Investigations on myelination in vitro: Thyroid hormone receptors in cultures of cells dissociated from embryonic mouse brain

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(Received 2 February 1981)

Brain cells dissociated from 15-day-old embryonic mice and grown in culture contain both cytosolic and nuclear receptors for L-3,5,3'-triiodothyronine (L-T<sub>3</sub>). K<sub>D</sub> values for L-T<sub>3</sub> of 3.05 x 10<sup>-9</sup> M and 4.2 x 10<sup>-9</sup> M were determined with the cytosolic and nuclear receptors respectively. These cultured cells, which are suitable for studying the regulation of myelination by T<sub>3</sub> in vitro, display a high specificity for L-T<sub>3</sub> in that the receptors for L-T<sub>3</sub> do not bind D-T<sub>3</sub>, D-thyroxine, L-diiodothyronine, or DL-thyronine, and bind only small amounts of L-thyroxine.

A number of studies have shown that the mammalian central nervous system is dependent for its normal growth and development, at an early critical age, on thyroid hormone (1-3). The anomalies which arise from a hypothyroid state can be rectified by hormone therapy during this early critical age. This critical period of maturation coincides with the start of active myelination. In the hypothyroid neonatal rat, the process of myelin formation is hampered (4,5) and a lesser amount of myelin deposition takes place (6,7). In contrast to this, a hyperthyroid state leads to precocious myelination in intact rats (8). Similar events have been induced in cultured cerebellar explants obtained from newborn rats (9), and in primary cultures of dissociated embryonic mouse brain cells (10,11).

The primary culture system of cells dissociated from embryonic mouse brain has proven particularly satisfactory for studying the regulation of the onset of myelination by thyroid hormone (11,12). No other type of culture system has provided sufficient tissue to permit biochemical measurements of the influence of thyroid hormone on myelination. The advantages of the culture system over the whole animal are that the primary role of a hormone can be assessed without interference from other hormones and that the mechanism of action of the hormone can be studied in greater depth at the molecular level. Because binding of a hormone to the cell (13,14) is perhaps the first of a series of important steps the hormone undergoes in regulating the metabolism of the cell, we initiated studies to detect the presence of nuclear and cytosolic receptors for L-3,5,3'-triiodothyronine (T<sub>3</sub>) in cultured cells dissociated from brain. A portion of this work has been previously presented (15).

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Materials and Methods

Calf serum (heat-inactivated), Dulbecco's modified Eagle's medium, and the antibiotic mixture were purchased from Gibco, Grand Island, NY. Serum from thyroidectomized calf was from Rockland Farms, Gilbertsville, PA. Sterile culture flasks as well as culture dishes were supplied by Fischer Scientific Co., Pittsburgh, PA. Polysine (Mr = 80 000) and T3 were from Sigma Chemical Co., St. Louis, MO. Activated charcoal was obtained from Bio-Rad, Rockville Centre, NY, and blue dextran was obtained from Pharmacia. Tris and B-mercaptoethanol were purchased from Bio-Rad. [125I]-3,5,3'-triiodothyronine (719 Ci/mmol) was from New England Nuclear, Boston, MA. Pregnant mice used in the present study, were supplied by Charles River Laboratories, Boston, MA.

Primary cultures. Fifteen-day-old mouse embryos numbering 10 to 14 per litter were removed under sterile conditions by caesarean section (16) and placed in Dulbecco's modified Eagle's medium supplemented with glucose (600 mg%), 0.23% sodium bicarbonate, 90 units/ml of penicillin, 90 µg/ml of streptomycin, and 0.225 µg/ml of fungizone, adjusted to pH 7.0. Cerebral hemispheres (cerebra) were dissected and temporarily placed in the medium. Cells were then dissociated mechanically (17) by passing through a nylon mesh (82 µm) and were placed in 250-ml polysine-coated plastic tissue-culture flasks as described earlier (10,11). The medium in the flasks together with unattached cells was carefully removed on the 4th day and replaced by 10 ml of the fresh medium containing 15% calf serum. Subsequently, the medium was changed once a week.

Subcellular fractionation. Nuclear and soluble fractions were obtained from cells grown 20 days in vitro, half of which were grown in the presence of either hypothyroid (1.2 µg of T4/ml and <25 ng of T3/100 ml) or normal (5.8 µg of T4/ml and 110 ng of T3/100 ml) calf serum from day 11 onward. The procedure followed to get these subcellular fractions was essentially that of Inoue et al. (18) with some modifications (19). The nuclear pellet was washed by first resuspending it in 2 vol. of buffer A (20 mM Tris, 0.25 M sucrose, 2 mM CaCl2, 1 mM MgCl2, 5% glycerol, 0.1 mM B-mercaptoethanol, pH 7.6) containing 0.5% Triton X-100. More buffer A containing 0.5% Triton X-100 was added to bring the volume-to-weight ratio to 5:1. The resuspended mixture was centrifuged at 800 x g for 7 min and this washing was repeated 2 times. This purified nuclear pellet was resuspended in buffer B (20 mM Tris, 0.25 M sucrose, 1 mM MgCl2, 0.1 mM B-mercaptoethanol, 5% glycerol, pH 7.6) and used in the binding experiments. The nuclei obtained in the fractionation procedure were of reasonably good purity and had a protein-to-DNA ratio of approximately 2.8, which agrees well with the value obtained by others (19).

Binding assay. The assay was similar to that used by Geel (20). The concentration of nuclear and cytosolic proteins used (70 µg protein) was in the range where binding was directly proportional to protein concentration. In principle, the binding studies were carried out by incubating cytosolic and nuclear suspensions with [125I]T3 along with increasing amounts of non-radioactive T3 hormone. The reaction mixture contained an aliquot of nuclear and cytosolic suspensions (~70
The reaction mixture was pre-incubated for 45 min at 25°C to dissociate any endogenously bound T₃, and then was incubated for 2 h at 0°C. The amount of non-specific binding to the nuclear and cytosolic receptors was obtained by incubating them with radioactive hormone along with a large excess (5 nmol/tube) of non-radioactive T₃ under conditions identical to that described above. The value thus obtained was subtracted from the total bound radioactivity to obtain specific binding. At the end of the incubation, the bound hormone was separated from the free hormone by the addition of 0.2 ml of Dextran-coated charcoal suspension (5% charcoal and 1% Dextran in 20 mM Tris-HCl, pH 7.5). The tubes were vortexed for 5 s, kept on ice for 10 min, and then centrifuged in an International PR-2 centrifuge at 2000 r.p.m. for 1 h. The clear supernatant containing radioactive hormone bound to receptor was removed by aspiration and an aliquot (0.2 ml) was counted in an Autogamma spectrometer to determine the radioactivity. The Dextran-coated-charcoal procedure effectively removed more than 98% of the free [¹²⁵I]T₃ at all concentrations used in the absence of the receptor proteins. The free hormone concentration was obtained by subtracting the total [¹²⁵I]T₃ bound from the total [¹²⁵I]T₃ c.p.m. added per assay.

Results

The data obtained from studies on the binding of [¹²⁵I]T₃ to nuclear and cytosolic receptor proteins was subsequently analyzed by drawing Scatchard plots to determine the equilibrium dissociation constant (K_D) and the number of receptor sites (n). Fig. 1 is representative of such a Scatchard plot depicting the binding of [¹²⁵I]T₃ to nuclear fractions isolated from cells grown in the presence of either normal or hypothyroid calf serum. Fig. 2 is a Scatchard plot of the data obtained from the binding of [¹²⁵I]T₃ to the cytosolic fraction isolated from the cultures grown under normal or hypothyroid conditions.

A summary of the results obtained from the Scatchard plots (Figs. 1 and 2) depicting the equilibrium dissociation constants (K_D) and the number of binding sites (n) is given in Table 1. The K_D for the T₃ binding as well as the number of receptor sites in the nuclear fractions from cells grown in the presence of either normal (4.20 x 10⁻⁹ M and 0.41 pmol/mg protein respectively) or hypothyroid (5.04 x 10⁻⁹ M and 0.44 pmol/mg protein) calf serum were similar. In comparison, although the K_D for T₃ in the cytosolic fractions in both normal and hypothyroid conditions was similar (3.05 x 10⁻⁹ M and 2.64 x 10⁻⁹ M respectively), the number of receptor sites was less in the cytosolic fractions. The number of receptor sites in the cytosolic fractions from the cells grown on hypothyroid serum was 0.20 pmol/mg protein and on normal (thyroid-hormone-containing) serum was 0.23 pmol/mg protein. The specificity of the cytosolic and nuclear
Fig. 1. Scatchard plot of the binding of $[^{125}\text{I}]T_3$ to nuclear receptors in dissociated cells from embryonic brain. Nuclear fractions were obtained from cells grown 20 d in culture, half of them in the presence of hypothyroid serum from day 11 onward. Binding studies were done using an aliquot of the nuclear suspensions along with $[^{125}\text{I}]T_3$ (50 fmol/tube) (719 Ci/mmol sp. act.) in the presence of various concentrations of unlabelled hormone. After preincubation for 45 min at 25°C, the reaction mixture was incubated for 2 h more at 0°C, after which 0.2 ml of a suspension of Dextran-coated charcoal (5% charcoal and 1% Dextran in 20 mM Tris-HCl, pH 7.5) was added: subsequently the contents were centrifuged to collect the clear supernatant containing the receptor-bound radioactivity, which was counted in a gamma-spectrometer. A tube containing a large excess of nonradioactive $T_3$ in the above reaction mixture served to determine non-specifically bound counts, and the value was subtracted from the rest of the experimental counts to determine the specific binding. A Scatchard plot was drawn from the binding data where the ratio $(B/F)$ of bound $[^{125}\text{I}]T_3$ to free $[^{125}\text{I}]T_3$ is plotted as a function of the concentration of bound $[^{125}\text{I}]T_3$. The number of binding sites, $n$, is determined from the intercept of the plot with the abscissa, and the equilibrium dissociation constant, $K_D$, was calculated from the slope of the line. Each value is the average of 6 observations. - ● -, normal; - □ -, hypothyroid.
receptors for the binding of L-T₃ was found to be very high (Tables 2 and 3). Radioactive L-T₃ was not displaced from the bound state by a 500-fold excess of either D-T₃, D-T₄ (D-3,5,3',5'-tetraiodothyronine), DL-thyronine, or L-T₂ (L-3,5-diiodothyronine), but it was displaced by non-radioactive L-T₃ (>70%) and slightly (10%) by L-T₄ (L-3,5,3',5'-tetraiodothyronine). The high specificity of the system suggests that the binding of T₃ measured in these cells is of physiological importance.

Discussion

The primary culture system of cells dissociated from embryonic mouse brain appears to be suitable for studying myelination (10) and its regulation by T₃ (11,12) in vitro. One of the advantages of this culture system over the intact animal is that the direct effects of T₃ on myelination can be determined at a more sophisticated molecular
Table 1. Nuclear and cytosolic receptors for thyroid hormone (T₃) in cells dissociated from embryonic brain

Nuclear and soluble fractions were obtained from cells grown 20 d in vitro, half of them in the presence of normal (110 ng T₃/100 ml) and the other half in the presence of hypothyroid sera (<25 ng T₃/100 ml) from day 11 onward. Binding studies were carried out using an aliquot of these fractions and [¹²³I]T₃ (50 fmol/tube) (719 Ci/mmol) in the presence of various concentrations of non-radioactive hormone. After preincubation for 45 min at 25°C, the reaction mixture was incubated for 2 h more at 0°C, after which the reaction was stopped by the addition of 0.2 ml of a suspension of Dextran-coated charcoal and the tubes were processed to determine the receptor-bound radioactivity as described in detail under 'Materials and Methods'. The binding data were used for Scatchard analysis to determine K_D and n.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Name of sample</th>
<th>K_D (1 x 10⁻⁹ M)</th>
<th>Number of sites (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nuclei (normal)</td>
<td>4.20</td>
<td>0.41</td>
</tr>
<tr>
<td>2</td>
<td>Nuclei (hypo)</td>
<td>5.04</td>
<td>0.44</td>
</tr>
<tr>
<td>3</td>
<td>Cytosol (normal)</td>
<td>3.05</td>
<td>0.23</td>
</tr>
<tr>
<td>4</td>
<td>Cytosol (hypo)</td>
<td>2.64</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Cells that are targets for thyroid hormone have been shown to bind T₃ to the receptors in cytosolic, nuclear, and mitochondrial fractions of the cell (21). Although the function of the cytosolic binder of T₃ is not clear, the mitochondrial and nuclear binders are probably associated with metabolic (respiration) and developmental functions of the hormone. Since the binding of T₃ to appropriate receptors might be considered the first of a number of subcellular metabolic events associated with the mechanism of regulation of myelination by T₃, we initiated studies on the binding of T₃ to cytosolic and nuclear receptors whose function is more likely aligned with the ontogenic (vs. respiratory) effects of T₃ on the cultured brain cells.

Prior studies on receptors for T₃ in brain appear to be limited to whole-animal systems (22,23,24). The K_D values of the cytosolic and nuclear binding receptors for T₃ from the cultured brain cells (~ 10⁻⁹ M) were ten-fold lower than the K_D values of 10⁻⁸ M obtained by Roye and Nayer (22) for the binding of T₃ to cytosolic and nuclear fractions from brains of rats. Murthy et al. (24), who studied the binding of radioactive T₃ to hepatic nuclear preparations, observed that both the K_Ds and the number of binding sites in thyroidectomized rats were similar to those obtained with normal animals. In contrast, cytosolic preparations (from thyroidectomized rats) showed some decrease in binding sites without any change in affinity. Tata (25) observed no significant changes in either fraction by thyroid hormone. Coulombe et al. (26) observed K_D values and numbers of binding sites
Table 2. Effect of various analogues on the binding of labelled L-T$_3$ to nuclear receptors

Incubation was carried out by using the assay mixture containing 50 fmol of radiolabelled L-T$_3$, a 500-fold excess of various thyroid hormone analogues, and an aliquot of nuclear suspensions, in a final total volume of 0.5 ml. After preincubation of the reaction mixture for 45 min at 25°C to exchange endogenously bound hormone, an additional incubation was carried out for 2 h at 0°C. After the incubation the tubes were processed as described in 'Materials and Methods'. L-T$_3$, L-3,5,3'-triiodothyronine; L-T$_4$, L-3,5,3',5'-tetraiodothyronine; L-T$_2$, L-3,5-diiodothyronine; D-T$_3$, D-3,5,3'-triiodothyronine; D-T$_4$, D-3,5,3',5'-tetraiodothyronine; DL-T, DL-thyronine.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Unlabelled analogue</th>
<th>Analogue concentration (fold excess)</th>
<th>[${}^{125}$I]T$_3$ bound (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>concentration</td>
<td>Nuclei (N)$^a$</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>-</td>
<td>100$^c$</td>
</tr>
<tr>
<td>2</td>
<td>L-T$_3$</td>
<td>500</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>L-T$_4$</td>
<td>500</td>
<td>89</td>
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<td>L-T$_2$</td>
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<td>6</td>
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<tr>
<td>7</td>
<td>DL-T</td>
<td>500</td>
<td>102</td>
</tr>
</tbody>
</table>

$^a$(N) = cells grown in the presence of normal serum containing thyroid hormone.

$^b$(Hypo) = cells grown in the presence of hypothyroid serum.

$^c$100% [${}^{125}$I]T$_3$ bound represents the binding of labelled T$_3$ to its receptors (750 c.p.m.) in the absence of any analogues.

$^d$100% [${}^{125}$I]T$_3$ bound represents the binding of labelled T$_3$ to its receptors (590 c.p.m.) in the absence of any analogues.

in hepatic solubilized nuclear extracts which are very similar to the values observed in our work. Our results from cultured cells broadly agree with the findings of Murthy et al. (24).

None of the values for K$_D$ in either fraction as well as the binding sites in either fraction were significantly altered by manipulating the amount of thyroid hormone present.

It is worth noting that the T$_3$ binding characteristics of the cultured dissociated brain cells were similar to the T$_3$ binding properties of fresh brain isolated from the intact animal. This similarity is another parameter demonstrating the efficacy of using this culture system to study the mechanism of action of T$_3$ in vitro. The relatively high binding specificity for L-T$_3$ also adds credence to the physiological importance of the T$_3$ receptors in these cultures.
Table 3. Effect of various analogues on the binding of labelled L-T_3 to cytosolic receptors

Incubation system is similar to the one described in Table 2 except that cytosolic subcellular fractions (instead of nuclear) were used in this case. L-T_3, L-3,5,3'-triiodothyronine; L-T_4, L-3,5,3',5'-tetraiodothyronine; L-T_2, L-3,5-diiodothyronine; D-T_3, D-3,5,3'-triiodothyronine; D-T_4, D-3,5,3',5'-tetraiodothyronine; DL-T, DL-thyronine.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Unlabelled compound added</th>
<th>Analogue concentration (fold excess)</th>
<th>[(^{125}\text{I})]T_3 bound (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cytosol (N)</td>
<td>Cytosol (Hypo)</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>-</td>
<td>100(^c)</td>
</tr>
<tr>
<td>2</td>
<td>L-T_3</td>
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<td>L-T_4</td>
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<td>100</td>
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<tr>
<td>6</td>
<td>D-T_4</td>
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</tr>
<tr>
<td>7</td>
<td>DL-T</td>
<td>500</td>
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</tbody>
</table>

\(^a\)(N) = cells grown in the presence of normal serum containing thyroid hormone.
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\(^d\)100% [\(^{125}\text{I}\)]T_3 bound represents the binding of labelled T_3 to its receptors (580 c.p.m.) in the absence of any analogues.

Acknowledgements

This work was supported by the Kroc Foundation and, in part, by Research Grants NS 10221, AI-05730, and DE04957 from the U.S. Public Health Service.

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