

Thyroid Hormone Action

DEMONSTRATION OF SIMILAR RECEPTORS IN ISOLATED NUCLEI OF RAT LIVER AND CULTURED GH₁ CELLS

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ABSTRACT High-affinity, limited-capacity nuclear binding activities, putative receptors for triiodothyronine, were detected after incubation of hormone with intact rat pituitary GH₁ cells in culture, isolated GH₁ cell nuclei, or rat liver nuclei. The total number of triiodothyronine binding sites per nucleus was similar in each case (approximately 8,000). The estimated equilibrium dissociation constants were virtually identical in isolated GH₁ cell nuclei and rat liver nuclei, and both values were similar to that determined in intact GH₁ cells. These results suggest that mechanisms of thyroid hormone action defined in cell culture could apply to thyroid hormone regulatory effects *in vivo*.

INTRODUCTION

Thyroid hormones have a profound effect on the growth, development, and metabolism of virtually all tissues of higher organisms (1). In spite of extensive *in vivo* studies, the mechanism of action of the thyroid hormones has not been defined. We have recently demonstrated that thyroxine (T₄)¹ and triiodothyronine (T₃) induce a maximal threefold increase in the rate of growth of GH₁ cells, a rat pituitary tumor cell line, in culture (2). The estimated free hormone concentrations in culture inducing a half-maximal biologic effect were 0.8×10^{-11} M for T₃ and 1×10^{-10} M for T₄ (2).

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¹Abbreviations used in this paper: K_d , equilibrium dissociation constant; STM buffer: 0.25 M sucrose, 20 mM tris, 1.1 mM MgCl₂, pH 7.85 at 25°C; STM-Triton buffer: 0.25 M sucrose, 20 mM tris, 1.1 mM MgCl₂, 0.5% Triton X-100, pH 7.85 at 25°C; T₃, triiodothyronine; T₄, thyroxine.

Studies on the binding of [¹²⁵I]T₃ and [¹²⁵I]T₄ to subcellular fractions after incubation of hormone with intact cells demonstrated high-affinity, limited-capacity binding sites in the nuclear fraction (3, 4). The estimated equilibrium dissociation constants (K_d) determined by Scatchard analysis were 2.9×10^{-11} M for T₃ and 2.5×10^{-10} M for T₄ (3, 4). These affinities correlate well enough with the hormone concentrations inducing a half-maximal biologic effect in this system to suggest that these high-affinity nuclear binding activities function as receptors for the thyroid hormones. This description of high-affinity, limited-capacity binding after incubation of the thyroid hormones with intact cells in culture is similar in nature to the observations of Oppenheimer, Koerner, Schwartz, and Surks after injection of hormone into intact rats (5).

Although detailed equilibrium dissociation constants (K_d) were not described, we have previously reported that high-affinity, limited-capacity T₃-binding sites could also be demonstrated by incubation of isolated GH₁ cell nuclei directly with hormone *in vitro* (3, 4).

This communication demonstrates that high-affinity, limited-capacity binding for thyroid hormone can be resolved in isolated nuclei from rat liver as well as GH₁ cells. The similarity in affinity and number of binding sites per nucleus suggests that our observations of the mechanisms of thyroid hormone binding in cell culture might also serve to define the details of thyroid hormone-receptor interaction *in vivo*.

METHODS

Cell suspensions and media. GH₁ cells were obtained from the American Type Culture Collection, Rockville, Md. Cell cultures were routinely grown in 95% air, 5% CO₂ with Ham's F-10 media supplemented to 15% with horse serum and to 2.5% with fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). The total hormone concentrations present in these sera are physiologic and are

approximately 2×10^{-9} M for T3 and 1×10^{-7} M for T4 as determined by gas-liquid chromatography and specific radioimmunoassay (2).

Binding studies with intact cells. For binding studies, the cells were harvested in the late logarithmic phase of growth with a rubber policeman and centrifuged at 500 *g* for 5 min. The cell pellet was washed three times with 10 ml of serum-free Ham's F-10 media by repeated dispersion and centrifugation. The cells (1.5×10^6) were then suspended in 1.0 ml of serum-free media with various concentrations of [125 I]T3 and incubated at 37°C for 2½ h. Nuclear binding of T3 in intact cells attains an equilibrium after 2–3 h of incubation (3, 4). [125 I]T3 (initial sp act 320 Ci/mmol) was obtained from Abbott Laboratories, North Chicago, Ill. All radioactive analysis was determined with a gamma spectrometer. After incubation with hormone, the cell suspensions were centrifuged at 500 *g* for 5 min, and the media supernate was saved for determination of hormone concentration. All further procedures were carried out at 0°C. The final pellet was homogenized in 10 vol of STM buffer (0.25 M sucrose, 20 mM tris, 1.1 mM MgCl₂, pH 7.85 at 25°C) by 15 strokes at 5,000 rpm with a motorized Teflon pestle (Tri R Instruments, Rockville Centre, N. Y.). The homogenate was centrifuged at 800 *g* for 10 min. The homogenate pellet was used to prepare nuclei by two successive suspensions and centrifugations in 10 ml of STM-Triton buffer (4, 6). Triton X-100 was obtained from Packard Instrument Co., Inc., Downers Grove, Ill.

As we have previously reported, nuclei prepared with Triton X-100 were free of cytoplasmic contamination as estimated by phase contrast microscopy and staining with aceto-orcein as well as azure C (4). Binding of [125 I]T3 to nuclei was not altered by isolation with Triton X-100, and the magnitude of limited-capacity T3 binding was similar when nuclei were isolated by ultracentrifugation with 2.2 M sucrose (4). Both methods of nuclear isolation prepared GH₁ cell nuclei with protein/DNA ratios of 2.0 and RNA/DNA ratios of 0.22 (4). The yield of nuclei with both methods was approximately 85–90% as calculated by DNA determination.

Protein, DNA, and RNA were determined as previously described (7–9). One million GH₁ cells contain 13.0 µg of DNA (2). Nuclear bound [125 I]T3 was determined with a gamma spectrometer.

Preparation of nuclei from GH₁ cells and rat liver. GH₁ cell nuclei were prepared by the homogenization procedure with Triton X-100 indicated above. To prepare rat liver nuclei, euthyroid male (Sprague-Dawley) rats were killed by subluxation of the cervical spine. Approximately 0.5 g of liver was excised and minced in chilled STM buffer and then homogenized with 15 strokes in at least 10 vol of chilled STM buffer at 5,000 rpm with a motorized pestle. The homogenate was centrifuged at 800 *g* for 10 min. The homogenate pellet was then suspended by a loose pestle at 3,000 rpm in STM-Triton buffer. This suspension was centrifuged at 800 *g* for 10 min. This procedure was repeated two additional times. The rat liver nuclei had protein/DNA ratios of approximately 3.0 and RNA/DNA ratios of approximately 0.3 (6).

Demonstration of nuclear receptors by direct incubation with isolated nuclei. The incubation mixture, prepared at 0°C, contained a nuclear suspension equivalent to 20–50 µg of DNA, 0.25 M sucrose, 20 mM Tris, 1.1 mM MgCl₂, pH 7.85 at 25°C, 2.0 mM EDTA, and 5 mM dithiothreitol in volume of 0.9 ml. [125 I]T3 was added to the incubation

mixture in 0.1 ml of serum-free Ham's F-10 media. Hormone binding was studied by incubating the nuclear preparation at 37°C. We have previously demonstrated that the binding reaction in isolated nuclei attains an equilibrium within 30–40 min at 37°C (3, 4). Therefore, after 45 min of incubation, the samples were chilled in an ice bath and then centrifuged at 1,000 *g* for 8 min. The supernate was saved for determination of hormone concentration. The nuclear pellet was suspended in 1.0 ml of STM-Triton buffer and centrifuged again. [125 I]T3 binding was determined in the resultant pellet with a gamma spectrometer.

To determine the nature of the bound 125 I radioactivity, the nuclear pellet was incubated in 0.5 M NaCl, 0.015 N NaOH, at 25°C. This dissociated greater than 98% of the radioactivity. The 125 I radioactivity was subjected to chromatographic analysis as previously described (4, 10). The 125 I radioactivity in the incubation buffer was also subjected to a similar analysis. As we previously reported for intact GH₁ cells, the nuclear bound radioactivity was greater than 97% [125 I]T3, and no [125 I]T3 degradation was detected in the buffer after the incubation procedure (4).

We also chromatographed the buffer at 4°C on Sephadex G-25 (fine) columns to exclude the possibility that nuclear binding activity leaked into the buffer during the incubation. This might diminish the free hormone concentration and result in an error in the estimation of the equilibrium dissociation constant. The incubation buffer, 0.45 ml, was applied to a 0.9 × 4.0-cm (2.5 ml) column in which the Sephadex had been preswollen with STM buffer. The columns were then eluted with 1.5 ml of STM buffer. This procedure can completely separate bound from free hormone. After a 45-min incubation with nuclei, more than 99.5% of the [125 I]T3 in the buffer remained in a free unbound form.

RESULTS

Binding of T3 to nuclei after incubation with intact cells. Fig. 1 illustrates an estimation of the maximal binding capacity and the apparent equilibrium dissociation constant (K_d) for T3 determined after incubation of intact cells for 2½ h with various concentrations of [125 I]T3 (11). The apparent K_d for T3 was 2.9×10^{-11} M and the maximal binding capacity was 100×10^{-15} mol of T3 bound per 100 µg DNA. This represents approximately 7,800 molecules of T3 bound per cell nucleus at saturation.

Binding of T3 after incubation with isolated nuclei. Fig. 2 illustrates an estimation of the apparent K_d for T3 determined by incubation of GH₁ cell nuclei with various concentrations of [125 I]T3 for 45 min at 37°C. The estimated K_d for T3 was 1.65×10^{-10} M, and the maximum binding capacity was 104×10^{-15} mol of T3 bound per 100 µg DNA. This represents approximately 8,100 binding sites for T3 per GH₁ cell nucleus. Fig. 3 illustrates an estimation of the K_d for T3 determined by incubation of rat liver nuclei with [125 I]T3 for 45 min at 37°C. The apparent K_d for rat liver nuclei was 2.1×10^{-10} M, which was similar in magnitude to that obtained for isolated GH₁ cell nuclei, and the maximal binding capacity was 100×10^{-15} mol of T3 bound per

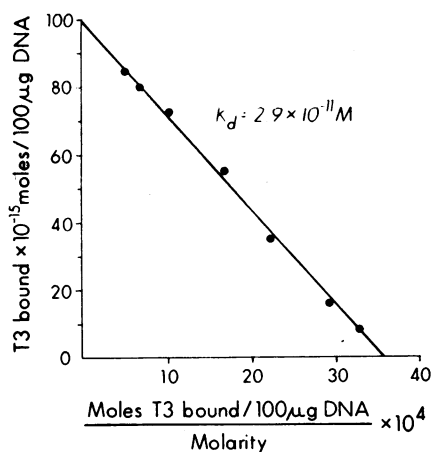


FIGURE 1 Scatchard analysis of nuclear binding of [¹²⁵I]T3 after incubation with intact GH₁ cells. GH₁ cells (1.5×10^6) were incubated in 1.0 ml of serum-free media with various concentrations of [¹²⁵I]T3 at 37°C for 2½ h. As described in the section on Methods, the nuclei were isolated and the media saved for determination of hormone concentration. The binding at each hormone concentration represents the average of three determinations which did not vary by more than 5%.

100 μg DNA. This represents approximately 7,800 binding sites for T3 per rat liver nucleus.

The total number of binding sites per cell nucleus determined after incubation of [¹²⁵I]T3 with intact GH₁ cells is similar in magnitude to that determined after incubation of [¹²⁵I]T3 with isolated GH₁ cell nuclei or rat liver nuclei. This suggests that the nuclear binding

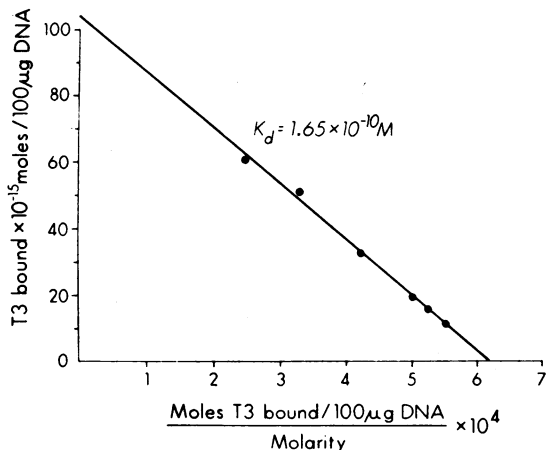


FIGURE 2 Scatchard analysis of nuclear binding of [¹²⁵I]T3 after incubation with isolated GH₁ cell nuclei. Isolated GH₁ cell nuclei were incubated with various concentrations of [¹²⁵I]T3 at 37°C for 45 min and then centrifuged and washed with STM-Triton buffer as described in the section on Methods. The binding at each hormone concentration represents the average of three determinations which did not vary by more than 5%.

activity determined in intact cells and isolated nuclei is likely to be identical and that the binding activity in isolated nuclei is stable at 37°C under the conditions of incubation. The magnitude of the K_d for T3 determined in intact GH₁ cells and isolated GH₁ cell nuclei or rat liver nuclei are sufficiently similar to also suggest that similar nuclear T3-binding activities are detected in intact cells and isolated nuclei. The difference in affinity is likely due to obvious differences in the nuclear environmental conditions of intact cells and the isolated nuclear binding assay.

DISCUSSION

We have reported that GH₁ cells contain high-affinity, limited-capacity binding sites for T3 in the cell nucleus but not in the crude cytosol or mitochondrial fractions (3, 4). The concentration of T3 inducing a half-maximal biologic effect on cell growth and glucose utilization and half-maximal nuclear binding are sufficiently similar to suggest that these high-affinity nuclear binding activities function as putative receptors for the thyroid hormones (2-4).

Early effects of T3 on increasing RNA-polymerase activity can be detected within 2-3 h after incubation of hormone with intact cells (12). This early effect of T3 on RNA-polymerase activity as well as the localization of putative receptors in the cell nucleus supports the concept that thyroid hormone modulates gene activity (13).

The current study utilized isolated GH₁ cell nuclei, intact GH₁ cells grown with euthyroid calf serum, and

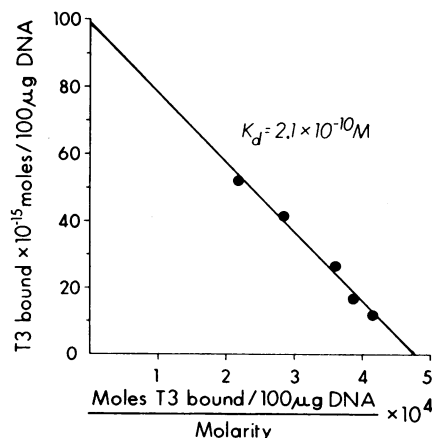


FIGURE 3 Scatchard analysis of nuclear binding of [¹²⁵I]T3 after incubation with isolated rat liver nuclei. Isolated rat liver nuclei were incubated with various concentrations of [¹²⁵I]T3 at 37°C for 45 min and then centrifuged and washed with STM-Triton buffer as described in the section on Methods. The binding at each hormone concentration represents the average of three determinations which did not vary by more than 5%.

liver nuclei obtained from a euthyroid rat. It is not likely, however, that hormone bound to nuclei endogenously before isolation significantly altered our *in vitro* estimations of the K_d and number of nuclear binding sites for T3. Our previous determination of the K_d for T3 nuclear binding and the total number of binding sites per nucleus were estimated with intact GH₁ cells depleted of thyroid hormone with hypothyroid calf serum (3, 4). From the concentration binding curves, it can be calculated that at estimated euthyroid-free T3 concentrations, 8×10^{-12} M, approximately 20% of the T3 nuclear binding sites are occupied and 80% are unoccupied (4). The half time of dissociation *in vitro* of T3 endogenously bound to nuclei before isolation was estimated to be 20–25 min (14). Therefore, during the 45-min *in vitro* incubation in the current study, approximately 75% of endogenously bound nonradioactive T3 would dissociate. This would, therefore, permit an estimation of approximately 95% of the total number of nuclear binding sites for T3. This is confirmed by the fact that a similar number of high-affinity nuclear T3-binding sites was estimated with intact GH₁ cells cultured with hypothyroid calf serum (4). It can also be estimated, by using nuclei equivalent to 20–30 μ g of DNA, that the final concentration resulting from complete dissociation of endogenously bound T3 would be less than 5% of the concentration of T3 that results in half-maximal nuclear binding *in vitro*. Therefore, T3 bound to nuclear sites before isolation should not significantly affect the *in vitro* estimation of the number of high-affinity T3 binding sites or the magnitude of the equilibrium dissociation constant.

As we have demonstrated, the number of high-affinity binding sites for T3 per nucleus are virtually identical for intact GH₁ cells, isolated GH₁ cell nuclei, and rat liver nuclei. The estimated K_d for T3 determined for rat liver nuclei *in vitro* is also in excellent agreement with that determined *in vivo* by Oppenheimer, Schwartz, Koerner, and Surks (15). This suggests that our studies of thyroid hormone action in cell culture might also serve to define the details of thyroid hormone-receptor interaction as well as the mechanisms of thyroid hormone regulatory effects *in vivo*.

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