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MULTIPLE THYROID HORMONE BINDING SITES ON RAT LIVER NUCLEAR ENVELOPES

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SUMMARY: Nuclear envelopes relatively free of plasma membrane contamination were isolated from the male rat liver. Equilibrium binding of T_3 to nuclear envelopes occurred after incubation for 3 h at 20°C. Scatchard analysis revealed two classes of binding sites; a high affinity site having a K_D of 1.8 nM with a maximum binding capacity of 14.5 pmol/mg protein and a low affinity site having a K_D of 152.1 nM with a maximum binding capacity of 346.8 pmol/mg protein. No degradation of the radioligand occurred during incubation with the nuclear envelope. T_4, rT_3 and Triac competed effectively for the binding of T_3 to the high affinity site whereas only T_4 competed well for binding to the lower affinity site. The binding site was protease sensitive but not salt extractable. Multiple T_3 binding sites having similar affinities have been reported on plasma membranes. An intriguing possibility is that membrane binding sites may be involved in translocation of thyroid hormone across membrane barriers.

Thyroid hormones help regulate mammalian metabolism and development and also govern levels of certain gene products in target tissues (1). Evidence from a number of laboratories suggests that thyroid hormone action is initiated by an interaction of the hormone with a specific chromatin-associated non-histone protein receptor (2). To reach this receptor within the nucleus, the thyroid hormone must traverse the nuclear envelope (NE) barrier, which separates cytoplasmic from nuclear components. Since the hormone may interact with NE components during this passage, we have begun determining the molecular mechanism of T_3 transport across the NE by investigating thyroid hormone binding to rat liver NE's. In this paper we report the presence of two classes of binding sites on the NE; a high affinity, low capacity site and a low affinity, high capacity site. Interestingly, these sites resemble two classes of T_3 binding sites identified previously on rat liver plasma membranes (3), which raises the possiblity that these sites are involved in transport of the hormone across membrane barriers.

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MATERIALS AND METHODS

Materials: L-[^{125}I]T_3 (3800 μCi/μg) was purchased from New England Nuclear (Montreal, Canada) T_3, 3, 3', 5-tri-iodothyroacetic acid (Triac) and L-T_4 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). rT_3 was purchased from Henning-Berlin (Berlin, Germany).

Preparation of NE's: The NE's were prepared from the purified nuclear pellet by subjecting the nuclei to deoxyribonuclease digestion and purified by layering on a discontinuous sucrose gradient as described (4).

Assays: The T_3 binding assay has been described (4). 5'-Nucleotidase (EC 3.1.3.5) activity was determined by the procedure described by Goldfine et al. (5). Protein content was determined by the method of Lowry et al. (6).

T_3 Metabolites: Pellets obtained after the binding assay were extracted with N-butanol four times, then the combined extracts were evaporated in vacuo at 37°C in the dark (7) and analyzed by thin layer chromatography using silica gel GF. The solvent used was ammonia (30% v/v)-methanol-chloroform (0.1:1:2 v/v) (8). The plates were sprayed with ceric sulfate-sodium arsenite solution followed by methylene blue, then exposed to ammonia vapor (9). The samples were located as blue spots. The chemical form of the radioactivity was determined after scraping 1 cm portions of silica from the plates.

RESULTS

The NE preparation was analyzed for 5'-nucleotidase activity. Low levels of 5'-nucleotidase (1.2% of the homogenate activity) indicated slight contamination of the NE with plasma membrane.

In a previous publication (4) we reported a NE binding site for T_3 with a K_D of 86 nM and a maximum capacity of 223 pmol/mg protein. At that time we suggested the possibility that there might be more than one class of binding site. This is a more detailed study in which binding parameters at lower concentrations of the hormone ligand were analyzed rigorously. First, conditions for the equilibrium binding of T_3 to the NE were re-examined. The temperature dependency of T_3 binding to NE's has been reported (4). However, in that study 2 h was the maximum incubation time tested. We wished to examine binding after longer incubation times. Fig. 1 shows that maximum binding occurred at 30°C after 3 h. However as less degradation occurred at 20°C and the binding was much more stable, we chose to do further experiments at that temperature.

Fig. 2a shows that T_3 competed for the binding of [^{125}I]T_3 to NE's. When the data was analyzed using the "Ligand" computer program (10) and plotted according to Scatchard, data values were compatible with the existence (Fig.

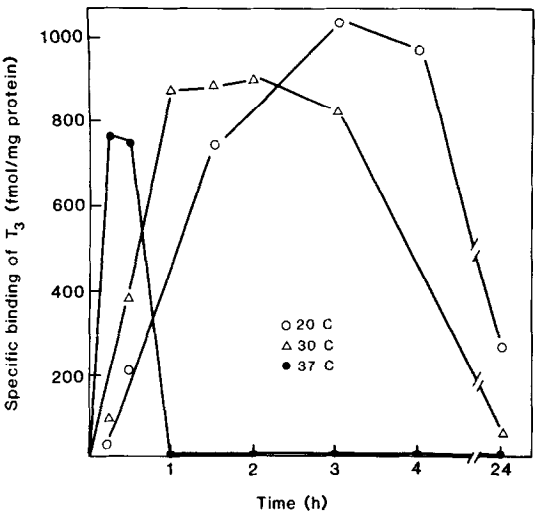

Fig. 1. Effect of incubation time and temperature on the binding of T_3 to rat liver nuclear envelopes. Nuclear envelopes (10 µg/assay tube) were incubated in triplicate with [^{125}I]T_3 (0.07 nM) and 0.5 nM T_3 in the presence and absence of 10 µM T_3 at 20°C, 30°C or 37°C for 0-24 h. Specific binding was calculated from the difference in binding in the presence and absence of 10 µM T_3.

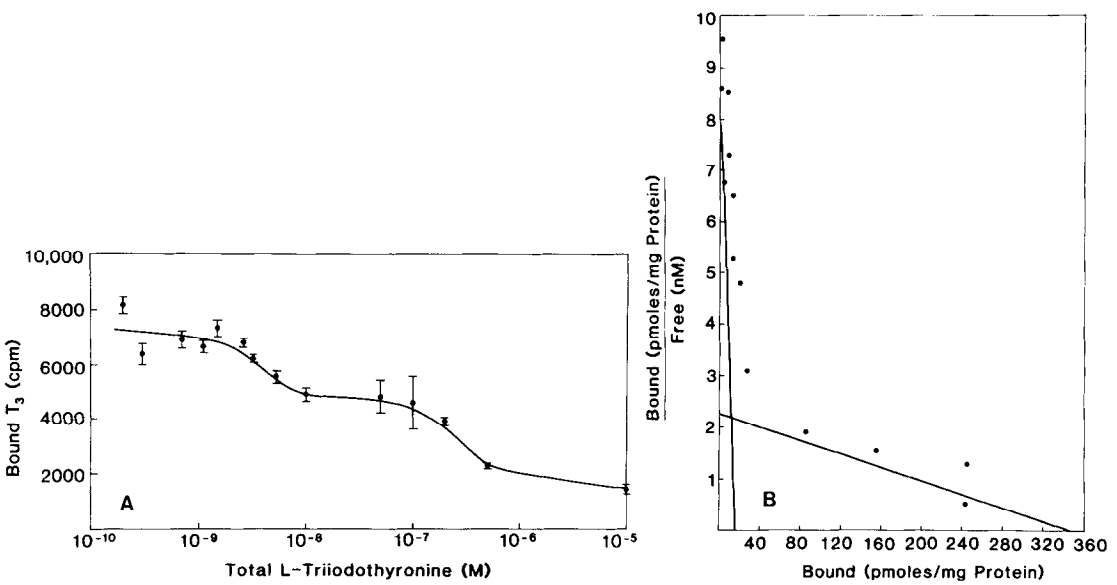

Fig. 2. A) Competition of [^{125}I]T_3 and unlabelled T_3 for binding of nuclear envelopes. Nuclear envelopes (10 µg protein/assay tube) were incubated with 0.07 nM [^{125}I]T_3 at 20°C for 3 h with various concentrations of T_3. Each point is the mean of three determinations. Non-specific binding was not subtracted.
B) Specific binding from two experiments was calculated from the difference in retained radioactivity in the absence and presence of unlabelled T_3. The data was analyzed using the "Ligand" computer program (10) and plotted according to Scatchard.

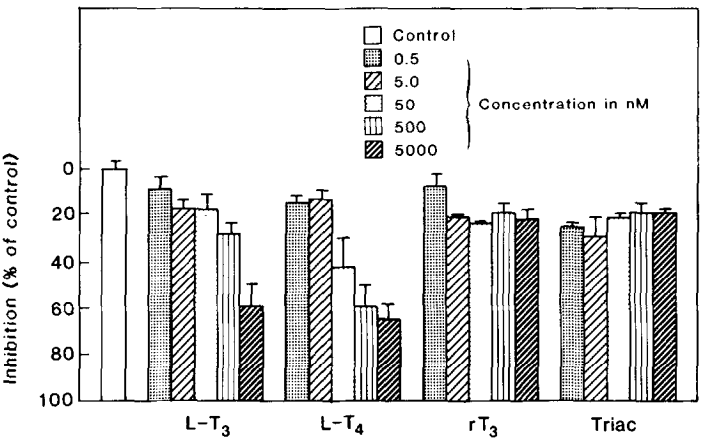

Fig. 3. Effect of unlabelled L-T_3, L-T_4, triac and rT_3 on specific binding of L-[^{125}I]T_3 to nuclear envelopes. Nuclear envelopes were incubated with [^{125}I]T_3 in the presence of 0.5 - 5000 nM concentrations of L-T_3 or T_3 analogues for 3 h at 20 C.

2b) of a high affinity site having a K_D of 1.82 ± 0.79 nM and a maximum capacity of 14.5 ± 4.6 pmol/mg protein as well as a lower affinity site having a K_D of 152.1 ± 54.8 nM and a maximum capacity of 346.8 ± 58.8 pmol/mg protein.

Competition experiments, in which the extent to which T_3 and T_3 analogues displaced [^{125}I]T_3 was examined, revealed (Fig. 3) that L-T_4 was as effective as L-T_3. Triac, the acetic acid analogue of T_3, and rT_3 competed for [^{125}I]T_3 binding at lower concentrations. They did not, however, compete any more effectively at higher concentrations. This finding suggests that the higher affinity site was less able to discriminate amongst the thyroid hormone analogues than was the lower affinity site. Our earlier studies (4) indicated that T_4 was not a good competitor, while Triac was 75% as potent as T_3 and rT_3 was an effective competitor of [^{125}I]-T_3. We are able to reproduce this order of analogue potency by carrying out the binding assay for 2 h at 30°C. We believe that proteolytic degradation may be responsible for the altered analogue affinities at the higher temperature. This observation warrants further investigation and may explain discrepancies in the literature.

Thin layer chromatographic analysis revealed no degradation of the radioligand during the incubation of the NE with [^{125}I]T_3. The radioactivity

Table 1. Effect of protease, deoxyribonuclease and KCl on specifically bound T_3 to nuclear envelopes

Treatment	Concentration	Specific Binding (cpm)	Specific Binding (% Binding)
Control	-	3870	100
Protease Type VI	1 mg/ml	1069	28
Deoxyribonuclease Type I	0.5 mg/ml	2951	76
KCl	0.6 M	3570	92

After incubation of nuclear envelopes (10 µg protein/assay tube) with 0.07 nM [^{125}I]-T_3 and 0.5 nM T_3, bound and free T_3 were separated by centrifugation and the pellets washed twice with 20 mM Tris-HCl, pH 7.4. The pellets were then incubated for 30 min at 2°C in 0.25 ml 20 mM Tris-HCl, pH 7.4 in the presence or absence of protease, DNAse type I or KCl. The incubation was stopped as described in Materials and Methods and the radioactivity retained by the pellet was determined. Values represent the means of triplicates from two experiments.

(97%) was recovered in the area of the thin layer plate where the standard T_3 ran.

Table 1 demonstrates that protease treatment of T_3 bound to NE's extracted 72% of the specifically bound T_3. Deoxyribonuclease extracted 24%, while KCl (0.6 M) extracted only 8% of the specific binding.

DISCUSSION

We have now identified two classes of T_3 binding sites on NE's from the male rat liver, a high affinity site having a K_D of 1.8 nM and a lower affinity site having a K_D of 152.1 nM. Interestingly, although the NE binding sites appear to be protein in nature, treatment of the membranes with DNAse released a significant proportion of the bound [^{125}I]T_3. It has been suggested that low amounts of DNA are required for the structural integrity of the NE (11). The protein binding sites for T_3 may be associated with this residual DNA.

The capacity of the NE high affinity site was 14.5 pmol/mg protein or 1.09 ng/g tissue. Reported values for binding to rat liver nuclei (12) are in this range as well. Nuclear binding studies, however, have been carried out using nuclei which have been treated with non-ionic detergents. This treatment removes not only the outer nuclear membrane but also some inner membrane

components (13), which may contain the binding sites that we have identified on the NE.

The relationship of the two rat liver NE T_3 binding sites to other previously described cellular binding sites is an important consideration. The high affinity site for T_3 on the NE resembles the nuclear or chromatin binding site in affinity (14). The specificity of this binding site is not, however, consistent with its being a nuclear receptor. Furthermore, although the T_3 receptor was extracted from nuclei with 0.4 M KCl (15,16) we were unable to extract the NE binding site with KCl at concentrations as high as 0.6 M. We conclude that the high affinity NE binding site is different from the nuclear receptor previously described. The lower affinity site appears to resemble the cytosolic T_3 binding site in affinity and specificity (17). Because extraction with high salt concentrations did not remove the binding, however, this site appears unlikely to come from cytoplasmic contamination of the nuclear envelope. Pliam and Goldfine (5) found two orders of saturable binding sites for T_3 on rat liver plasma membranes; a high affinity, low capacity site with a K_D of 3.2 nM and a lower affinity, higher capacity site with a K_D of 220 nM. The affinities of the two plasma membrane binding sites are similar to the two NE binding sites. In addition, competition studies also showed that the high affinity plasma membrane binding site did not discriminate between T_4 and L-T_3. As the capacities of the two plasma membrane sites are lower than that of the NE binding sites, plasma membrane contamination of the NE cannot account for the binding observed in the NE. A possiblity is that the two sets of binding sites on the plasma and nuclear membranes are involved in a similar function, perhaps a transport function.

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