Saturable binding of thyroid hormone to isolated rat hepatocytes

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The binding of [^{125}I]triiodothyronine (T_3) to freshly prepared rat hepatocytes was studied at 0° C. The abundant non-saturable binding could be suppressed by washing the cells with alkaline buffer, pH 10.5 at 0° C, without loss of cell viability, thus allowing detection of saturable binding. Three classes of binding sites were identified from analysis of the saturable T_3 binding in the presence and absence of bromosulfophthalein (BSP). One of these classes was inhibited by BSP. The T_3 dissociation constants were 3.5, 35 and 115 nM and the number of sites was respectively 0.9, 20 and 36×10^6 sites/cell. L-T_3 had a 10-times higher affinity than D-T_3 and a 50-times higher affinity than triiodothyroacetic acid. Saturable T_3 binding was associated with plasma membrane-containing subcellular fractions. These binding sites may be related to those previously described in isolated plasma membranes from rat liver and could be involved in the entry of T_3 into the hepatocyte.

Triiodothyronine *Thyroid hormone receptor* *Bromosulfophthalein* *Cell membrane binding site (Isolated hepatocyte)* *Thyroid hormone action*

1. INTRODUCTION

Although thyroid hormones are thought to exert their effects at more than one initial locus, the cell nucleus is considered to be the main site of action, where triiodothyronine (T_3) influences gene expression (review [1]).

In the liver, intranuclear T_3 has a plasma origin [2]. The translocation from plasma to the intracellular compartment was at first presumed to take place by passive diffusion. However, active transport into isolated rat liver cells was described [3,4] and recently, receptor-mediated endocytosis of T_3 was reported to occur in GH_3 cells and mouse fibroblasts [5,6].

Specific T_3-binding sites have been detected in purified rat liver plasma membrane [7,8] and were suggested to be implicated in the cellular entry of T_3.

The aim of the present work was to identify saturable T_3 binding to unfractionated isolated hepatocytes at 0°C, since thyroid hormones were reported not to be internalized at low temperature [6,9,10]. Such binding sites would presumably be located at the cell surface and their identification and characterization is a prerequisite for the study of their function and regulation in intact cells.

2. MATERIALS AND METHODS

2.1. *Materials*

3,3,5′-Triiodo-L-thyronine (L-T_3), 3,3′,5-triiodo-D-thyronine (D-T_3), L-thyroxine (T_4), 3,3′,5-triiodothyroacetic acid (triac), 3,5-diiodo-L-thyronine (T_2), DL-thyronine (T_0), bovine serum albumin (type V) (BSA), collagenase (type I), soybean trypsin inhibitor and bromosulfophthalein (BSP) were purchased from Sigma; 3,3′,5′-triiodo-L-thyronine (r-T_3) from Calbiochem; Hepes from Boehringer; Eagle's basal medium from Gibco; Percoll from Pharmacia; Dowex (AG1X8)

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from Bio-Rad; [^{125}I]T_3 (spec. act. >44 MBq/μg) from Amersham.

All polystyrene tubes and pipet tips, used for handling thyroid hormones, were siliconized (Sigmacote, Sigma).

2.2. *Methods*

Male Wistar rats (~200 g body wt) were thyroidectomized and, 4–10 weeks later, hepatocytes were isolated by the collagenase perfusion method of Seglen [11] modified by Oldberg et al. [12]. Cells were incubated in oxygenated medium A (Eagle's basal medium supplemented with 2.6 g/l glucose and 10 mM Hepes, pH 7.4) for 30 min at 37°C, centrifuged (70 × *g*, 2 min) and resuspended in isotonic Percoll diluted with medium A (d = 1.09). After centrifugation at 400 × *g* for 5 min, the pellet of viable cells was resuspended in medium A and kept at 0°C.

Viability was determined by the trypan blue exclusion test. It was over 97% after purification through Percoll and was maintained for at least 4 h at 0°C.

Unless otherwise stated, standard conditions for measurement of binding were as follows: 4×10^5 hepatocytes suspended in 1 ml medium A were incubated with [^{125}I]T_3 and other additives in siliconized polystyrene tubes for 4 h at 0°C.

Incubation was terminated by addition of 10 μM unlabelled T_3 and centrifugation at 70 × *g* for 2 min. Unbound T_3 was determined on an aliquot of the supernatant. The cell pellet was washed by successive resuspension/centrifugation (70 × *g*, 2 min), once with 1.5 ml glycine buffer (50 mM glycine, 150 mM NaCl, 3% (w/v) BSA, pH 10.5) and twice with 2 ml Hepes buffer (137 mM NaCl, 4.7 mM KCl, 0.65 mM $MgSO_4$, 1.2 mM $CaCl_2$, 8.5 mM Hepes, pH 7.4). These buffers contained 10 μM unlabelled T_3 and all operations were performed at 0–4°C. Bound radioactivity was measured on cell pellets with an efficiency of 80%. Saturable binding was obtained by subtraction of the binding measured in the presence of 10 μM unlabelled T_3 from those measured in its absence.

3. RESULTS AND DISCUSSION

3.1. *Evidence for saturable binding of T_3 to isolated hepatocytes at 0°C*

In preliminary experiments, hepatocytes (2×10^6 cells/ml) were incubated with 0.1 nM [^{125}I]T_3 ± 1 μM unlabelled T_3 at 0°C and bound radioactivity was measured by a rapid oil centrifugation technique as in [6]. No saturable binding was observed: indeed, bound radioactivity in the presence of unlabelled T_3 was 20–30% higher than in its absence, irrespective of the incubation time (ranging from 1 to 90 min) (not shown). Similar absence of saturability of [^{125}I]T_3 uptake by hepatocyte was recently reported [13], when measured by an oil centrifugation technique.

To improve the procedure for separation of bound and unbound T_3, cells were washed by successive centrifugations/resuspensions (see section 2). Since the solubility of iodothyronines increases at extreme pH, the effect of the washing buffer pH on the binding of T_3 to isolated cells was examined. Contrary to the results obtained with the oil centrifugation technique, specific binding was detectable at pH 7.4. In addition, fig.1A shows that, when the pH of the first washing buffer was increased, the amount of non-saturable binding decreased markedly from ~70% of total binding at pH 7.5 to less than 10% at pH 11. Consequently, the differential saturable binding increased and reached a plateau at pH ~10.

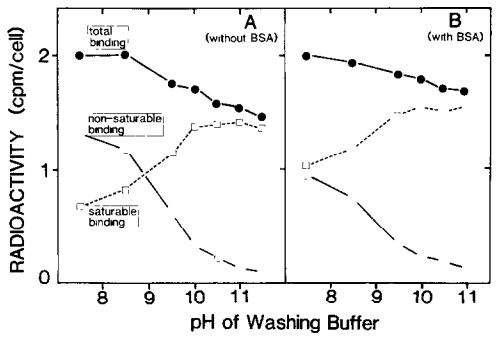

Fig.1. Effect of pH of washing buffer on binding of T_3 to hepatocytes. 2×10^6 cells/ml were incubated with 0.1 nM [^{125}I]T_3, in the absence (total binding) or presence (non-saturable binding) of 10 μM unlabelled T_3, for 2 h at 0°C. Cells were washed by successive centrifugations/resuspensions (see section 2), once with 1.5 ml buffer at various pH values (Hepes buffer, pH 7.5; 150 mM NaCl/10 mM Tris buffer, pH 8.5; glycine buffer, pH 9.5–11.5), without (A) or with (B) 3% (w/v) BSA, and twice with 2 ml Hepes buffer, pH 7.4. Each point is the mean of triplicates (coefficient of variation <2.5%). Saturable binding was calculated as the difference between total and non-saturable binding.

Cell viability was not affected by the contact with alkaline buffer (up to pH 11) provided the cells were processed at a temperature close to 0°C.

The addition of 3% (w/v) BSA to the washing buffer increased the amount of saturable binding measured by 20–40% (fig.1B). The effect was particularly noticeable when the pH was close to neutrality. Increasing the concentration of BSA up to 12% (w/v) did not effect further improvement (not shown). The use of acidic glycine buffer (pH 2.5) resulted in a considerable increase (250%) in non-saturable binding (not shown). Therefore, washing with glycine buffer (pH 10.5) containing 3% BSA was routinely used for characterization of T_3 binding to the cells (see section 2).

3.2. *Association kinetics of T_3 to hepatocytes at 0°C*

The time course of saturable binding to intact cells at 0°C was studied using the alkaline washing procedure.

Fig.2 shows that residual, alkaline-resistant, non-saturable binding varied very little throughout the incubation time and was between 5 and 10% of the total binding. Apparent equilibrium for saturable binding was not reached before ~10 h of incubation. Similar slow kinetics of association of T_3 to cultured fibroblasts at 0°C were reported by others [10]. In contrast, the alkaline labile, non-saturable binding proceeded more rapidly, reaching a maximum after ~5 h and slowly declining thereafter. Non-equilibrium conditions (i.e. a 4 h incubation) were adopted to characterize the binding since cell viability was not affected (>97%), whereas it decreased to ~95% and 85–90% after 8 and 20 h respectively, at 0°C. Furthermore, in this condition, hepatocytes remained able to translocate T_3 to the nucleus in a manner similar to freshly prepared cells, when the temperature was raised to 25°C (not shown).

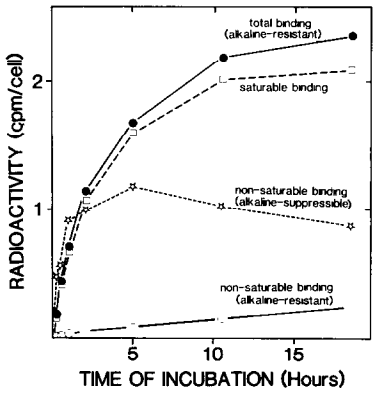

Fig.2. Time course of association of T_3 to hepatocytes at 0°C. 4×10^5 cells/ml were incubated with 0.1 nM [^{125}I]T_3 in the absence (total, alkaline-resistant binding) or presence (non-saturable, alkaline-resistant binding) of 10 μM unlabelled T_3. Cells were washed by the standard procedure described in section 2. Each point is the mean of triplicates (coefficient of variation <2%). Saturable binding was calculated as the difference between total and non-saturable binding. Non-saturable, alkaline-suppressible binding was calculated by subtracting unbound (measured as described in section 2) and bound, alkaline-resistant T_3 from total T_3 added to the cells.

3.3. *Characteristics of T_3 binding*

Fig.3 illustrates a Scatchard analysis of [^{125}I]T_3 binding at 0°C to isolated hepatocytes, indicative of either multiple categories of non-interacting binding sites or negative cooperativity [14]. The effect of BSP, a dye known to inhibit thyroid hormone binding to serum proteins [15], deiodination enzymes [16] and liver cytosol proteins [17], was examined. BSP (100 μM) partly inhibited binding of T_3 to intact hepatocytes and increased the curvature of the Scatchard plot (fig.3). Subtraction of the curve obtained in the presence of BSP from that in its absence, along radial axes of the Scat-

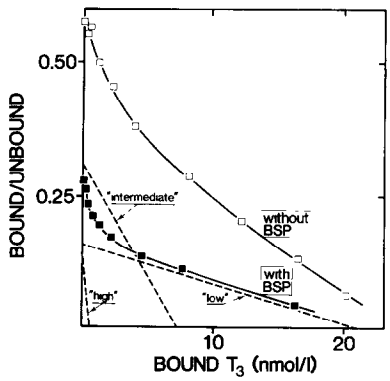

Fig.3. Scatchard plot of binding of T_3 to hepatocytes at 0°C. Saturable binding was measured under the standard conditions described in section 2, in the presence and absence of 100 μM BSP. T_3 concentration varied from 0.2 to 500 nM. Dashed lines represent the high-, intermediate- and low-affinity sites derived as described in the text.

chard plot [14,18], yielded a straight line representing the BSP-suppressible class of T_3-binding sites. This was characterized by a dissociation constant (K_d) of 36 ± 22 nM and a number of binding sites of 20 ± 5 (sites × 10^6)/cell (mean ± SD of 4 independent determinations). The binding not suppressed by BSP could be resolved into two categories of binding sites by an iterative procedure. The 'high'- and 'low'-affinity sites had K_d values of 3.5 ± 1.1 and 115 ± 30 nM and numbers of binding sites of 0.9 ± 0.4 and 36 ± 7 (sites × 10^6)/cell, respectively (mean ± SD of 4 independent determinations). The three categories of binding sites are represented by dashed lines in fig.3.

T_3 binding studies conducted with liver plasma membrane preparations [7,8] indicated the existence of 2 classes of binding sites: one with a K_d of 6–9 nM and the other with a K_d of 220–240 nM. These values are close to those measured for the high- and low-affinity sites in the present work. Heterogeneity of binding of thyroid hormone to isolated liver plasma membranes was also suggested by photoaffinity labelling experiments [19] in which 3 distinct binding proteins were detected.

The significance of the BSP inhibition of the 'intermediate'-affinity site is unknown: BSP has been reported to inhibit T_3 and T_4 uptake by rat liver in vivo and in vitro [20]. BSP is known to bind to the liver plasma membrane transporter involved in the hepatic uptake of bilirubin and various organic anions [21]. This raises the possibility that T_3 could bind to this transporter in the plasma membrane of the hepatocyte.

3.4. *Specificity of hepatocyte binding sites for structural analogues of T_3*

Competition experiments, illustrated in fig.4, demonstrated that T_3 binding to intact hepatocytes at 0°C was specific. Unlabelled analogues were allowed to compete with [^{125}I]T_3 for binding; thyronine was ineffective and triac, D-T_3, T_2, T_4, r-T_3 were all less effective than unlabelled T_3 in displacing bound [^{125}I]T_3. From the molar concentration of unlabelled iodothyronines required to depress the binding of tracer [^{125}I]T_3 by 50%, it could be roughly estimated that hepatocytes bound T_3 2.5-, 3.5-, 5-, 10- and 50-times as avidly as r-T_3, T_4, T_2, D-T_3 and triac, respectively.

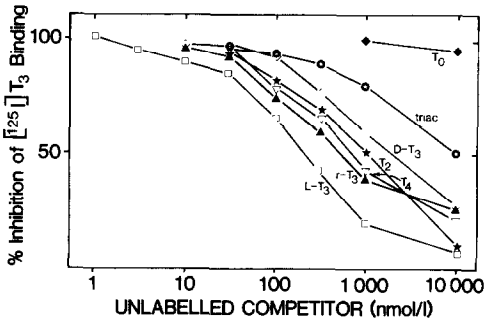

Fig.4. Binding specificity of thyronine analogues. Cells were incubated at 0°C for 4 h with 0.2 nM [^{125}I]T_3 in the presence of increasing concentrations of unlabelled analogues. Saturable binding was determined as described in section 2 and expressed as the percentage of binding in the absence of unlabelled competitor.

However, since multiple categories of binding sites were revealed by the Scatchard plot and since they all contribute to the overall binding at the concentration of [^{125}I]T_3 used, this experiment does not detail the specificity of each category of binding site. The situation is also complicated by the fact that T_4 and T_3 bind with cross-reactivity to separate sites in isolated liver plasma membranes [8].

The fact that D-T_3 has 10-times less apparent affinity for hepatocytes than L-T_3 indicates a high degree of stereospecificity. Quantitatively similar specificity was reported for binding of the two enantiomers to cultured fibroblasts [10] and to purified plasma membranes from rat liver [7]. This can be related to the enhanced entrance of L as compared with D-T_3 into various tissues in vivo [22] and into isolated hepatocytes in vitro [13]. The specificity of receptors in isolated nuclei from rat liver is quite different: triac is 4-times as effective as L-T_3 and D-T_3 is equal to L-T_3 [23]. The cytosol-binding protein in liver, kidney [24] and brain cells [25] also lacks stereospecificity towards L- and D-T_3.

3.5. *Subcellular localization of T_3 binding*

Only the intermediary, BSP-inhibited site had a K_d (36 nM) compatible with that of cytosol-binding protein [24,26]. However, the latter was reported to have negligible affinity for BSP [26].

The competition experiments indicated that specificity of iodothyronine binding by the whole hepatocyte at 0°C was different from those of the

nuclear receptor and cytosol-binding protein, but resembled those of sites in isolated plasma membrane. Negligible nuclear binding was detectable after a 4 h incubation of hepatocytes with T_3 at 0°C, followed by preparation of nuclei according to Cheng [10] (not shown). Table 1 shows the distribution of saturable binding sites to subcellular fractions of hepatocytes after incubation of whole cells at 0°C. Irrespective of the concentration of T_3 in the incubation medium, saturable sites were distributed equally between the 2000 × *g* pellet and supernatant. A study of [^{3}H]concanavalin A binding to whole hepatocytes, according to Record et al. [27], indicated that plasma membranes were present to a similar extent in both fractions (not shown). Further centrifugation of the supernatant at 100000 × *g* for 45 min gave a microsomal pellet with which two thirds of the 2000 × *g* supernatant radioactivity was associated, the remaining third being found in the cytosol. However, treatment of the cytosol with Dowex [28] indicated that more than 90% of the cytosol T_3 was unbound. This could originate from dissociation of particulate-bound T_3, although some dissociation from cytosol-binding protein during the ultracentrifugation step cannot be excluded. Therefore, after homogenization, more than 80% of the hepatocyte-bound T_3 remained associated with particulate material, probably plasma membranes, since T_3 does not enter intact cells at low temperature [6,9,10]. This distribution is independent of the concentration of T_3 over a wide range, indicating a similar subcellular localization of the various classes of saturable binding sites.

In conclusion, preparation of high viability hepatocytes combined with alkaline washing of cells and partial inhibition of binding by BSP have permitted characterization of binding sites on whole cells at 0°C. These saturable sites, specific for T_3, could be involved in entry of the hormone into the hepatocyte through the plasma membrane. Preliminary experiments indicate that part of the alkaline-resistant, specifically bound T_3 can be transferred to the nucleus after subsequent incubation of the hepatocytes at 25°C.

About 2×10^5 molecules of T_3/cell were bound to saturable sites at 0°C when the free extracellular T_3 concentration was 0.2 nM. This is ~100-times more than the corresponding occupied nuclear sites at 37°C since, at this concentration, the nuclear receptors in intact hepatocytes are half-saturated [13]. It can be calculated that, at 0°C, occupancy of high-, intermediate- and low-affinity binding sites was ~5, 0.5 and 0.2%, respectively. Therefore, binding to these sites does not seem to be a limiting step for access of T_3 to the cell interior. However, they might be implicated in stereospecific, facilitated diffusion of thyroid hormone through the cell surface.

Table 1

Distribution of saturable T_3 binding to subcellular fractions of hepatocytes preincubated with various concentrations of [^{125}I]T_3

	T_3 concentration			
	0.2 nM	5 nM	50 nM	200 nM
Whole cells	0.100 (100)	2.46 (100)	18.78 (100)	43.52 (100)
2000 × *g* pellet	0.045 (45)	1.14 (46)	8.89 (47)	19.11 (44)
2000 × *g* supernatant	0.052 (52)	1.23 (50)	9.07 (48)	19.43 (45)
100000 × *g* pellet	0.033 (33)	0.81 (33)	5.95 (32)	13.01 (30)
Cytosol	0.019 (19)	0.44 (18)	3.30 (18)	6.70 (15)
Cytosol Dowex	0.0013 (1.3)	0.033 (1.3)	0.22 (1.2)	0.44 (1.0)

Cells were incubated and washed as described in section 2, then Dounce-homogenized (20 strokes) in 1 ml Hepes buffer and subcellular fractions prepared by differential centrifugation. Treatment of the cytosol with Dowex was according to [28]. Results are expressed as the number of T_3 molecules ($\times 10^{-6}$) bound per cell to saturable sites and are the mean of duplicate experiments. The percentage relative to whole cells is given in parentheses

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