Relationship of Receptor Affinity to the Modulation of Thyroid Hormone Nuclear Receptor Levels and Growth Hormone Synthesis by L-Triiodothyronine and Iodothyronine Analogues in Cultured GH_1 Cells

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ABSTRACT We have previously demonstrated that L-triiodothyronine (L-T3) induces an increase in growth hormone synthesis and messenger RNA in cultured GH_1 cells, a rat pituitary cell line. In addition to regulating the growth hormone response, L-T3 elicits a time- and dose-dependent reduction in the level of its nuclear receptor, which is a direct function of the occupancy of the receptor binding site. In this study we have compared the relative affinity of L-T3, triiodothyroacetic acid, D-triiodothyronine (D-T3), and L-thyroxine (L-T4) for the receptor with the induction of the growth hormone synthesis and the ability of these compounds to elicit a reduction in thyroid hormone nuclear receptor levels. Triiodothyroacetic acid and D-T3 were specifically examined because the biologic effect of these compounds in the intact rat is significantly lower than predicted by their affinity for the receptor using isolated rat liver nuclei in vitro. In intact cells each compound demonstrated an excellent relationship between the relative receptor affinity, the induction of growth hormone production, and the concentration-dependent reduction in nuclear receptor levels. With the exception of D-T3, the relative affinity of iodothyronine was identical for the receptor using intact cells in serum-free media, or isolated GH_1 cell nuclei in vitro. The apparent receptor affinity of D-T3 with intact cells was 5.5-fold lower than with isolated nuclei, which suggests a decrease in cell entry of D-T3 relative to the other iodothyronines. Quantitation of the [^{125}I]iodothyronine associated with the receptor in GH_1 cells after a 36-h incubation with L-^{125}I-T4 was 90% L-T4 and 10% L-T3, which indicates that the major effect of L-T4 in GH_1 cells is a result of intrinsic L-T4 activity. Studies with dispersed rat anterior pituitary cells demonstrated that L-T3 induces growth hormone synthesis and elicits a reduction in nuclear receptor levels in the same fashion as GH_1 cells. The observation that thyroid hormone influences dispersed rat pituitary cells in a fashion qualitatively similar to GH_1 cells may have implications for the growth hormone response of the somatotroph cell in vivo to different thyroidal states.

INTRODUCTION

We have previously documented that GH_1 cells, a growth hormone-producing rat pituitary cell line, is a valid culture system which can be used to define the molecular action of physiological concentrations of thyroid hormone in mammalian cells (1–8). In this system, L-triiodothyronine (L-T3)[1] stimulates a 3- to 10-fold increase in the rate of growth hormone synthesis (2, 5, 7) which is paralleled by changes in total cytoplasmic growth hormone messenger RNA (mRNA) levels (8). The physiological relevence of this effect in GH_1 cells is underscored by the observation that thyroid hormone plays an important role in controlling the production of growth hormone in the rat pituitary in vivo (9–12), although thyroid growth hormone has been reported to have an equivocal effect on growth hormone production in dispersed anterior pituitary cells in vitro (13).

Portions of this work were presented at The 59th Annual Meeting of the Endocrine Society, Chicago, Ill., 8 June 1977. Abstract 295.

Received for publication 7 August 1978 and in revised form 15 January 1979.

[1] *Abbreviations used in this paper*: D-T3, D-triiodothyronine; L-T3, L-triiodothyronine; L-T4, L-thyroxine; mRNA, messenger RNA; triac, triiodothyroacetic acid.

The induction of growth hormone synthesis and mRNA in GH_1 cells appears to be mediated by the thyroid hormone nuclear receptor. This is based on the good agreement between the concentration-dependent occupancy of the receptor by L-T3 and the induction of the growth hormone response (6, 7). In addition to inducing the growth hormone response, we have demonstrated that L-T3 elicits a time- and dose-dependent reduction of the thyroid hormone receptor to approximately one-half of the initial value (6, 7). This hormone-mediated receptor regulation appears to require the association of L-T3 with the receptor binding site (7), and the decrease in nuclear receptor levels is associated with a decrease in the induced biological response (7).

In addition to the GH_1 cell studies, a wide body of evidence indicates that the high affinity nuclear binding protein for L-T3 and L-thyroxine (L-T4) functions as a biologically relevant cellular receptor. This is based primarily on the good agreement between the relative affinity of thyroid hormone analogues for isolated liver nuclei in vitro and their reported in vivo biologic effects in terms of anti-goiter activity and O_2 consumption (14–19), and induction of α-glycerophosphate dehydrogenase in rat liver (20). Two hormonal analogues which show a higher affinity for the receptor, using isolated nuclei in vitro (21–23) as compared with their in vivo biological activity, are triiodothyroacetic acid (triac) and D-triiodothyronine (D-T3). It has been argued that this discrepancy indicates that the cell nucleus is not the only site of action of thyroid hormone in the cell (24). Goslings et al. (25) attempted to resolve this question for triac and demonstrated, in the rat, a more rapid fractional metabolic clearance rate for triac than L-T3. This might result in a lower triac:L-T3 potency ratio than predicted with isolated nuclear binding studies.

To further clarify this problem, we have compared the relative affinity of the nuclear receptor in intact GH_1 cells for L-T3, triac, D-T3, and L-T4 with the induction of growth hormone synthesis and the modulation of the nuclear receptor by these analogues. We demonstrate that the regulation of growth hormone and nuclear receptor levels is a direct function of the occupancy of the receptor binding site by these compounds. In addition, studies with isolated GH_1 cell nuclei suggest that a decrease in cell entry of D-T3 relative to L-T3 may in part explain the discrepancy between in vivo biologic responses and the in vitro isolated nuclear binding assay for this analogue.

METHODS

Hormones and chemicals. L-[^{125}I]T3 (300–400 Ci/mmol) and L-^{125}I-T4 (700 Ci/mmol) were obtained from Abbott Laboratories, Chemical Div., North Chicago, Ill. ^{125}Iodide (carrier free) for iodination was from New England Nuclear, Boston, Mass. L-[^{35}S]methionine (1,110 Ci/mmol) was from Amersham/Searle Corp., Arlington Heights, Ill. Ham's F-10 culture medium, fetal calf serum, and horse serum were from Grand Island Biological Co., Grand Island, N. Y. Hypothyroid calf serum was obtained from Rockland, Inc., Gilbertsville, Pa. Before thyroidectomy the serum L-T3 was 130 ng/dl and L-T4 was 7 μg/dl (26). After thyroidectomy L-T4 was 0.05 μg/dl and the L-T3 was below the sensitivity of the assay of the procedure (27) (<2 ng/dl). L-T3, L-T4, D-T3, and triac were from Sigma Chemical Co., St. Louis, Mo. Both L-^{125}I-T4 and L-[^{125}I]T3 were purified before use as previously described (1).

GH_1 cell culture conditions. The growth of GH_1 cells in monolayer culture using 25-cm^2 plastic flasks or 2-cm^2 multiwell plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) was carried out as previously described (1–8). The cells were inoculated at initial cell densities between 2 and 4 $\times$ 10^4 cells/cm^2 with growth medium (Ham's F-10 medium which contained 2.5% fetal calf serum and 15% horse serum) and were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for 48–72 h. The medium was replaced with Ham's F-10 medium which contained 10% hypothyroid calf serum, and the cells were incubated for an additional 24–48 h. This insured complete cellular depletion of thyroid hormone, which resulted from the growth of the cells with medium that contained euthyroid fetal calf and horse serum (4, 26).

To study the growth hormone response, the medium was replaced with Ham's F-10 medium which contained 10% hypothyroid calf serum along with the concentrations of the iodothyronine analogues in the total medium as indicated in the text. For dose-response studies, the medium was replaced with identical medium which contained the same iodothyronine concentrations at 24-h intervals, and the growth hormone accumulating in the medium between 24 and 48 h was used to estimate the rate of growth hormone production. We have shown that the accumulation of growth hormone in the medium is an accurate assessment of the rate of growth hormone synthesis and is similar to that determined by a 10- to 15-min incubation with L-[^{35}S]methionine or L-[^{3}H]leucine followed by a selective immunoprecipitation with specific anti-growth hormone antibody (5–8). After removing the medium, the cells were rinsed three times with 0.14 M NaCl at 4°C, and the cells were saved for quantitation of cell protein (28) or intracellular growth hormone by radioimmunoassay (2, 8).

Rat anterior pituitary cell culture. Rat pituitaries were obtained from 200-g male Sprague-Dawley rats, dispersed as described by Vale et al. (29), and inoculated into 2-cm^2 multiwell plates at an initial cell density of 2–4 $\times$ 10^4 cells/cm^2 as described for GH_1 cells. The influence of L-T3 on growth hormone production was determined with the protocol described above for GH_1 cells. This was determined by radioimmunoassay as well as by a 15-min incubation with L-[^{35}S]-methionine followed by selective immunoprecipitation of the intracellular radiolabeled growth hormone as described for GH_1 cells (5).

Quantitation of the relative affinity of hormonal analogues for the thyroid hormone nuclear receptor in GH_1 cells and isolated nuclei. The protocol for cell inoculation and for exchange of medium that contained hypothyroid calf serum was exactly as described above with the studies on growth hormone production rates. Hormone binding studies with serum-supplemented medium were carried out under identical conditions as the biologic response was examined. An assessment of the relative affinity of L-T3, triac, D-T3, and L-T4 for the nuclear receptor was determined by incubating cells with 2 nM L-[^{125}I]T3 along with different concentrations of the analogues for 3 h. In other studies, the hypothyroid calf-serum medium was first removed, and the cells were washed twice with serum-free Ham's F-10 media. The relative affinity was

then determined with serum-free conditions by incubating cells with 0.2 nM L-[^{125}I]T3 along with different concentrations of analogues for 3 h. The nuclear binding in the intact-cell studies was quantitated exactly as previously described (6, 7).

An additional study assessed the relative affinity of the analogues for the nuclear receptor with isolated GH_1 cell nuclei in vitro with 0.25 nM L-[^{125}I]T3 along with different concentrations of the analogues. The nuclei were incubated for 45 min at 37°C. The conditions for incubation and washing of nuclei were carried out as previously described (30). The magnitude of nonspecific binding, determined by simultaneous incubation with a 500- 1,000-fold molar excess of nonradioactive T3, was <4% of the total L-[^{125}I]T3 bound in the intact-cell studies and 12% with the isolated nuclear studies. These values were subtracted from the total L-[^{125}I]T3 bound to determine the magnitude of hormone bound to the receptor. Nuclear DNA content was determined by the method of Burton (31).

Influence of hormonal analogues on nuclear thyroid hormone receptor levels. In a previous study (6) we demonstrated that the influence of L-T3 on reducing thyroid hormone nuclear receptor levels could be determined either by incubating cells with only radiolabeled hormone, or by first incubating cells with nonradioactive L-T3 followed by the addition of 5 nM L-[^{125}I]T3 and a second incubation for 3 h. 5 nM L-[^{125}I]T3 occupies >95% of the receptor and allows an estimate of total nuclear receptor levels (6, 7). Calculation of the total level of receptor binding sites by the second procedure is dependent upon rapid exchange between the L-T3 and L-[^{125}I]T3 present in the medium and that bound to cell components such that the final specific activity of the system can be predicted knowing the nonradioactive L-T3 and the L-[^{125}I]T3 concentrations. The validity of this exchange procedure was confirmed in a study in which identical dose-dependent depletion of nuclear bound receptor was determined with only L-[^{125}I]T3, or L-T3 followed by an exchange reaction with 5 nM L-[^{125}I]T3 (6). The femtomoles of receptor, assuming one receptor binding site per receptor molecule, was calculated from the formulation given in Eq. 1, in which the femtomoles of L-[^{125}I]T3 bound was determined from the initial L-[^{125}I]T3 specific activity. This value is multiplied by the sum of the L-[^{125}I]T3 and L-T3 concentrations and divided by the concentration of L-[^{125}I]T3. All concentrations, as indicated by the parenthesis in Eqs. 1 and 2 are in nanomolar. This gives an assessment of the total iodothyronine associated with the receptor binding site.

$$\text{fmol receptor} = \text{fmol L-[}^{125}\text{I]T3 bound} \times \left(\frac{\text{L-[}^{125}\text{I]T3} + \text{L-T3}}{\text{L-[}^{125}\text{I]T3}}\right). \quad (1)$$

This formulation can be extended (Eq. 2) to estimate total nuclear receptor levels using iodothyronines other then L-T3, i.e., triac, D-T3, and L-T4, if the affinity of these compounds relative to L-T3 is determined.

$$\text{fmol receptor} = \text{fmol L-[}^{125}\text{I]T3 bound} \times \left(\frac{\text{L-[}^{125}\text{I]T3} + \dfrac{\text{iodothyronine}}{\text{iodothyronine affinity relative to L-T3}}}{\text{L-[}^{125}\text{I]T3}}\right). \quad (2)$$

To examine the influence of iodothyronine analogues on reducing thyroid hormone nuclear levels, the protocol for cell inoculation and medium exchange was as described for growth hormone induction studies. The cells were then incubated with different concentrations of hormone analogues for 24 h. This incubation was followed by a second 3-h incubation with 5 nM L[^{125}I]T3. The nuclear bound L-[^{125}I]T3 was determined and the total receptor was determined with Eq. 2 above.

Estimation of free L-T3 and L-T4 concentrations. L-[^{125}I]T3 and L-^{125}I-T4 were purified by chromatography as previously described (1, 32). Estimation of free hormone concentrations was determined by equilibrium dialysis (33) with 1-ml acrylic plastic dialysis cells. 1 ml of Ham's F-10 medium which contained 10% hypothyroid calf serum and L-^{125}I-T4 or L-[^{125}I]T3 was dialyzed against 1 ml of serum-free Ham's F-10 media at 37°C for 24 h in a humidified incubator in an atmosphere of 95% air and 5% CO_2 while shaking on a rocker platform. Equilibration occurred within 24 h and identical results were obtained if L-^{125}I-T4 or L-[^{125}I]T3 was initially added to the serum-free Ham's F-10 media.

Influence of L-[^{125}I]T3 on thyroid hormone nuclear receptor levels with dispersed rat pituitary cells. Rat pituitary cells were dispersed as described above for the growth hormone studies and were inoculated into 12 25-cm^2 flasks at a cell density of 3×10^4 cells/cm^2. The protocol of media exchange was as described in the section on GH_1 cell culture conditions. 24 h after the cells received their last replacement with media which contained hypothyroid calf serum, 5 nM L-[^{125}I]T3 was added to four flasks, and two flasks received 5 nM L-[^{125}I]T3 plus a 1,000-fold molar excess of nonradioactive L-T3. The cells were incubated for an additional 24 h. At the end of the 24-h incubation period, four flasks which did not initially receive hormone were incubated for 1.5 h with 5 nM L-[^{125}I]T3 to estimate total receptor levels, and two flasks received 5 nM L-[^{125}I]T3 plus 1,000-fold molar excess of nonradioactive L-T3 to assess the nonspecific binding fraction. As previously described (6, 7) the magnitude of receptor depletion was measured by the difference between the 1.5-h and 24-h incubations.

Quantitation of L-[^{125}I]T3 and L-^{125}I-T4 in cells and medium after a 36-h incubation of L-^{125}I-T4 with GH_1 cells. L-^{125}I-T4, purified as previously described (1, 32), was incubated with GH_1 cells at 50 nM with and without a 500-fold molar excess of nonradioactive L-T4. The flasks were then chilled to 4°C, the medium removed, and the cell monolayers were rinsed four times with 0.14 M NaCl at 4°C. Nuclear and extranuclear cells fractions were isolated as previously described (1, 6, 7). The samples were adjusted to 0.015 N NaOH - 0.5 M NaCl, and 0.3 ml of the cellular material was applied to a 5-ml Sephadex G-25 (fine) column (Pharmacia Fine Chemicals, Inc., Div. Pharmacia Inc., Piscataway, N. J.) preequilibrated with the same buffer. The column was then eluted stepwise with 0.9-ml fractions of 0.1 N NaOH - 5 mM NaCl. 100 μl of media was adjusted to 0.015 N NaOH - 0.5 M NaCl and chromatographed in the same fashion. With this procedure (1, 34) ^{125}iodide elutes with the first column volume, L-[^{125}I]T3 elutes in the second column volumes, and L-^{125}I-T4 elutes in the third to fifth column volumes.

RESULTS

Dose-response induction of growth hormone synthesis in GH_1 cells. Fig. 1 illustrates the dose-response induction of growth hormone synthesis induced by L-T3, triac, D-T3, and L-T4. In medium supplemented with 10% hypothyroid calf serum, L-T3 and triac showed almost identical dose-response relationships with half-maximal responses occurring at 0.17 nM for L-T3 and 0.19 nM for triac. This value for L-T3 is in good agreement with the value (0.22 nM) that we reported previously which was determined with L-[^{35}S]-

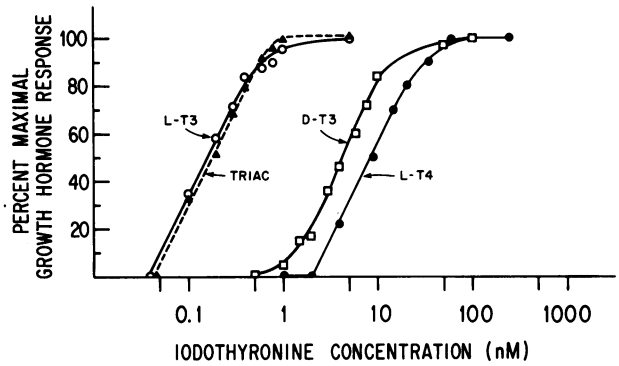

FIGURE 1 Relative growth hormone response of iodothyronines. Induction of growth hormone production with different concentrations of L-T3, triac, D-T3, and L-T4. 40,000 GH_1 cells were inoculated into 2-cm² multiwells and were cultured and exchanged with Ham's medium which contained 10% hypothyroid calf serum as described in Methods. The cells were incubated with the concentrations of L-T3 (○), triac (▲), D-T3 (□), and L-T4 (●), as indicated in the figure. The medium was changed at 24-h intervals, and the dose-response curves were determined by the growth hormone which accumulated in the medium between 24 and 48 h of incubation. Cell cultures which received no hormone served as controls. The growth hormone production in the control cells was 1±0.13 μg growth hormone/24 h per 100 μg cell protein. The maximal induced level was 8±0.5 μg growth hormone/24 h per 100 μg cell protein. In construction of the dose-response curve, the growth hormone production of the control cells was subtracted from the response at each hormone concentration. The results reflect the mean of triplicate cultures with <±10% variation.

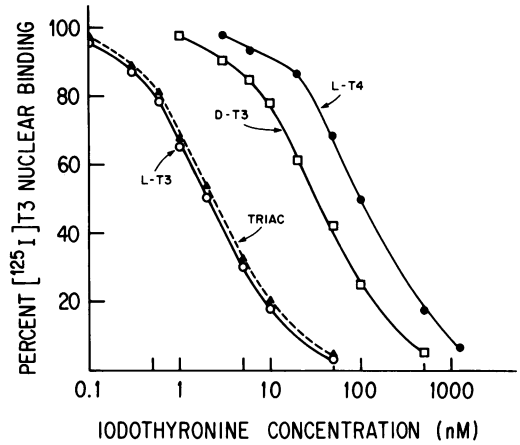

FIGURE 2 Relative binding of indothyronines to the nuclear receptor. Inhibition of L-[125I]T3 nuclear receptor binding by L-T3, triac, D-T3, and L-T4. 500,000 GH_1 cells were inoculated into 25-cm² plastic flasks and were cultured and exchanged with Ham's F-10 medium which contained 10% hypothyroid calf serum as described in Methods. The L-[125I]T3 concentration was 2 nM, and the concentrations of L-T3 (○), triac (▲), D-T3 (□), and L-T4 (●) were as indicated in the figure. The L-[125I]T3 bound to receptor, without added nonradioactive hormone, was 130 fmol/100 μg DNA±5%, and the analogue results are expressed as a percentage of this value. The nonspecific bound fraction of L-[125I]T3 determined by incubating 2 nM L-[125I]T3 plus a 500-fold molar excess of nonradioactive L-T3 was 5 fmol±1.2/100 μg DNA. This value was subtracted from the L-[125I]T3 bound from each point in the figure to determine the L-[125I]T3 bound to the receptor. The results reflect the mean of triplicate determinations with <±5% variation.

methionine incorporation followed by immunoprecipitation with highly selective anti-growth hormone antibody (6). D-T3 induced a half-maximal growth hormone response at 4 nM, and L-T4 induced a half-maximal response at 9 nM. Therefore, under the conditions in which the medium is supplemented with 10% hypothyroid calf serum, triac has 89.5%, D-T3 has 4%, and L-T4 has 2% of the activity of L-T3.

Relative affinity of hormonal analogues for the thyroid hormone nuclear receptor in serum-containing medium. An estimation of the relative affinity of L-T3, triac, D-T3, and L-T4 for the thyroid hormone nuclear receptor was performed with intact cells and medium that contained hypothyroid calf serum to allow for a relative comparison with the growth hormone dose-response curves.

Fig. 2 illustrates the results of this study in which cells were incubated with 2 nM L-[125I]T3 plus different concentrations of nonradioactive L-T3, triac, D-T3, and L-T4 for 3 h. Triac was 87%, D-T3 was 5.8%, and L-T4 was 2% as effective as L-T3 in inhibiting the binding of L-[125I]T3 to the receptor. Therefore, the relative affinity of these compounds for the thyroid hormone nuclear receptor demonstrated excellent agreement with the relative ability of these compounds to induce growth hormone synthesis (Fig. 1).

Dose-dependent depletion of the thyroid hormone nuclear receptor by hormonal analogues. With GH_1 cells in medium that contained 10% hypothyroid calf serum, we previously reported that the concentration of L-T3 which results in half-maximal occupancy of the thyroid hormone nuclear receptor was 0.51 nM (6). Based on this value and the relative inhibition data of Fig. 2, the iodothyronine concentrations which would result in half-maximal occupancy of the nuclear receptor was calculated to be 0.57 nM for triac, 9.8 nM for D-T3, and 25 nM for L-T4. From the iodothyronine concentrations which result in half-maximal induction of growth hormone synthesis in Fig. 1, it is apparent that the growth hormone response curves are shifted leftward of the receptor occupancy curves by a factor of ≅2.5. In previous studies with L-T3 (6, 7) this shift in the growth hormone response curve from the receptor occupancy curve appeared to result from a dose-dependent hormone-mediated reduction in nuclear receptor levels, and the L-T3 concentration which resulted in one-half of the maximal level of receptor depletion was 0.17 nM. Therefore, the results of Figs. 1 and 2 would suggest that triac, D-T3, and L-T4, would be expected to elicit a dose-dependent reduction in

thyroid hormone nuclear receptor levels. Fig. 3 illustrates the results of a study in which this was examined. The cells were initially incubated with the concentrations of iodothyronine indicated in Fig. 3 for 24 h, followed by an incubation with 5 nM L-[^{125}I]T3 for 3 h. Total nuclear receptor levels were calculated using Eq. 2 described in Methods and the affinity of each compound for the receptor relative to L-T3 derived from Fig. 2. Fig. 3 indicates that each compound elicits a 40% decrease in total nuclear receptor levels, and the dose-dependent reduction of receptor parallels the affinities of the analogues for the receptor. One-half of the maximal reduction in nuclear receptor levels occurred at 0.15 nM for L-T3, 0.17 nM for triac, 2.5 nM for D-T3, and 9 nM for L-T4. Based on these concentrations, triac has 88%, D-T3 has 6.0%, and L-T4 has 1.7% of the activity of L-T3.

Quantitation of L-T3 and L-T4 in media and cells after long-term incubation with L-[^{125}I]T4. Silva and Larsen (35) recently reported that L-T4 is rapidly converted to L-T3 in the pituitary in vivo, and after L-^{125}I-T4 injection >90% of the nuclear bound radiolabeled iodothyronine was L-[^{125}I]T3. In a previous study in GH_1 cells, we demonstrated that after a 2.5-h incubation with L-^{125}I-T4 >97% of the [^{125}I]iodothyronine bound to the nuclear receptor remained as L-^{125}I-T4 (1). The experiment in Figs. 1 and 3, however, assessed cellular responses after a 24- to 48-h incubation and, therefore, it remains possible that the L-T4-mediated induction of growth hormone synthesis and nuclear receptor depletion is a result of L-T3 formed as a result of L-T4 conversion. This possibility was examined by incubating GH_1 cells with Ham's F-10 medium which contained hypothyroid calf serum and 50 nM of purified L-^{125}I-T4, or 50 nM L-^{125}I-T4, plus a 500-fold molar excess of nonradioactive L-T4 for 36 h. After the incubation the medium was saved, and the nuclear and extranuclear fractions of the cells were isolated (1, 6, 7). The ^{125}iodothyronine was dissociated from binding protein with 0.015 N NaOH – 0.5 M NaCl, and the samples were chromatographed on Sephadex G-25 (fine) columns to separate iodide, L-T3, and L-T4, as previously described (34).

Fig. 4 illustrates the results of this study for media (4A), cellular extranuclear material (4B), and nuclei (4C). The elution positions of ^{125}iodide and L-[^{125}I]T3 were determined from parallel studies with GH_1 cells and ^{125}iodide and L-[^{125}I]T3. Fig. 4A and B illustrate that

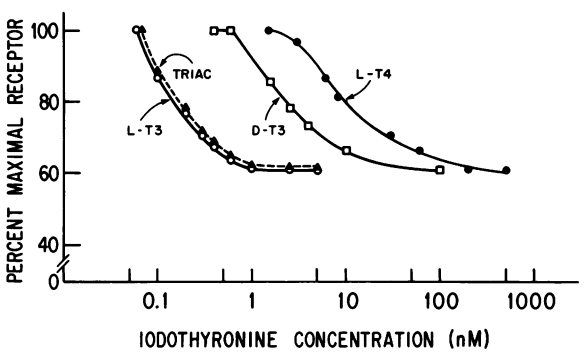

FIGURE 3 Relative effect of iodothyronines on receptor depletion in GH_1 cells. Influence of L-T3, triac, D-T3, and L-T4 on total thyroid hormone nuclear receptor levels. 600,000 cells were inoculated into 25-cm^2 plastic flasks, and the cells were cultured and exchanged with Ham's F-10 medium which contained 10% hypothyroid calf serum, as described in Methods. The cells were incubated for 24 h with the concentrations of L-T3 (○), triac (▲), D-T3 (□), and L-T4 (●) indicated in the figure. This was followed by a second 3-h incubation with 5 nM L-[^{125}I]T3. The nuclear bound L-[^{125}I]T3 was determined, and the total receptor was determined by estimating the total iodothyronine bound to the receptor binding sites using Eq. 2. The iodothyronine affinity values relative to L-T3 were derived from Fig. 2. Maximal receptor levels (160±5.5 fmol/100 μg DNA) were determined with cells which received 5 nM L-T3 for 3 h which had previously received no hormone during the earlier 24 h incubation. The nonspecific bound L-[^{125}I]T3, determined by incubating cells with 5 nM L-[^{125}I]T3 plus a 500-fold molar excess of nonradioactive L-T3, was 5 fmol±1.3/100 μg DNA. The results reflect the mean of triplicate determination with <±5% variation.

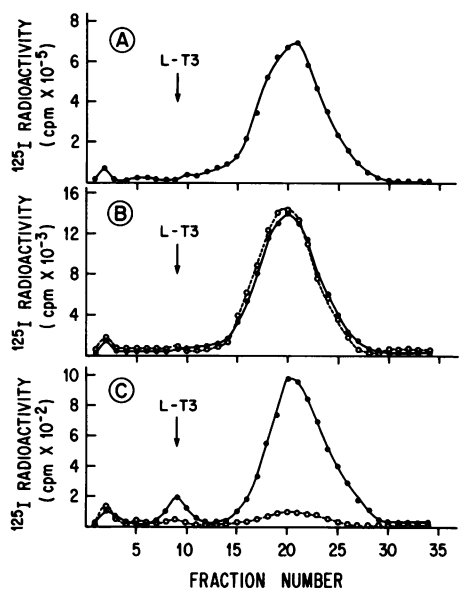

FIGURE 4 Quantitation of L-[^{125}I]T3 and L-^{125}I-T4 in media and cell extranuclear and nuclear fractions. 600,000 cells were inoculated into 25-cm^2 plastic flasks and cultured and exchanged with Ham's F-10 medium which contained hypothyroid calf serum as described in Methods. The cells were then incubated for 36 h with either 50 nM L-^{125}I-T4 (●) or 50 nM L-^{125}I-T4 plus a 500-fold excess of nonradioactive L-T4 (○). The media, nuclear, and extranuclear fractions were isolated and chromatographed on Sephadex G-25 (fine) columns to separate L-T3 and L-T4 as described in Methods. (A) medium; (B) extranuclear fractions; (C) nuclear fractions. The arrows indicate the elution position of L-[^{125}I]T3, which was determined with a parallel column. In a parallel column, ^{125}iodide eluted in fractions 4 through 6.

no detectable L-[^{125}I]T3 was identified in the medium or extranuclear fraction. In addition, the absolute level of L-^{125}I-T4 associated with the extranuclear compartment was not altered by the 500-fold excess of nonradioactive L-T4. The elution pattern of the ^{125}I radioactivity from nuclei showed a radiolabeled peak which eluted in the L-T3 position and a peak of L-T4. The radiolabeled peak of L-T3 accounted for 5% of the total ^{125}iodothyronine. No iodide peak was observed. The peak in fraction 2 is at the void volume of the column and represents residual radioactivity associated with protein. Both the L-T3 and the L-T4 peaks were reduced by 90% in the cells incubated with the 500-fold excess of nonradioactive L-T4. Because the ^{125}iodide is localized to the 3′ position of L-T4 and the deiodination of the 3′ and 5′ iodide is likely a random process, the nuclear receptor-bound iodothyronine was calculated to be 90% L-T4 and 10% L-T3.

The concentration of L-T4 used in this study (50 nM) induced a maximal growth hormone response (Fig. 1) and elicited a reduction in thyroid hormone nuclear receptor levels to 90% of the final steady-state value (Fig. 3). With this concentration of L-T4, the occupancy of the nuclear receptor by L-T3 represents a maximum of 10% of the total receptor binding sites (Fig. 4). This degree of occupancy of the receptor by L-T3 elicits a growth hormone response and receptor depletion which are <20% of the values obtained with full receptor occupancy by L-T3 (6, 7). Therefore, the major biologic effects of L-T4 in GH_1 cells on receptor depletion and growth hormone reduction appear to be mediated by L-T4 and not L-T3 formed as a result of conversion.

Quantitation of media-free L-T3 and L-T4 and comparison to serum-free studies. To make a direct comparison between the relative affinity for the receptor, the growth hormone response, and receptor depletion, the cellular responses were studied with Ham's F-10 medium supplemented with 10% hypothyroid calf serum. Although this allows a valid relative comparison between iodothyronine analogue binding characteristics and growth hormone induction and regulation of the receptor, it does not allow a direct measurement of the intrinsic differences in biological activity as a result of inherent differences in the serum binding of the analogues. In an earlier study with intact GH_1 cells and serum-free Ham's F-10 media, the K_d for nuclear binding was determined to be 0.029 nM for L-T3 and 0.26 nM for L-T4 (1). This 9.7-fold difference between L-T4 and L-T3 differs from the ≅50-fold difference in the L-T4 response determined in Figs. 1–3 relative to that of L-T3. Therefore, the free L-T3 and L-T4 fractions were quantitated in Ham's F-10 medium which contained 10% hypothyroid calf serum to allow for direct comparison with the affinities determined with intact-cell studies with serum-free media. Between 0.01–5 nM L-T3 and 0.1–50 nM L-T4 the percentage of total media hormone concentrations which was in the free fraction was estimated to be 5% for L-T3 and 1% for L-T4. With these values and the demonstration that 0.51 nM L-T3 in serum-containing medium occupies 50% of the receptor binding sites (6), it is possible to compare the L-T3 and L-T4 results in this study with earlier serum-free experiments (1). The estimated free hormone concentration which results in half-maximal occupancy of the thyroid hormone receptor was calculated to be 0.026 nM for L-T3 and 0.26 nM for L-T4. These results are essentially identical, therefore, to the K_d values determined for L-T3 and L-T4 binding to nuclear receptor in intact cells using serum-free conditions (1).

Relative receptor affinity of thyroid analogues with intact cells in serum-free medium and with isolated GH_1 cell nuclei in vitro. Studies with isolated liver nuclei by Koerner et al. (21) and DeGroot and Torresani (23) have demonstrated that triac has a 164 (21) to 400% (23) greater affinity than L-T3, whereas the relative affinity of D-T3 was equal to (23) or 60% (21) of that of L-T3. These results differ significantly from the relative affinity determined in intact cells with 10% hypothyroid calf serum medium in which triac had an affinity which was slightly less then L-T3, and D-T3 had only 5.8% of the affinity as compared with L-T3. These differences may reflect a greater avidity of serum-binding proteins for these analogues, intrinsic differences in cell entry, or differences in the nuclear receptor derived from GH_1 cells and liver.

To explore these possibilities, we compared the relative affinity of the iodothyronine analogues for nuclear receptors in intact cells in serum-free conditions (Fig. 5), and in isolated GH_1 cell nuclei in vitro (Fig. 6). In intact cells, triac had 306%, D-T3 had 9.6%, and L-T4 had 8% of the affinity relative to L-T3. The apparent increase in relative affinities for triac and L-T4 are consistent with the greater serum binding of triac and L-T4 (25) as compared with L-T3. With serum-free conditions, however, the D-T3 relative affinity in intact cells is only slightly greater then with serum-containing medium (Fig. 2). Fig. 6 illustrates the relative affinities of the analogues for the nuclear receptor using isolated GH_1 nuclei in vitro. In this study triac had 400%, D-T3 had 53%, and L-T4 had 10% of the affinity for the receptor as compared with L-T3.

These results with isolated GH_1 cell nuclei are similar to those previously reported with isolated rat liver nuclei (21, 23). Furthermore, the affinity of triac and L-T4 as compared with L-T3 are also almost identical with isolated nuclei to that determined in the serum-free intact-cell study indicating no apparent differences in cell permeability of L-T3, triac, and L-T4. In contrast, the relative affinity of D-T3 is 5.5-fold lower in

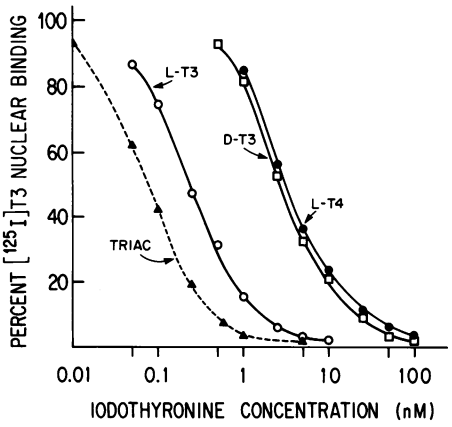

FIGURE 5 Binding of iodothyronines to nuclear receptors in serum-free media. Inhibition of L-[^{125}I]T3 nuclear receptor binding by L-T3, triac, D-T3, and L-T4 with serum-free conditions. 500,000 cells were inoculated into 25-cm^2 plastic flasks and were cultured and exchanged with Ham's F-10 medium which contained 10% hypothyroid calf serum as described in Methods. The cells were then washed twice with serum-free Ham's F-10 medium before the binding analysis. The L-[^{125}I]T3 concentration was 0.2 nM, and the concentrations of L-T3 (○), triac (▲), D-T3 (□), and L-T4 (●) were as indicated in the figure. The L-[^{125}I]T3 bound to receptor without added nonradioactive hormone, was 120 fmol/100 μg DNA±6%, and the analogue results are expressed as a percentage of this value. The nonspecific bound fraction of L-[^{125}I]T3 determined by incubating 0.2 nM L-[^{125}I]T3 plus a 500-fold molar excess of nonradioactive L-T3 was 4 fmol±1.6/100 μg DNA. This value was subtracted from the L-[^{125}I]T3 bound from each point in the figure to determine the L-[^{125}I]T3 bound to the receptor. The results reflect a mean of triplicate determinations with <±6% variation.

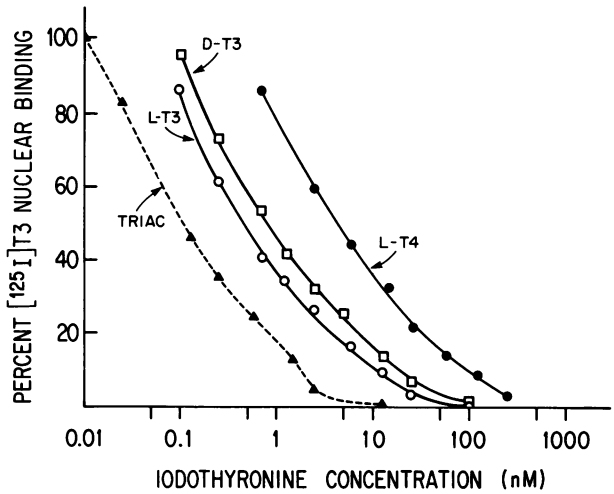

FIGURE 6 Binding of iodothyronines to nuclear receptors in isolated nuclei. Inhibition of L-[^{125}I]T3 nuclear receptor binding by L-T3, triac, D-T3, and L-T4 with isolated GH_1 cell nuclei in vitro. GH_1 cell nuclei were prepared from cells that were cultured and exchanged with Ham's F-10 medium which contained hypothyroid calf serum as described in Methods. Purified nuclei equivalent to 50 μg of DNA were incubated with L-[^{125}I]T3 at 0.25 nM and the concentrations of L-T3 (○), triac (▲), D-T3 (□), and L-T4 (●) as indicated in the figure. The L-[^{125}I]T3 bound to receptor without added nonradioactive hormone, was 103 fmol/100 μg DNA±6%, and the analogue results are expressed as a percentage of this value. The nonspecific bound fraction of L-[^{125}I]T3, determined by incubating 0.25 nM L-[^{125}I]T3 plus a 1000-fold excess of nonradioactive L-T3, was 12 fmol±2.2/100 μg DNA. This value was subtracted from the L-[^{125}I]T3 bound from each point in the figure to determine the L-[^{125}I]T3 bound to the receptor. The results reflect the mean of triplicate determinations with <±8% variation.

intact cells under serum-free conditions as compared with isolated GH_1 cell nuclei, which implies a relative decrease in cell entry of D-T3 as compared with the other hormonal analogues.

Influence of L-T3 on growth hormone induction and thyroid hormone receptor depletion in dispersed rat anterior pituitary cells. In the rat, thyroidectomy markedly lowers the pituitary and serum growth hormone content, and this is rapidly restored by thyroid hormone administration (9–12). The onset and time-course of growth hormone accumulation in GH_1 cells (36) and in the rat pituitary in vivo (12) are essentially identical, which suggests that thyroid hormone induces this response by the same mechanism in GH_1 cells and the pituitary somatotroph. It has been reported (13) that thyroid hormone does not induce an increase in growth hormone production in dispersed anterior pituitary cells. The medium used in these studies, however, was supplemented with fetal calf serum which contains high endogenous thyroid hormone levels. Therefore, with medium that contained hypothyroid calf serum, we examined whether L-T3 induces growth hormone production in dispersed rat pituitary cells, and we also studied whether L-T3 elicits a reduction in pituitary nuclear receptor levels.

Table I illustrates the effect of L-T3 on the growth hormone response in rat pituitary cells as determined by L-[^{35}S]methionine incorporation and radioimmunoassay and compares this with a parallel study in GH_1 cells. L-T3 induced a fivefold increase in growth hormone production in GH_1 cells, and the medium growth hormone represents 98% of the total growth hormone in the culture. In rat pituitary cells, L-T3 induced a twofold increase in the growth hormone content per culture (medium plus cells), and in contrast with GH_1 cells, ≅70% of the growth hormone of the culture accumulated in the medium. The difference in distribution between GH_1 cells and rat pituitary cells most likely reflects the fact that pituitary cells store growth hormone (37) whereas GH_1 cells do not store significant amounts of growth hormone and the intracellular $t_{1/2}$ of growth hormone in GH_1 cells is ≅60–70 min (5). The net increase in growth hormone accumulation was

TABLE I

Comparison of Growth Hormone Induction by L-T3 in Dispersed Rat Pituitary Cells and GH_1 Cells

	Dispersed rat pituitary cells				Cultured GH_1 cells		
	Growth hormone synthesis*	Growth hormone production			Growth hormone production		
	Cells	Cells	Medium	Total	Cells	Medium	Total
	cpm/100 μg DNA × 0.001	*μg/24 h/100 μg cell protein*			*μg/24 h/100 μg cell protein*		
Control	157.7	5.80	12.14	17.94	0.05	2.3	2.35
L-T3	296.9	9.50	24.55	34.05	0.26	11.15	11.41

50,000 GH_1 cells or dispersed rat pituitary cells were inoculated into 2-cm^2 multiwell culture plates and were treated with Ham's F-10 media which contained 10% hypothyroid calf serum as described in Methods. The L-T3 concentration was 5 nM, and the growth hormone production rates reflect the growth hormone which accumulated in the medium between 24 and 48 h of incubation. The intracellular growth hormone levels reflect that at 48 h of incubation. Cell protein was 23 μg±10% in the rat pituitary cell cultures and 30 μg±10% in the GH_1 cell cultures. The growth hormone production responses reflect the mean of triplicate cultures with <±8% variation.

* In a separate study at 48 h, growth hormone synthetic rates were determined by a 15-min incubation with L-[^{35}S]methionine (50 μCi/ml) followed by selective immunoprecipitation of the intracellular radiolabeled growth hormone as previously described for GH_1 cells (5). The incorporation of L-[^{35}S]methionine into total cell protein per 100 μg DNA was 398.9 × 10^4±10% for the control cells and 430.9 × 10^4±12% for the L-T3-incubated cells.

higher for the pituitary cells (16.11 μg/100 μg protein) than for the GH_1 cells (9.06 μg growth hormone/100 μg cell protein). In previous studies using L-[^{35}S]methionine incorporation followed by selective immunoprecipitation of the radiolabeled protein with anti-rat growth hormone antibody, we demonstrated that the growth hormone response in GH_1 cells determined by radioimmunoassay was a valid measure of the rate of growth hormone synthesis (5, 8, 36). Table I also illustrates that the induction of the growth hormone response in pituitary cells, as measured by radioimmunoassay, parallels the growth hormone synthetic rate determined by L-[^{35}S]methionine incorporation, which indicates that the regulation of growth hormone production in the rat pituitary cells reflects changes in growth hormone synthetic rates.

Table II illustrates an experiment in which we compared the L-T3-mediated receptor depletion in GH_1 cells and rat pituitary cells after a 24-h incubation with 5 nM L-[^{125}I]T3. Based on a short-term incubation (1.5 h), the number of receptors in GH_1 cells was 1.65-fold that of dispersed pituitary cells. In both cases, the 24-h incubation with 5 nM L-[^{125}I]T3 elicits a reduction in nuclear receptor levels which was 25% with the dispersed pituitary cells and 48% with GH_1 cells.

DISCUSSION

In this study with GH_1 cells and serum-containing medium we have examined the detailed relationship between the relative affinity of L-T3, triac, D-T3, and L-T4 for the nuclear receptor and the induction of growth hormone synthesis. An excellent relationship was observed between the relative receptor affinity and induced biological responses, which is supportive of a nuclear mechanism of action for triac and D-T3. Half-maximal growth hormone induction occurred at 0.17 nM L-T3, 0.19 nM triac, 2.5 nM D-T3, and 9 nM L-T4. The value determined for L-T3 (0.17 nM) is in good agreement with the value we previously reported in GH_1 cells (0.22 nM) (6).

Subsequently, similar values for L-T3 were reported in different cell culture systems. Gershengorn (38) recently reported that 0.2 nM L-T3 resulted in half-maximal inhibition of thyroid-stimulating hormone produc-

TABLE II

L-T3-Mediated Reduction in Thyroid Hormone Nuclear Receptor Levels in Dispersed Rat Pituitary Cells and GH_1 Cells

	Thyroid hormone nuclear receptor levels	
Incubation time	Dispersed rat pituitary cells	GH_1 cells
h	*fmol L-[^{125}I]T3 bound/100 μg DNA*	
1.5	68.0±1.7	132.2±2.2
24	51.1±1.3	63.5±1.6

The description of cell inoculation, culture, and media exchange with Ham's F-10 medium which contained 10% hypothyroid calf serum are described in Methods. The results represent the mean±range of quadruplicate flasks and are corrected for the magnitude of nonspecific binding which was equal to 5.1 fmol L-[^{125}I]T3 bound/100 μg DNA in the dispersed rat pituitary cells and 4.6 fmol L-[^{125}I]T3 bound/100 μg DNA in the GH_1 cells. The DNA content was 20±2.2 μg/flask in the dispersed rat pituitary cells and 30±3 μg/flask in the GH_1 cell cultures.

tion in mouse thyrotrophic tumor cells. In addition, Tsai and Chen (39) recently reported that L-T3 induces β-adrenergic receptors in cultured rat myocardial cells, and a half-maximal increase in β-adrenergic receptor levels was induced by 0.3 nM L-T3. Therefore, the dose-response characteristics for L-T3 induction of growth hormone synthesis appear to be a general response characteristic for L-T3-inducible functions in cell culture and are not unique for the growth hormone response.

In addition to regulating the growth hormone response, we have demonstrated that L-T3 elicits a time- and dose-dependent reduction in thyroid hormone nuclear receptor levels in GH_1 cells (6, 7). We have presented evidence that the nuclear receptor exists as two functional populations: one which represents 40–50% of total receptor, is depletable by L-T3, and is involved in the regulation of growth hormone response; and a nondepletable fraction which is not involved in the control of growth hormone synthesis but may have other regulatory functions (7). The main implications of this observation is that a partial reduction of the total receptor (depletable plus nondepletable) would result in a significant reduction in the magnitude of the growth hormone response (7). Both putative populations of receptor had identical affinities for L-T3, and the initial rate of disappearance of the depletable receptor population was a direct function of the occupancy by L-T3 and new steady-state levels of receptor were achieved within 24 h of incubation (6, 7). In this study we demonstrated that both putative populations of receptor also have identical affinities for each analogue examined (Fig. 2), and modulation of nuclear receptor levels also occurs with each analogue as a direct function of the iodothyronine fractional occupancy of the receptor binding site (Fig. 3).

Although studies with serum-containing medium demonstrate an excellent relationship between the relative association of iodothyronine with the receptor and the growth hormone response, the relative affinities as compared with L-T3 differ markedly from that reported with isolated hepatic nuclei in vitro (21–23). These differences may reflect differential serum binding of iodothyronine analogues, intrinsic differences in cell entry, or an intrinsic difference in the nuclear receptor derived from GH_1 cells and rat liver. Based on the estimation of the free L-T3 and L-T4 levels in serum-containing medium, the discrepancy for L-T4 appears to reflect enhanced serum binding of L-T4 relative to L-T3.

To further examine these questions we compared the relative affinity of iodothyronine analogues with nuclear receptors in intact GH_1 cells, using serum-free conditions, and in isolated GH_1 cell nuclei in vitro. The results obtained with isolated GH_1 cell nuclei are similar to that previously reported with isolated rat liver nuclei (21, 23). Triac had 400%, D-T3 had 53%, and L-T4 had 10% of the affinity for the receptor as compared with L-T3. The affinity of triac and L-T4 relative to L-T3 in intact cells was essentially identical with that observed in isolated GH_1 cell nuclei. In contrast, the relative affinity of D-T3 is 5.5-fold lower in intact cells with serum-free conditions as compared with isolated GH_1 cell or liver nuclei, which implies a relative decrease in cell entry of D-T3 as compared with the other hormonal analogues. This suggests that differences in iodothyronine cell entry may in part explain a discrepancy between the relative receptor affinity determined with isolated hepatic nuclei in vitro and the biologic response elicited after in vivo injection. Furthermore, if selective differences in tissue permeability to iodothyronines occurred, certain analogues may be more effective in eliciting thyroid hormone effects in certain tissues after in vivo injection. This is suggested by the observations of Oppenheimer et al. (40) in which D-T3 was 2.5-fold more effective in inhibiting L-[^{125}I]T3 binding to hepatic nuclei as compared with heart nuclei after in vivo injection.

Thyroid hormone increases the pituitary content of growth hormone in the thyroidectomized rat (12) with the same onset and time-course as in GH_1 cells (36). This suggests that thyroid hormone acts to stimulate growth hormone production directly at the level of the somatotroph in vivo and not through intervening hypothalamic factors. In this study, with medium supplemented with hypothyroid calf serum, we demonstrate that L-T3 induces growth hormone synthesis and modulates thyroid hormone nuclear receptor levels in dispersed rat pituitary cells in the same fashion as in GH_1 cells. The net increase in growth hormone production per 100 μg of protein (L-T3-induced minus control) in dispersed pituitary cells is twofold greater than GH_1 cells, and this is likely greater because the somatotroph population represents ≅50% of the total pituitary cell population (41).

In addition, L-T3 incubation elicits a 25% reduction in nuclear receptor levels in dispersed pituitary cells. Because dispersed anterior pituitary cells are a heterogeneous cell population we do not know whether the depletion of the receptor represents a uniform decrease in all cell types or is restricted to the somatotroph population. Because the depletion of the receptor does not appear to occur in all cells, e.g., hepatic cells (42, 43), it remains possible that the depletion of receptor in the anterior pituitary population is restricted to the somatotroph cell. The somatotroph cell represents ≅50% of the anterior pituitary cell population (41). If receptor modulation were restricted to these cells, the 25% decrease of receptor in the dispersed pituitary cells would represent a 50% reduction of receptor in

the somatotroph population, a value similar to that of GH_1 cells (6, 7). It remains possible that the L-T3 reduction in receptor in dispersed pituitary cells reflects an absolute reduction in the thyrotroph population. This seems unlikely, however, because thyrotropic cells represent <11% of the pituitary cell population (41); have less nuclear receptor than somatotrophic cells (38); and, as estimated by immunoperoxidase staining, grow poorly under culture conditions (44).

If thyroid hormone controlled the level of thyroid hormone nuclear receptors in the somatotroph in vivo, extrapolation of our observations on hormone-mediated reduction of the receptor and the growth hormone response in GH_1 cells (6, 7) may allow prediction of the growth hormone response in different thyroidal states in vivo. In the hypothyroid rat thyroid hormone nuclear receptor levels would be high, and the somatotroph would be expected to show a marked increase in the growth hormone response to a single injection of thyroid hormone. This has been observed by Hervas et al. (12). In addition, with chronic thyroid hormone administration in euthyroid rats sufficient to induce thyrotoxicosis, reduction of the receptor would be expected to lower the magnitude of the growth hormone response relative to that of the euthyroid state.

Only a limited number of studies, however, have examined the effect of chronic thyroid hormone administration on the growth hormone response in the rat (9, 45). In early studies relating thyroid function and the growth hormone response, Solomon and Greep (9) estimated the growth hormone content of the rat pituitary to be 26 μg/mg pituitary±36.1% in the thyroidectomized rat, and 170 μg/mg pituitary±36% in the euthyroid rat. After thyroid hormone administration sufficient to induce thyrotoxicosis, the growth hormone content of the pituitary decreased to 75.2 μg/mg pituitary ±35.5%. Recently, Coulombe et al. (45) reported that daily injection of thryoid hormone in euthyroid rats, sufficient to produce thyrotoxicosis, decreased the pituitary growth hormone content as compared with euthyroid controls. Therefore, the growth hormone response parameters in the rat as a function of the thyroidal state approximates a bell-shaped curve with a reduction in pituitary growth hormone content both in the hypothyroid and thyrotoxic rate.

Studies on the influence of thyroid hormone on the physiology of growth hormone in man tend to parallel the observations in the rat although the studies demonstrate some variability in the response (46, 47). In a series of reported studies, between 40 and 80% of hypothyroid patients demonstrated a blunted growth hormone response to insulin-induced hypoglycemia, and in the majority of these patients the response normalized after thyroid hormone administration and restoration of the euthyroid state (46–48). In thyrotoxicosis, insulin-induced hypoglycemia tends to result in subnormal growth hormone responses as compared with euthyroid controls, and this is restored to normal levels after restoration of euthyroidism (49, 50). Finkelstein et al. (50) quantitated the growth hormone plasma half-life and 24-h secretory rates in hyperthyroid patients and euthyroid controls. The plasma half-life was unchanged in hyperthyroidism but the 24-h secretory rates were reduced ≅43%, and this was restored to normal after restoration of the euthyroid state. Therefore, although studies in man do not allow estimation of pituitary growth hormone content, the general pattern of growth hormone response as a function of thyroidal state is qualitatively similar to that determined experimentally in the rat.

Modulation of the thyroid hormone nuclear receptor level in the somatotroph provides a plausable explanation for the influence of the thyroidal state on the growth hormone response in vivo. By this mechanism, alterations in receptor levels would be associated with changes in the production rates and steady-state levels of growth hormone mRNA. Variations in the growth hormone response in different thyroidal states, however, may be secondary to changes in the turnover or degradation of pituitary growth hormone or growth hormone mRNA. Resolution of the detailed aspects of these parameters should provide an understanding of the influence of the thyroidal state on the growth hormone response.

ACKNOWLEDGMENTS

We wish to thank Mary McCarthy for expert secretarial assistance.

These studies were supported by National Institutes of Health grant AM 16636.

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