Regulation of Type II Iodothyronine 5'-Deiodinase by Thyroid Hormone

INHIBITION OF ACTIN POLYMERIZATION BLOCKS ENZYME INACTIVATION IN CAMP-STIMULATED GLIAL CELLS

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The cellular events mediating the rapid, thyroid hormone-dependent modulation of membrane-bound, type II iodothyronine 5'-deiodinase were studied in dibutyryl cyclic AMP (dBcAMP)-treated brain astrocytes. Unstimulated cells had undetectable type II 5'-deiodinating activity. Treating the cells with dBcAMP and hydrocortisone induced enzyme expression by increasing transcription of the enzyme or an essential enzyme related protein(s), with steady-state levels of type II 5'-deiodinase attained after 8 h of dBcAMP treatment. Glial cells grown in the absence of thyroid hormone had 10-15-fold higher levels of 5'-deiodinating activity than cells grown in the presence of serum. The increased type II 5'-deiodinating activity observed in serum-free cultures was due to a prolonged enzyme half-life with no change in the rate of enzyme synthesis. Addition of thyroxine or 3,3',5'-triiodothyronine to the serum-free culture medium resulted in a concentration-dependent fall in steady-state enzyme levels, with ECso values of ~0.4 nM. 3,3',5'-Triiodothyronine was at least 100-fold less effective. Chloroquine, NH4Cl, tunicamycin, colchicine, and monodansylcadaverine had no effect on the Ts of the enzyme, while both carbonyl cyanide m-chlorophenylhydrazone and cytochalasin blocked the inactivation of the type II 5'-deiodinase. These data indicate that in glial cells, an intact actin-cytoskeleton is required for thyroid hormone to modulate the energy-dependent regulation of the half-life of the short-lived, membrane-bound enzyme, type II 5'-deiodinase.

Thyroid hormone metabolism plays a fundamental role in determining the intracellular levels of bio-active 3,5,3'-triiodothyronine (T3) in the brain. Recent studies have shown that >90% of the T3 bound to brain cell nuclei is derived from intracerebral T3 levels. The cellular mechanism for this thyroid hormone response has been difficult to study in intact animals, and cell culture models expressing thyroid hormone metabolizing enzymes such as dispersed fetal (11) or neonatal rat brain (12, 13), and the neuroblastoma cell line NB41A3 (14), sufer from cell heterogeneity and/or low 5'-deiodinase levels. Our demonstration that enzyme levels are more sensitive to T3 than to T4 (7, 10), together with the demonstration that this action of thyroid hormone is not blocked by inhibitors of transcription or translation (8), suggest a novel, extranuclear site of action for thyroid hormone in the brain.

The cellular mechanism for this thyroid hormone response has been difficult to study in intact animals, and cell culture models expressing thyroid hormone metabolizing enzymes such as dispersed fetal (11) or neonatal rat brain (12, 13), and the neuroblastoma cell line NB41A3 (14), suffer from cell heterogeneity and/or low 5'-deiodinase levels. Our demonstration that cAMP induces abundant type II 5'-deiodinase in cultured astrocytes (15) has been exploited to examine the Ts-dependent regulation of this short-lived, membrane-bound enzyme. The data show that Ts rapidly modulate the rate of enzyme inactivation in these cells, whereas T3 is a much less effective hormone. In addition, inactivation and/or degradation of this membrane-bound enzyme was found to be energy dependent and to depend on the structural integrity of the actin-cytoskeleton.

MATERIALS AND METHODS

Dulbecco’s modified Eagle’s medium (DMEM), antibiotics, Hank’s salt solution, glucose, and trypsin were obtained from Gibco. Fetal bovine serum (heat-inactivated) was obtained from Hyclone, Inc. Culture flasks and plasticware were purchased from MA Bioproducts. L-T4 and L-T3 were from Sigma; L-rT3 was from Behring Diagnostica, and 1-3,3'-diiodothyronine was from Henning GmbH. BtscAMP, hydrocortisone, insulin, actinomycin D, progesterland F20, and protresine were purchased from Sigma. Fibroblast growth factor was obtained from Collaborative Research Inc. Na235I (~17 Ci/mg) was purchased from Du Pont-New England Nuclear. L-[3' or 5'-35S]rT3 (~2200 Ci/mmol) was prepared by the method of Weeke and Orskov.
(16) and purified as previously described (17). Radiodinated rT3 was >98% pure with 35S as the major contaminant. Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories. All other reagents were of the highest purity commercially available.

Culture Conditions—Glial cells were prepared from cerebral hemispheres of 1-day-old rats as described by McCarthy and De Vellis (16). Dissociated cells were seeded at 200,000 cells/cm² and grown in a humidified incubator at 37 °C under an atmosphere of 5% CO₂, 95% air. Culture medium was replaced after 3 days and on an alternate day schedule thereafter. Glial cells were subcultured (20–30 × 10⁶ cells/cm²) every 7–10 days; in all experiments, cells were used between the 2nd and 6th passage. “Euthyroid medium” consisted of DMEM containing 10% fetal bovine serum, while “hypothyroid medium” consisted of either serum-free DMEM, chemically defined medium (19) lacking thyroid hormones, or in DMEM containing 5% hypothyroid rat serum or 10% hormone-stripped fetal bovine serum (20); all yielded equivalent result. Cells were grown in “hypothyroid” medium for 24–48 h prior to enzyme induction. As indicated, thyroid hormone replacement was done by adding the iodothyronine to serum-free DMEM containing 1 mg/ml bovine serum albumin 24 h prior to dbcAMP treatment. Free (available) iodothyronine concentrations were determined by equilibrium dialysis.

Growth characteristics during log-phase growth were determined by cell counts after trypanosin (0.25% w/v in Hamaker’s salt solution). Cell counts were done daily until cells reached confluence.

Induction of Type II 5’-Deiodinase Activity—Enzyme activity was induced by treating cells with 1 μM dbcAMP for 10 h as previously described (15). Glial cells grown in hypothyroid medium were stimulated with 1 μM dbcAMP after an initial 24–48-h period in the absence of iodothyronines.

Analytical Procedures—Iodothyronine 5’-deiodinase activity was determined in cell homogenates prepared by sonication in 10 mM Hepes buffer, pH 7.0, 1 mM EDTA, and 10 mM dithiothreitol. Enzyme activity was determined by measuring the release of radioiodide from L-33’-131I[1,2]rT3 or 5’-131I[rT3, 20 mM dithiothreitol, and 1 mM propylthiouracil as previously described (6, 21). Under these experimental conditions, equivalent amounts of iodide and 3,3’-diiodothyronine are produced. Enzymatic activity is reported as femtomoles of iodide released per hour.

Determination of Cell Filamentous Actin Content—Confluent monolayers were washed with ice-cold sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and treated for 5 min with 0.5% Triton in 50 mM Tris buffer, pH 6.8, containing 2 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride (22). Cells were then scraped from the dish and the Triton-insoluble cytoskeleton was collected by centrifugation at 12,000 × g for 10 min at 4 °C. Cytoskeleton proteins were dissolved in polyacrylamide gel electrophoresis sample buffer composed of 50 mM Tris buffer, pH 6.8, 1% (w/v) sodium dodecyl sulfate, 140 mM β-mercaptoethanol, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue, denatured by heating in a boiling water bath for 10 min, and separated by electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gels according to Laemmli (23). Gels were stained with Coomassie Blue (R-250). Filamentous actin (F-actin; M, 43,000 (monomer)) was identified by its electrophoretic mobility and its characteristic insolubility in detergent.

Miscellaneous Methods—Protein was determined by the method of Bradford (24) using human γ-globulin as the protein standard. DNA was measured by the method of Hill and Whatley (25).

RESULTS

Cell monolayers consisted of flat polygonal cells interspersed with occasional clusters of arborized cells. Greater than 95% of the cells contained glial fibrillary acidic protein (data not shown), an intermediate filament characteristic of astrocytes (26). Glial cells, under our culture conditions, showed a doubling time of 25 ± 2 h that was unaffected by 1 mM dbcAMP for up to 48 h.

In agreement with earlier work (15), dbcAMP treatment resulted in the time-dependent appearance of type II 5’-deiodinase activity that plateaued after 8 h of stimulation (Fig. 1). DbcAMP was required to maintain steady-state levels of type II 5’-deiodinase, since removal of the cyclic nucleotide resulted in the progressive loss of the enzyme after a 60-min lag period. In subsequent studies, dbcAMP was present throughout the experimental period.

Effects of Thyroid Hormone on Type II 5’-Deiodinase in DbcAMP-stimulated Glial Cells—The effects of T₄, T₃, and rT₃ on steady-state type II iodothyronine 5’-deiodinase levels are shown in Fig. 2. Cells grown in hypothyroid medium showed 10–15-fold higher steady-state levels of 5’-deiodinating activity than cells grown in euthyroid medium. Addition of increasing amounts of either T₄ or T₃ resulted in the concentration-dependent fall in steady-state enzyme levels with an EDbO of 0.4 nM or ~60 pM “free” hormone (determined by equilibrium dialysis, Fig. 2). T₃ was at least 100-fold less effective in modulating steady-state enzyme levels.

Effects of Individual Growth Factors on DbcAMP Induction of Type II 5’-Deiodinase—To examine whether one or more of the components in the chemically defined medium contributed to the increased expression of type II 5’-deiodinase, components were individually examined for their ability to influence 5’-deiodinating activity. In the absence of dbcAMP, none of the compounds tested induced type II 5’-deiodinase (data not shown). Data in Table I show that fibroblast growth factor, putrescine, and prostaglandin F₂α also had no effect on dbcAMP-induced enzyme activity. In contrast, hydrocortisone increased 5’-deiodinating activity ~2-fold, whereas insulin depressed enzyme levels ~40%.

Hydrocortisone stimulation of type II 5’-deiodinase was concentration dependent (Fig. 3A) with an EDbO of 4 nM. As shown in Fig. 3B, the fractional disappearance of 5’-deiodinating activity in cycloheximide-blocked, dbcAMP-stimulated cells was unchanged by hydrocortisone, suggesting that the glucocorticoid-dependent increase in type II 5’-deiodinase levels was most likely due to increased enzyme production.

The effect of actinomycin D on enzyme induction by dbcAMP is shown in Fig. 4. At the start of the experiment, 1 mM dbcAMP was added to the medium of all cells followed, at 30 min intervals, by addition of 10 μM actinomycin D. Actinomycin D-blocked cells were incubated for an additional 60 min and 5’-deiodinating activity determined. Type II 5’-deiodinase activity was undetectable for the first 45–60 min...
Fig. 2. Effects of T₄, rT₃, and T₃ on steady-state levels of type II 5′-deiodinase (5′-D-II) activity in btCAMP-stimulated astrocytes. Triplicate flasks (25 cm²) of confluent glial cells were grown in serum-free DMEM containing 100 nM hydrocortisone, antibiotics, and supplemented with increasing concentrations of T₄, T₃, or rT₃, dissolved in Hank’s solution containing 1 mg/ml bovine serum albumin, for 24 h prior to 1 mM btCAMP stimulation. Cells were harvested after 16–18 h of stimulation and enzyme activity was determined as described under “Materials and Methods.” Each point represents the mean of triplicate flasks and results were expressed as percentage of the activity in serum-depleted control cultures (2000 units/mg protein). Data are reported as mean ± S.E.

Effects of individual components of the chemically defined medium on expression of type II 5′-deiodinase in btCAMP-stimulated glial cells

Cells (25-cm² flasks) were stimulated for 16 h at 37°C with 1 mM btCAMP in serum-free DMEM containing additives as listed. Cells were harvested and enzyme activity determined as described under “Materials and Methods.” Data are reported as the percent of control enzyme activity (mean ± S.E., n = 3) in cells grown in the presence of 10% serum (304 ± 16 unit/mg protein, X ± S.E., n = 3). Data evaluated by Student’s t test.

<table>
<thead>
<tr>
<th>Individual component added</th>
<th>Type II 5′-deiodinase activity of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. DMEM - 10% fetal bovine serum</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>B. DMEM - serum free</td>
<td>1150 ± 120a</td>
</tr>
<tr>
<td>C. + PGF₂α (500 ng/ml)</td>
<td>980 ± 30</td>
</tr>
<tr>
<td>D. + fibroblast growth factor (100 ng/ml)</td>
<td>1060 ± 45</td>
</tr>
<tr>
<td>E. + putrescine (100 nM)</td>
<td>930 ± 70</td>
</tr>
<tr>
<td>F. + insulin (90 μg/ml)</td>
<td>680 ± 60a</td>
</tr>
<tr>
<td>G. + hydrocortisone (50 nM)</td>
<td>2190 ± 120a</td>
</tr>
</tbody>
</table>

*p < 0.01 B versus A; G versus B.

*p < 0.05 F versus B.

Effects of Thyroid Status on the Turnover of Type II 5′-Deiodinase—The influence of thyroid hormone on the tₙₕ of type II 5′-deiodinase is shown in Fig. 5. Cycloheximide-dependent inhibition of protein synthesis in cells grown in euthyroid medium resulted in the exponential disappearance of type II 5′-deiodinase with a tₙₕ of 20 min, while the enzyme in cells grown in hypothyroid medium showed a tₙₕ of 300 min. Enzyme production rates, calculated from steady-state enzyme levels and the disappearance rate constant (k), were essentially the same in euthyroid and hypothyroid media indicating that thyroid hormone had little or no effect on enzyme synthesis (Table III). The data in Table III also show a modest reduction in btCAMP-inducible type II 5′-deiodinase in cells after prolonged culture (i.e. passage 6). This decrement in enzyme activity was routinely observed and was progressive so that after passage 8, inducible type II 5′-deiodinating activity was only 20% of that observed at passage 2 (data not shown). For this reason, experiments were done on cells between the 2nd and 6th passage.

Effects of Antimetabolites and Cytoskeletal Inhibitors on the Turnover of Type II 5′-Deiodinase—The clearance of many integral membrane proteins is often initiated by internalization, followed by delivery to lysosomes. Since the degradation pathway for type II 5′-deiodinase was unknown, we examined the energy dependence, cell structural requirements, and role of lysosomes in the turnover of this short-lived enzyme. BtCAMP-stimulated cells, at steady state with respect to type II 5′-deiodinase, were exposed to selected inhibitors in the presence or absence of cycloheximide and incubated for 30 min at 37°C. As shown in Table IV, cycloheximide reduced enzyme activity by 78% at 30 min and 91% at 60 min. ATP depletion by addition of 20 μM carbonyl cyanide m-chlorophenylhydrazone, resulted in a 45% decrease in type II 5′-deiodinase, and cycloheximide did not further decrease 5′-deiodinating activity in carbonyl cyanide m-chlorophenylhydrazone-treated cells. Lysosomotropic agents (chloroquine and NH₄Cl), inhibitors of endocytosis (monodansylcadaverine), or glycosidation (tunicamycin), and microtubule disruption (nocodazole) had little or no effect on enzyme levels either in the absence or presence of cycloheximide. On the other hand, colchicine rapidly reversed the btCAMP-stimulated contraction of the glial cell borders, so that within 30 min the morphology of the btCAMP-treated cell was indistinguishable from that of unstimulated controls (data not shown).
T. Modulation of the $t_\alpha$ of Type II 5'-Deiodinase

Fig. 4. Effects of actinomycin D on appearance of type II 5'-deiodinase (5'-D-II) activity in b2cAMP-stimulated glial cells. Confluent monolayers of glial cells were grown in DMEM containing 10% fetal bovine serum and antibiotics. At the start of the experiment, all flasks received b2cAMP (final concentration, 1 mM) and at the times indicated by arrows, triplicate flasks received actinomycin D (final concentration, 10 $\mu$M) and the cultures then incubated for an additional 60 min at 37 °C. Cells in individual flasks were harvested by scraping and type II 5'-deiodinase determined in triplicate as described under "Materials and Methods." Data are reported as the means of closely agreeing (+10%) triplicate cultures. (□) control cultures without actinomycin D; (■) cultures treated with actinomycin D.

Table II
Effects of actinomycin D and hydrocortisone on steady-state levels of type II 5'-deiodinase in b2cAMP-stimulated glial cells

<table>
<thead>
<tr>
<th>Treatment conditions</th>
<th>Time (min)</th>
<th>Type II 5'-deiodinase activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>30</td>
<td>518 ± 25</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>518 ± 25</td>
</tr>
<tr>
<td>100 nM hydrocortisone</td>
<td>30</td>
<td>634 ± 29</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>645 ± 84</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>518 ± 56</td>
</tr>
</tbody>
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Table IV
Depolymerization of the actin-cytoskeleton selectively blocked the loss of type II 5'-deiodinase without altering the synthetic pathway for the integral membrane enzyme.
In this study, we have shown that the btpcAMP-stimulated glial cell is an excellent model system for the characterization of the molecular events regulating the turnover of type II 5'-deiodinase in the central nervous system. Steady-state levels of type II 5'-deiodinase, 50-100 times those observed in the presence of thyroid hormone, similar to the appearance of type II 5'-deiodinating activity stimulated by cyclic nucleotides in cycloheximide-blocked cells. Steady-state levels of type II 5'-deiodinase were induced as described in legend to Fig. 5. Production rate = steady-state enzyme activity (units) × fractional turnover (% enzyme/h).

**DISCUSSION**

In this study, we have shown that the btpcAMP-stimulated glial cell is an excellent model system for the characterization of the molecular events regulating the turnover of type II 5'-deiodinase in the central nervous system. Steady-state levels of type II 5'-deiodinase, 50-100 times those observed in the cerebral cortex of hypothyroid rats (5, 7), are readily achieved by the inducer of type II 5'-deiodinase, since btpcAMP did not slow the growth rate of the cultured astrocytes and previous work has shown that butyrate, a differentiating agent (28), did not induce type II 5'-deiodinase (15, 27).

The appearance of type II 5'-deiodinating activity stimulated by cyclic nucleotides was preceded by a transcriptional event(s) and synthesis of new mRNA encoding either the enzyme or an essential enzyme regulatory protein quickly reached non-rate limiting levels. Interestingly, enzyme induction was amplified by hydrocortisone by a mechanism requiring increasing transcription. However, the glucocorticoid effect was only seen in the presence of btpcAMP indicating an absolute requirement for cyclic nucleotide for the expression of type II 5'-deiodinase in the cultured astrocyte.

Thyroid hormone had a marked influence on glial cell type II 5'-deiodinase with 10-20 fold increases in activity achieved in cells grown in the absence of thyroid hormone, similar to
that found in rat brain (5–8, 29). Comparison of the enzyme production rates in cells grown in euthyroid and hypothyroid media revealed that thyroid hormone had no effect on synthesis; the increased 5′-deiodinating activity observed in hypothyroid cultures was entirely accounted for by diminished enzyme degradation/inactivation. These data are in agreement with earlier work that showed that the $t_{1/2}$ for cerebrocortical type II 5′-deiodinase was prolonged 10–15-fold in the hypothyroid rat (8) and further that the acute effects of thyroid hormone on the enzyme $t_{1/2}$ did not require continued transcription or translation (8). Thus, cAMP-stimulated gial cell faithfully mimics the intact rat brain with respect to thyroid hormone-dependent regulation of this key membrane-bound enzyme.

The influence of individual iodothyronines on steady-state enzyme levels in the stimulated astrocyte was identical to those previously determined in vivo. $T_3$ and $T_4$ had the most potent effect on the turnover of type II 5′-deiodinase remaining to be determined. Comparison of the enzyme catalyzing type II 5′-deiodinase in astrocytes grown in the presence of $T_4$ and $T_3$, a metabolically inactive iodothyronine, were ~100-fold more potent than $T_3$ in modulating enzyme levels, as had been reported by Silva and Leonard (7) and Kaiser et al. (10) in vivo, and by St. Germain (29) in NB41A3 cells, and half-maximal hormonal effects were observed with biologically relevant concentrations. The rank order of potency of this limited series of iodothyronines differed markedly from that for nuclear $T_3$ receptor (for reviews see Refs. 30, 31) and is consistent with the proposed extra-nuclear site of action.

Additional support for an extranuclear site of thyroid hormone action is provided by the observation that under our culture conditions few, if any, nuclear $T_3$ receptors are present in glial cells (32). In contrast, others have reported modest levels of nuclear $T_3$ receptors in cultured glial cells obtained from primary dispersions of fetal mouse cerebral cortex grown without further subculture (33) and in C-6 astrocytomas cells (34, 35). Thus, it seems likely that continued subculture of astrocytes may result in loss of the nuclear $T_3$ receptor, without affecting the ability of the cell to respond to thyroid hormone by modulating type II 5′-deiodinase.

Our preliminary survey of potential cellular mechanisms mediating the $T_4$-dependent modulation of the degradation/inactivation of the membrane-bound enzyme revealed that this was an energy-dependent process requiring an intact actin-cytoskeleton. Lysosomotropic agents and inhibitors of endocytosis and glycodelysis had little or no effect on type II 5′-deiodinase suggesting that neither enzyme internalization by selective endocytosis nor glycodelysis during synthesis contributed to the short half-life of the enzyme. The failure of colchicine to influence the dynamics of type II 5′-deiodinase induction and/or inactivation, despite a profound effect on the morphology of the brain, the $T_4$-dependent regulation of biological half-life of the enzyme is mediated by an energy-dependent process requiring an intact actin-cytoskeleton. Characterization of the interactions between thyroid hormone and the actin-cytoskeleton, without involvement of the nuclear $T_3$ receptor, should provide new insights into the potential mechanisms available for this metabolically potent hormone to modulate neuronal arborization, cell-cell communication, and the structural abnormalities observed in the brain in congenital hypothyroidism.

**REFERENCES**


* C. J. Zuckerman and J. L. Leonard, unpublished observations.
T₄ Modulation of the t₄₄ of Type II 5'-Deiodinase

Regulation of type II iodothyronine 5'-deiodinase by thyroid hormone. Inhibition of actin polymerization blocks enzyme inactivation in cAMP-stimulated glial cells.

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