Metabolic Effect of 3,3',5'-Triiodothyronine in Cultured Growth Hormone-producing Rat Pituitary Tumor Cells
Evidence for a Unique Mechanism of Thyroid Hormone Action

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Abstract
Physiologic levels of 3,3',5'-triiodothyronine (rT3) are generally believed to have minimal metabolic effects in the pituitary gland and other tissues. In the present studies, the regulatory role of rT3 and other thyroid hormones on iodothyronine 5'-deiodinase (15D) activity was studied in a growth hormone-producing rat pituitary tumor cell line (GH3 cells). 15D activity was thiol-dependent and displayed nonlinear reaction kinetics suggesting the presence of two enzymatic processes, one having a low Michaelis constant (K_m for thyroxine [T4] of 2 nM) and a second with a high K_m value (0.9 μM). Growth of cells in hormone-depleted medium resulted in a two- to 3.5-fold increase in low K_m 15D activity (P < 0.001). The addition of thyroid hormones to the culture medium resulted in a rapid, dose-dependent inhibition of low K_m 15D activity with the following order of analogue potency: rT3 ≫ T4 > 3,5,3'-triiodothyronine (T3). Using serum-free culture conditions, rT3 was ~50 times more active than T3. These inhibitory effects were noted within 15 min of hormone addition and could not be attributed to substrate competition with T4. These findings suggest that the control of T4 to T3 conversion by thyroid hormones in the anterior pituitary gland is mediated by a unique cellular mechanism that is independent of the nuclear T4 receptor; and under some circumstances, rT3 may play a regulatory role in controlling this enzymatic process.

Introduction
The metabolic actions of thyroid hormones appear to be mediated primarily through the interaction of 3,5,3'-triiodothyronine (T3) with specific nuclear receptors (1). In the various growth hormone-producing rat pituitary tumor cell lines (GH cells) for example, T3 is the most potent thyroid hormone analogue in stimulating glucose consumption (2), growth hormone production (3), and amino acid transport (4), and these effects correlate well with the proportion of nuclear binding sites occupied by T3. Although 3,3',5'-triiodothyronine (rT3) also has agonist activity in GH cells, 1,000-fold greater concentrations are required, presumably due to the nuclear receptor having a significantly lower affinity for rT3. In other systems, the metabolic effects of rT3 also appear to be minimal (5).

In the present studies, we used the GH3 cell line to investigate the regulatory effects of thyroid hormones on a low Michaelis constant (K_m) iodothyronine 5'-deiodinase (15D) process (type II). Our finding that rT3 is a potent and rapid inhibitor of this enzymatic process suggests that thyroid hormone control of T3 formation in anterior pituitary tissue is mediated through a unique cellular mechanism that is independent of the nuclear T3 receptor.

Methods
GH3 cells were obtained from the American Type Culture Collection (Rockville, MD) and were routinely grown in 75-cm² plastic flasks and 100-mm plastic culture dishes using Ham's F-10 culture medium supplemented with 2.5% fetal calf serum (FCS), 15% horse serum (HS), and gentamycin (50 μg/ml) in a humidified atmosphere of 5% CO2/95% air. Medium was routinely changed twice weekly. In some experiments cells were grown to near confluence in standard medium and then transferred and maintained for 4 d in serum-free Ham's F-10 or in Ham's F-10 supplemented with FCS (10%) that previously had been treated with either charcoal (6) or an ion exchange resin (7). According to the manufacturer's (HyClone Laboratories, Logan, UT) specifications, the T4 and T3 levels were 12.7 μg/dl and 118 ng/dl, respectively, in the FCS and 2.9 μg/dl and 47 ng/dl, respectively, in the HS. Treatment of FCS either with charcoal or with an ion exchange resin was demonstrated to remove >99% of the iodothyronines.

Experiments were performed with cells at the near confluent stage of growth and 48 h after the last medium change. Cells were harvested by scraping with a rubber policeman, washed twice with ice-cold buffer (0.25 M sucrose, 0.02 M Tris-HCl, pH 7.6, 1 mM MgCl2, 2 mM CaCl2, 0.1 mM dithiothreitol [DTT], 5% glycerol), then resuspended in the same buffer and sonicated using a sonic dismembrator (Artex Systems Corp., Farmingdale, NY) for 25 s at a setting of 45. The sonicate was centrifuged at 800 g for 10 min and the supernatant was used immediately for determination of 15D activity as previously described (8, 9). T3 production rates were quantified by radioimmunoassay (RIA) using stable thyroxine (T4) as a substrate at concentrations of 0.002-3.0 μM in the presence of added DTT. The T4 content of the supernatant fraction, before the addition of stable T4 or enrichment with DTT, was determined by RIA in ethanol extracts (8). Protein concentration was determined by the method of Lowry et al. (10).

Several experiments were performed to characterize and define the optimal conditions for quantifying 15D activity in GH3 cells: (a) enzyme activity in supernatants was found to be a linear function of both protein concentration (up to 11 mg/ml) and incubation time (up to 45 min) and was dependent on the presence of sulphydryl reducing agents (maximum activity at 20 mM DTT). (b) No T3 formation was noted in supernatants heated to 80°C for 45 min before incubation with T4 and DTT. (c) Incubation of supernatants with known concentrations of stable T3 (at 37°C for 45 min in the presence of 20 mM DTT) failed to disclose any evidence of T3 degradation. (d) Enzyme activity determined 1–4 d after a medium change varied by <15% and was the same during either the log or near confluent stages of growth.

The uptake of rT3 by GH3 cells was determined by incubating cells in medium containing a known specific activity of 125I-rT3 (New England Nuclear, Boston, MA). Cells were then harvested, washed, and sonicated as above, and the amount of radioactivity in the supernate fraction was determined. The rT3 content was calculated after correcting for the proportion of counts due to 125I as determined by electrophoresis (11). Stable rT3 was obtained from Calbiochem-Behring Corp., La Jolla, CA.

Kinetic data were analyzed using Eadie-Hofstee plots, and nonlinear plots were resolved into two components as previously described (9). Statistical comparison was performed using the unpaired t test. All results are given as mean±SE.

Results

15D activity in GH3 cell supernatants demonstrated nonlinear reaction kinetics suggesting the presence of two enzymatic processes having $K_m$ values of 2.4±1.0 nM ($n = 4$ experiments) and 0.9±0.3 μM (Fig. 1). Corresponding values for the maximum velocity were 23±7 fmol T3/min mg protein and 32±6 fmol T3/min mg protein. The addition of 0.5 mM 6-n-propyl-2-thiouracil to cell sonicates (in the presence of 5 mM DTT) inhibited T3 production by <10% at low substrate concentrations (10 nM T4) but by 53% ($P < 0.001$) at T4 concentrations of 3 μM (data not shown). In subsequent experiments 15D activity was determined using supernatant substrate concentrations of 10 or 50 nM T4 and 20 mM DTT; these conditions permit quantification of primarily the low $K_m$ process.

The effect on 15D activity of culturing cells in medium supplemented with different serum preparations is shown in Fig. 2. Cells grown in Ham's F-10 medium supplemented with 10% FCS manifested only 46% of the activity noted in cells cultured in standard medium. However, cells maintained in serum-free medium or in the presence of 10% charcoal-treated FCS showed a two- to 3.5-fold increase in 15D activity, suggesting that one or more components of serum exerted inhibitory effects on enzyme activity. The addition of T4, T3, or rT3 to the culture medium resulted in significant inhibitory effects on 15D activity. Dose response curves, as determined in serum-free medium, are shown in Fig. 3. In this experiment, rT3 was somewhat more active than T4 and 50 times more active than T3 in inhibiting 15D activity. Additional experiments ($n = 5$) using both serum-free conditions and medium supplemented with resin-treated FCS confirmed that the order of potency of these analogues was $rT3 > T3 > T4$. In medium supplemented with resin-treated FCS, T4 concentrations of >10^-8 M were required to inhibit 15D activity. Thyronine, at concentrations up to 10^-6 M, inhibited this process by <10%.

The potential of $rT3$ acting as a competitive substrate with $T4$ in the reaction mixture was assessed by determining the rate of $T3$ formation in the presence of both added $T4$ (50 nM) and $rT3$. Concentrations of $rT3$ of up to 10 nM had no effect on $T3$ production rates. Quantitation of $rT3$ cellular uptake revealed that 4 h after the addition of 10^-4 M $rT3$ to the culture medium, supernatant concentrations of $rT3$ were ~5 nM in cells grown in serum-free medium and 0.4 nM in cells maintained in medium supplemented with resin-treated FCS. Thus, even at high medium $rT3$ concentrations, the supernatant concentrations of $rT3$ were insufficient to affect $T3$ production rates when 50 nM $T4$ was provided as substrate in the reaction mixture. Furthermore, the concentration of $T4$ (as determined by RIA) in supernatants derived from cells grown in medium supplemented with resin-treated FCS and either 10^-6 M or 10^-7 M $T4$ were 0.7 nM and 7 nM, respectively; concentrations that would not add significantly to the rates of $T3$ formation in the reaction mixture.

2. Based on the measured sonicate protein concentration and the known values of protein content and cellular volume of GH3 cells (12), the actual intracellular concentrations of $rT3$ and $T4$ can be calculated. For cells incubated for 4 h in medium containing 10% FCS, intracellular $rT3$ and $T4$ concentrations were equal to the total medium concentrations of these iodothyronines. Under serum-free medium conditions, the intracellular concentrations were ~30-fold greater than the medium concentrations.

**Figure 1.** Kinetic analysis, utilizing an Eadie-Hofstee plot, of 15D activity in GH3 cells. Cells were cultured in standard medium containing 2.5% FCS and 15% HS. Enzyme activity was determined using $T4$ concentrations of 0.002–1.0 μM and 20 mM DTT. $T4$ concentrations on the abscissa are expressed in nanomoles per liter. Points represent the mean of duplicate values, which differed <15%. Prot, protein.

**Figure 2.** The effect of different medium conditions on 15D activity in GH3 cells. Cells were grown to near confluence in standard medium (FCS/HS) and then transferred and maintained for four additional days in the medium indicated. 15D activity was determined using a $T4$ concentration of 10 nM and 20 mM DTT. Values represent the mean±SE of triplicate determinations. *P < 0.001 compared with cells maintained in untreated FCS. Prot, protein.
Figure 3. Dose response curves of the inhibitory effects of thyroid hormones on 15'D activity in GH3 cells. Cells were grown to near confluence in standard medium and then transferred and maintained for four additional days in serum-free Ham's F-10 medium. Iodothyronines were added to the culture medium 4 h before harvesting. 15'D activity was determined using a T4 concentration of 50 nM and 20 mM DTT. Points represent the mean of triplicate determinations, which differed <5%.

The regulatory effect of rT3 on 15'D activity was shown to be quite rapid (Fig. 4). Significant inhibition was noted within 15 min of adding rT3 to the medium and was near maximal at 2 h. A similar time course of inhibition was shown for T4.

Discussion

15'D activity in GH3 cells was shown in the present studies to be a thiol-dependent process with nonlinear reaction kinetics suggesting the presence of two enzymatic pathways having markedly different Km values for T4. A differential sensitivity of these two processes to the inhibitory effects of PTU was also noted. These findings are similar to those previously described in homogenates of rat anterior pituitary glands (9, 13) and cerebral cortex (14). Our findings differ, however, from a prior report using GH3 cells where only a high Km process (3.8 μM) was identified (15). The failure to detect a low Km process in this latter study may have been secondary to the low concentrations of DTT used (2 mM) and the relative insensitivity of the T3 RIA (3.75 pg/tube lower limits of detection vs. 0.2 pg/tube in the present studies [8, 9]).

Recent studies have shown that low Km 15'D activity is responsible for the vast majority of "local" T3 formation in the pituitary and depends on thyroid hormone status (8, 13). In hypothyroid animals, the activity of this process is markedly elevated, whereas thyroid hormone administration induces a rapid decrease in T4 to T3 conversion. Our finding that low Km 15'D activity in GH3 cells rapidly responds to medium thyroid hormone concentrations supports the use of this cell line as a model system.

The effects of thyroid hormones on 15'D activity in various tissues are complex (16). In liver and kidney homogenates, rT3 inhibits T3 formation by substrate competition with T4 for a high Km (type I) 15'D process (17). The suppressive effects of thyroid hormones on low Km (type II) 15'D activity in GH3 cells, however, cannot be attributed to this mechanism. Our demonstration that rT3 is a potent inhibitor of this process contrasts sharply with the weak agonist activity of this analogue on other metabolic processes (2, 5) and strongly suggests that a unique regulatory mechanism, independent of the nuclear T3 receptor, is responsible for this effect. Further evidence for this is the finding that relatively high concentrations of T3 (10-8 M in medium containing serum), which have been shown by other investigators to stimulate significantly growth hormone production and maximally suppress thyrotropin-releasing hormone receptors in GH3 cells (18), had no effect on 15'D activity. A recent report (19) demonstrating that the injection of T4 or rT3 in the rat is more effective than T3 in inhibiting pituitary and cerebral cortex 15'D activity, suggests that the order of iodothyronine potency found in GH3 cells is also applicable in vivo. The cellular processes responsible for these regulatory effects are uncertain. Leonard et al. (20), however, have recently suggested that the T3-dependent inhibition of 15'D activity in rat pituitary and cerebral cortex is mediated by a posttranscriptional mechanism that increases the rate of degradation or inactivation of the enzyme.

To date, a 5-deiodinase process has not been described in pituitary tissue and none could be demonstrated in the present study in GH3 cells when T3 was used as a substrate. This finding, together with the near equal potency of T4 with rT3, suggests that the regulatory effects of T4 on 15'D activity are direct and not mediated by conversion to rT3.

An intriguing question raised by these studies is the possible role of rT3 in the control of T4 to T3 conversion in the pituitary and brain. Although rT3 appears to be a potent regulator of this metabolic process, its free concentration in normal human serum is ~40-fold lower than that of T4 (21). Thus, T4 rather than rT3 is likely to exert the principle physiologic control over this process. This thesis is consistent with reports that the administration of rT3 to normal human subjects has no effect on the serum levels of T4, T3, thyrotropin, or the thyrotropin response to thyrotropin-releasing hormone (22, 23). In nonthyroidal illness, however, marked alterations occur in thyroid hormone metabolism, which may result in decreased serum free T4 levels and increases in serum free rT3 levels of up to 15-fold (21). Under such circumstances, inhibitory effects of rT3 on 15'D activity may become significant.

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