease in D2 activity. These results suggest that D2 is inactivated by proteasomal uptake and that substrate reduces D2 activity by accelerating degradation through this pathway. This is the first demonstration of a critical role for proteasomes in the post-translational regulation of D2 activity. (*J. Clin. Invest.* 1998. 102:1895–1899.) Key words: selenocysteine • thyroid hormone metabolism • reverse T3 • MG132 • selenium

Introduction

Thyroid hormone metabolism plays an essential role in determining the intracellular levels of bioactive 3,5,3'-triiodothyronine (T3)¹ in the brain and pituitary. More than 80% of the nuclear T3 in the brain and ~ 50% of that in rat pituitary is

derived from intracellular thyroxine (T4) to T3 conversion, rather than from circulating T3 (1). The critical enzyme in this pathway is type 2 iodothyronine (D2) deiodinase (2, 3). In rats, D2 activity shows an inverse correlation with serum T4 concentration (4). Cycloheximide and actinomycin D fail to prevent the reduction of D2 in cerebral cortex and pituitary induced by supraphysiological doses of T3 (5). Furthermore, the most potent iodothyronines in decreasing D2 activity are the D2 substrates, T4 and the metabolically inactive 3,3',5'-triiodothyronine (reverse T3 or rT3), indicating that this effect does not require thyroid hormone receptors (6–8). There is also a marked increase in D2 mRNA (9–12) in hypothyroidism, indicating that iodothyronines regulate D2 activity through both pre- and posttranslational mechanisms.

The mechanism for inactivation of D2 has been studied in both rat pituitary tumor cells and primary cultures of rat glial cells. In hypothyroid GH3 cells, the half-life of D2 activity is 50 min in the presence of cycloheximide and the addition of rT3 further decreases the D2 half-life to 26 min (8). Other D2 substrates (T4 and iopanoic acid) have similar effects. Primary cultures of rat glial cells grown in the absence of thyroid hormone have two- to fivefold higher levels of D2 activity than cells grown in the presence of normal serum (13). The addition of cycloheximide or rT3 results in a rapid decrease in D2 activity in cAMP-stimulated glial cells (14, 15). ATP depletion with carbonylcyanide m-chlorophenylhydrazone (CCCP) blocked the rate of decrease in D2 activity with cycloheximide (15).

Eukaryotic cells contain multiple proteolytic systems, including the ATP-ubiquitin-proteasome-dependent pathway, lysosomal proteases, and calpains (reviewed in 16). The fact that ATP depletion blocked the decrease in D2 activity induced by cycloheximide in earlier studies suggested to us that an ATP-dependent step, such as conjugation with ubiquitin and proteasome uptake, might be rate-limiting in D2 degradation. The goal of the present study was to determine if the recently developed proteasome uptake inhibitor carbo-benzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) would inhibit D2 inactivation in GH4C1 cells (16, 17). We found that MG132 indeed completely blocks the acute cycloheximide or rT3-induced decrease in D2. This is the first evidence that D2 degradation occurs in the proteasome and that a D2 substrate accelerates proteasomal D2 uptake.

Methods

Reagents. MG132 was obtained from the Peptide Institute, Inc. (Osaka, Japan) and dissolved in DMSO. CCCP, chloroquine, and cytochalasin B were from Sigma Chemical Co. (St. Louis, MO). Cycloheximide and rT3 were from Calbiochem (La Jolla, CA). CCCP was dissolved in 100% ethanol and cytochalasin B and cycloheximide in DMSO. rT3 was dissolved in 70% ethanol. Outer ring-labeled [¹²⁵I]-

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1. Abbreviations used in this paper: CCCP, carbonylcyanide m-chlorophenylhydrazone; D2, type 2 iodothyronine deiodinase; MG132, carbo-benzoxy-L-leucyl-L-leucyl-L-leucinal; PTU, 6n-propylthiouracil; reverse T3 (rT3), 3,3',5'-triiodothyronine; T3, 3,5,3'-triiodothyronine; T4, thyroxine.

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T4 (specific activity: 4400 Ci/mmol) was from New England Nuclear Corp. (Boston, MA). All other reagents were of analytical grade.

Culture conditions. Rat pituitary tumor cells (GH4C1) were grown in Ham's F10 supplemented with 10% FBS for 2–4 wk until 50% confluence. They were switched to Ham's F10 supplemented with 10% FBS, which had been depleted of thyroid hormones with charcoal 24 h before each experiment to deplete thyroid hormone from the cells. After 6 h, the medium was replaced with DME supplemented with 0.1% BSA. We determined that exposure of GH4C1 cells to charcoal-stripped medium for 6 h (vs. longer periods of time) as well as to BSA for 18 h results in stable D2 activity over the time of the subsequent experiments. Each experiment was performed with triplicate dishes for each control group and each experimental condition. At each time point, cells were harvested and D2 activity measured. The final concentrations of DMSO and ethanol used for the various reagents were 0.2% and 0.1%, respectively. These were included in control incubations as appropriate.

Type 2 deiodinase assays. Cells were harvested, washed, sonicated briefly in 0.1 M potassium phosphate-1 mM EDTA, pH 6.9 (PE buffer) containing 10 mM DTT and 0.25 M sucrose. Cell homogenates were then assayed for deiodination of 2 nM [¹²⁵I]-T4 (DuPont-NEN, Boston, MA) under varying conditions as described previously but without 6*n*-propylthiouracil (PTU), since GH4C1 cells do not express type 1 iodothyronine deiodinase (D1) (18, 19). The freshly purified [¹²⁵I]-T4 contained < 0.5% iodide. Deiodination was linear with both the time and protein concentrations under the conditions of the assay, and the quantities of protein assayed consumed < 30% of the substrate. Protein determinations were by Bradford using BSA as standard (20). D2 activity is reported as femtomoles of T4 deiodinated per milligram of protein per minute and is corrected for random deiodination.

Expression of ⁷⁵Se-labeled D2. ⁷⁵Se-labeled human D2 was transiently expressed in HEK-293 cells as previously described (21). In brief, paired plates incubated in 10% charcoal-stripped FBS were transfected with a D10 eukaryotic expression vector containing KD2-SELP (K indicates a Kozak consensus sequence that was engineered 5' to the initiator ATG of the human D2 coding region and SELP a selenocysteine insertion sequence from the SELP gene, 21) and then incubated with Na₂[⁷⁵Se]O₃ in 1% FBS for 18 h (21). Plates were then washed and exposed to vehicle (DMSO, 0.5%), cycloheximide (100 μM), or rT3 (100 nM) with or without MG132 (50 μM) for 4 h in 0.1% BSA in DME. Cells were harvested, sonicated, and 200 μg of protein electrophoresed and the SDS gel autoradiographed. The densities of the 31-kD D2 protein band (21) were compared by densitometry.

Statistical analysis. Results are expressed as mean ± SE of the triplicate plates assayed at each time point for each condition. Because there were variations in basal D2 activities among various groups of cells (from 2.8 ± 0.2 to 11.8 ± 0.8 fmol of T4 deiodinated/mg/min) used for these experiments, we normalized results of a given perturbation to the mean control values for that day's experiment cells (see Table I). We could then determine the variance of the triplicate controls and experimental values and pool the normalized values to assess the statistical significance of a given treatment using different groups of cells. Student's *t* test was used for single comparisons of values between different treatments and Dunnett's test for multiple comparisons against a single control group. *P* < 0.05 was considered significant.

Results

Cycloheximide or rT3 cause rapid decreases in D2 activity. Cycloheximide (100 μM) was added to examine the half-life of D2 activity which decreased rapidly but plateaued at ~ 25% of basal activity after 2–3 h (Fig. 1). Taking this residual activity into account, the *t*_{1/2} was 47 min. Higher concentrations of cycloheximide (500 μM) had no greater effect. Preliminary experiments with rT3 at concentrations of 0.03–30 nM showed a

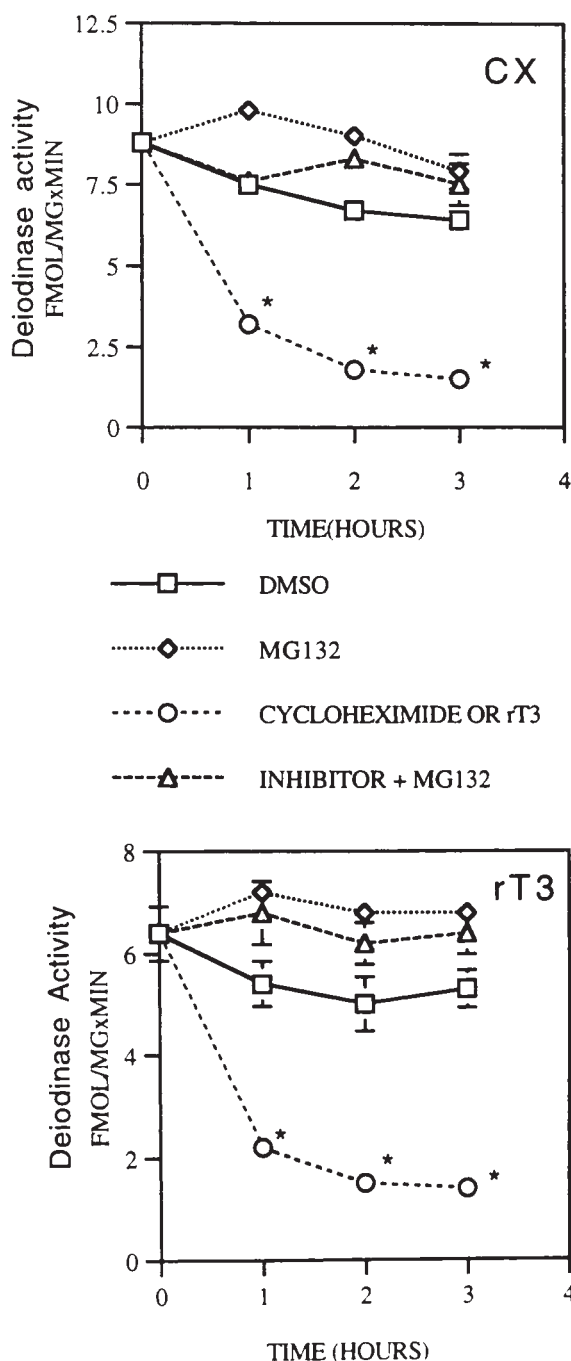


Figure 1. Effects of MG132 on cycloheximide (CX) or reverse T3 (rT3)-induced changes on D2 activity in GH4C1 cells. MG132 (10 μM) was added 10 min before cycloheximide (100 μM). rT3 (3 nM) was introduced simultaneously with cycloheximide. Values are the mean ± SE of two different experiments, each performed in triplicate. SE was too small to depict where SE bars are not shown. **P* < 0.05 vs. MG132 + cycloheximide (top panel) or MG132 + rT3 (bottom panel), at each time point (*t* test).

concentration and time-dependent decrease in D2 activity which was maximal at 3 nM in 0.1% BSA. This concentration of rT3 also caused a rapid decrease in D2 activity which again plateaued between 2 and 3 h at ~ 26% of control (Fig. 1). Taking this residual activity into account, the apparent half-life of D2 activity during rT3 treatment was ~ 60 min.

Table I. Effects of CCCP, Chloroquine, or Cytochalasin B on D2 Activity in GH4C1 Cells Treated with Cycloheximide or rT3

Inhibitor	Cycloheximide		rT3	
	D2 activity	% control	D2 activity	% control
Control	5.2±0.30	100±5.7	4.6±0.12	100±2.6
Inhibitor	2.1±0.23	40±2.9	1.9±0.13	42±2.2
CCCP	4.2±0.29*	100±6.9	3.1±0.35*	100±11.3
CCCP + inhibitor	2.5±0.17	60±5.7 [‡]	1.4±0.12	47±3.2 [‡]
Chloroquine	3.6±0.4*	100±11.1	3.6±0.40 [‡]	100±11.1
Chloroquine + inhibitor	1.6±0.29	43±3.4 [‡]	1.7±0.23	49±1.8 [‡]
Cytochalasin B	5.0±0.10 [‡]	100±1.2	ND	
Cytochalasin B + inhibitor	2.0±0.20	42±2.9 [‡]	ND	

Cells were exposed for 1 h to CCCP (10 μ M), chloroquine (100 μ M), cytochalasin B (15 μ M), or vehicle (controls, 0.1% ethanol) with or without cycloheximide (100 μ M) or rT3 (3 nM). Values are mean±SE of $n = 3-7$ different experiments, each in triplicate. Percentage values in each group are expressed relative to the respective control. D2 activity is expressed in femtomoles of T4 deiodinated per milligrams of protein per minute. * $P < 0.05$, for individual inhibitors vs. the respective control (t test). [‡]NS, vs. the respective control (t test). [‡] $P < 0.05$, for inhibitor + CCCP/CCCP or inhibitor + chloroquine/chloroquine vs. inhibitor/control (Dunnett's). [‡]NS, vs. inhibitor alone (Dunnett's). [‡]NS, for inhibitor + CCCP/CCCP or inhibitor + chloroquine/chloroquine vs. inhibitor/control (Dunnett's).

Effects of CCCP, chloroquine, or cytochalasin B on cycloheximide- or rT3-induced reductions in D2 activity. We used 1-h time periods to examine the effects of several agents which could affect the loss of D2 during these treatments. ATP depletion by CCCP alone caused modest decreases in D2 activity (Table I; CCCP, 4.2/5.2 = 79% of control, or 3.1/4.6 = 67% of control, $P < 0.05$). Chloroquine alone also decreased D2 activity, but cytochalasin B alone had no effect (Table I). The ratios of D2 activities between CCCP, chloroquine, or cytochalasin B alone and the test compound plus cycloheximide or rT3 were determined, expressed as a percentage, and compared with the effects of cycloheximide or rT3 alone (Table I). Cycloheximide causes a 60% decrease in D2 activity over 1 h, and this is partially blocked by coincubation with CCCP, but is not affected by chloroquine or cytochalasin B. However, neither CCCP nor chloroquine significantly affected the 58% decrease in D2 activity observed with rT3 (Table I).

MG132 blocks the loss of D2 activity induced by cycloheximide or rT3. The effect of CCCP to block the degradation of D2 suggested the possible involvement of the ATP-dependent ubiquitinylation/proteasome pathway in D2 degradation in GH4C1 cells. Therefore, we examined the effect of the proteasomal pathway inhibitor MG132 on D2 activity. MG132 (10 μ M) increased D2 activity 40, 36, and 29%, respectively, during 1, 2, or 3 h of treatment, suggesting that it caused at least partial inhibition of D2 degradation (Table II). MG132 had striking effects when combined with cycloheximide and rT3 (Fig. 1). It completely blocked the decrease in D2 activity after inhibition of protein synthesis and/or that during rT3 exposure. The effects of MG132 were reversible; when it was removed from the medium after blocking the rT3-induced de-

Table II. Concentration and Time Dependence of MG132 Effects on D2 Activity in GH4C1 Cells

Time (h)	Condition	n	D2 activity (fmol of T4 deiodinated/mg/min)	% of control
1	Control	6	7.8±1.22	100±15.6
	MG132 (3 μ M)	3	7.1±1.10	132±10.0*
	MG132 (10 μ M)	4	9.5±1.75	140±6.6*
2	Control	11	7.5±0.69	100±9.2
	MG132 (3 μ M)	5	9.0±1.65	135±4.7*
3	MG132 (10 μ M)	7	10.2±0.87*	136±5.0*
	Control	4	6.9±1.50	100±21.7
	MG132 (3 μ M)	2	10, 4.5	151, 162
	MG132 (10 μ M)	3	8.7±2.43	129±4.3*

Cells were exposed to the indicated concentrations of MG132 or 0.1% DMSO (control) for 1, 2, or 3 h. Values are expressed as the mean±SE or as percentage of control for 3–11 different experiments, except MG132, 3 μ M for 3 h for which $n = 2$ ($n =$ number of different experiments, each performed in triplicate). Control means at each time point include the pooled controls for both concentrations of MG132 (3 μ M and 10 μ M). The percentage values are the means of the activities expressed relative to the respective control for each individual experiment. * $P < 0.05$, vs. the respective control (Dunnett's test).

crease in D2 activity for 2 h, reintroduction of rT3 again decreased D2 activity (data not shown).

We also assessed the effects of MG132 on the combination of cycloheximide and rT3. D2 activity was reduced to 8% of control over 1 h in the presence of both inhibitors, which is the predicted level if these two agents act independently (Fig. 2). MG132 completely blocked the 92% decrease in D2 activity observed in the presence of both inhibitors, indicating again

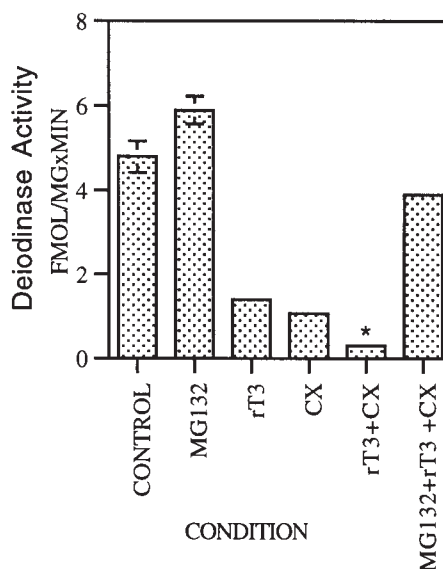


Figure 2. Effects of rT3 and cycloheximide in the presence or absence of MG132 on D2 activity in GH4C1 cells. MG132 (10 μ M) and/or rT3 (3 nM) were introduced into the culture medium 10 min before cycloheximide (100 μ M). D2 activity was measured after 1 h of treatment with the indicated agents. Bars depict the mean±SE of three different experiments, each performed in triplicate. SE was too small to depict where bars are not shown. * $P < 0.05$ vs. rT3 or cycloheximide alone (t test).

that proteasomal proteolysis is a rate-limiting step in both basal and induced D2 degradation.

MG132 blocks the degradation of transiently expressed ⁷⁵Se-labeled D2 in HEK-293 cells. To prove that MG132 blocks the degradation of D2, as opposed to having other effects to increase D2 activity, we transiently expressed human (h) D2 in the presence of Na₂[⁷⁵Se]O₃ in HEK-293 cells (21). Preliminary studies showed that the half-life of D2 activity in such cells is 2–3 h with cycloheximide and that 50 μM MG132 blocks its loss. Addition of cycloheximide caused a reduction of 35% in the ~ 31 kD ⁷⁵Se-labeled D2 band over 4 h. When cells were treated with a combination of MG132 and cycloheximide, the density of ⁷⁵Se-hD2 was fivefold higher than in cells exposed to cycloheximide alone. Preliminary experiments showed that the decrease in labeled D2 caused by 100 nM rT3 is also blocked by MG132.

Discussion

The short half-life of D2 in GH4C1 cells, about 47 min in T4-deprived cells, is similar to that reported earlier in hypothyroid GH3 cells (8). The reason(s) for the plateau of activity at ~ 25% of control between 2 and 3 h is not clear but could be explained by cycloheximide inhibition of cellular protein degradation processes. The pathway for the rapid inactivation and/or degradation of D2 has not been previously defined. Earlier studies of enzyme activity changes in astroglial cells showed that ATP depletion reduced the rate of loss of D2 activity in cycloheximide-treated cells (15). Our results are similar in that CCCP reduced the rate of D2 activity loss and, as with glial cells, chloroquine had no effect (Table I). The ATP dependence and lack of effect of chloroquine suggest the possible involvement of the 26S proteasome, as opposed to lysosomal proteases, in D2 degradation (16, 22, 23).

The ATP and ubiquitin-dependent pathway is important in intracellular proteolysis (reviewed in (16, 22)). A characteristic of proteolysis by this mechanism is its inhibition by one of several peptide aldehydes, such as MG132, which are substrate analogs (17, 24, 25). This, or other proteasome inhibitors, such as lactacystin, inhibit the degradation or processing of a number of proteins including the cystic fibrosis transmembrane conductance regulator, the MHC class 1 antigens, and NF-κB (reviewed in (16)). MG132 has reversible effects, as we observed, and has minimal toxicity. The stable levels of D2 in the presence of cycloheximide and MG132 indicates a complete inhibition of D2 degradation by this agent. The similar effects on ⁷⁵Se-labeled D2 in HEK-293 cells confirms that MG132 preserves the D2 protein per se. While incubation of GH4C1 cells with MG132 causes an increase of 25–30% in D2 activity (Figs. 1 and 2 and Table II), this increase is not progressive as would be predicted from its total blockade of D2 degradation. Possible explanations for this include the presence of other pathways for D2 degradation, which are activated when proteasomes are blocked or a modest inhibition of D2 synthesis by MG132, which we have observed in preliminary studies during an 18-h exposure.

Hypothyroxinemic conditions were used to allow detection of the effect of rT3 on D2 inactivation. Its posttranslational effects to decrease D2 activity have been demonstrated in many systems (6–8, 26, 27). The fact that it is more potent than T3 in accelerating D2 degradation indicates that its effects are not mediated through thyroid hormone receptors. Nevertheless,

supraphysiologic quantities of T3 can also act by the same mechanism in addition to suppressing D2 mRNA synthesis (5, 6, 11, 26, 28). rT3 causes the expected rapid decrease in D2 activity in GH4C1 cells with an apparent D2 half-life about the same as that during cycloheximide treatment (Table I, Fig. 1). However, the effects of rT3 and cycloheximide are additive (Fig. 2), which is in agreement with results in GH3 cells (8). Thus, the effect of rT3 is to accelerate the degradation of D2, not to block its synthesis. The effect of rT3 is inhibited by 10 μM MG132 (Fig. 1), alone or in combination with cycloheximide (Fig. 2). Thus, both normal D2 proteolysis and the accelerated degradation induced by D2 substrate occur through proteasomal uptake. The results indicate that persistence of a covalently linked substrate–D2 complex does not explain the loss of D2 activity induced by rT3. Rather, interaction with substrate appears to target the enzyme for more rapid proteolytic destruction. The degradation of D2 in GH3 cells is accelerated by diamide, which depletes reduced glutathione and is slowed by incubation of cells with DTT (8). Thus, oxidation of the putative selenoyl residue by interaction with substrate could be the signal for accelerated degradation of this enzyme.

These results have important implications for the physiological regulation of T4 activation. The short half-life of D2 in euthyroid states points to a requirement for its continued synthesis to maintain normal activity. In rats, D2 activity is increased threefold in the cerebral cortex within 1 d of thyroidectomy and is maximally elevated (2.5-fold) in GH4C1 cells within 3 h of thyroid hormone withdrawal (10, 29). Such rapid changes in D2 activity could well occur due to a decrease in the rate of D2 degradation. This would provide a rapidly responsive homeostatic mechanism to enhance the efficiency of T4 to T3 conversion when T4 production is reduced, such as in iodine deficiency. A pathophysiological stimulus that either impairs D2 gene transcription or inhibits D2 synthesis would be followed by a rapid fall in D2 activity due to its short half-life. If D2 is a significant source of circulating T3 in euthyroid humans, as seems likely, given its wide expression and the insensitivity of peripheral T4 to T3 conversion to PTU, then inhibition of D2 synthesis by stress could easily lead to the rapid decrease in serum T3 concentration associated with acute illness (11, 21, 30).

Acknowledgments

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