Preparation of unoccupied thyroid-hormone receptor

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Thyroid hormone (3,5,3'-tri-iodothyronine; T_3) regulates gene expression through binding to its specific receptor in the nucleus. In euthyroid animals, roughly half of all receptors are occupied by the hormone. Nuclear extracts thus yield mixtures of occupied and unoccupied receptors. We present here a simple method for transforming occupied receptors into unoccupied ones. *In vitro*, the T_3 -receptor complex dissociated in a half-dissociation time exceeding 100 h at 0 °C, and at temperatures that accelerated the dissociation the receptor was quickly inactivated. Long-chain-fatty-acyl-CoAs, on the other hand, greatly accelerated the dissociation of T_3 -receptor complex at 0 °C. The receptor was extracted from rat liver nuclei, incubated with oleoyl-CoA to release the bound hormone, and passed through a small column

of Lipidex, which strongly adsorbed both oleoyl-CoA and the dissociated hormone. The receptor was recovered in the flow-through fraction in its unoccupied form, as seen by the results of DEAE-Sephadex column chromatography and the loss of all previously bound [125 I] T_3 . The maximum T_3 -binding capacity of the unoccupied receptor was about 1.5-fold that of the untreated sample, and the dissociation constant was unaltered. The results suggest that most nuclear thyroid-hormone receptors occupied by the hormone were transformed into unoccupied ones. From the T_3 -binding capacity before and after oleoyl-CoA treatment, the *in vivo* T_3 occupancy of the receptor was estimated. The procedure is easy to perform, and the method should be useful for studies of unoccupied receptors.

INTRODUCTION

Nuclear thyroid-hormone receptors are non-histone proteins associated with chromatin that regulate the expression of target genes [1–3]. When these proteins are isolated from cell nuclei, some are occupied by the hormone and, to an extent that depends on the kind of cell, on physiological conditions. In most tissues of the euthyroid rat, 35–50 % of the receptors are occupied by the hormone [4]. Unoccupied receptors can bind to the responsive elements of the target genes and suppress the expression of the genes [5,6]. Unoccupied receptors compete with retinoic acid receptors (RARs) for retinoid X receptors, thereby also suppressing RAR-dependent transactivation [7].

Unoccupied thyroid-hormone receptors could be studied to elucidate the receptor structure and the mode of thyroid-hormone action. However, to isolate unoccupied receptors or to separate them from occupied ones is not easy, for receptors are unstable during fractionation and at the high temperatures needed to accelerate dissociation of the hormone.

Non-esterified long-chain fatty acids inhibit T_3 binding by the solubilized nuclear receptors and also T_3 binding to the nuclei of HTC-rat hepatoma cells in culture [8]. Fatty acyl-CoAs are more inhibitory than non-esterified fatty acids and promote dissociation of the hormone-receptor complexes [9]. Using this dissociation reaction we have developed a convenient method for the preparation of unoccupied receptor.

EXPERIMENTAL

Materials

125 I-labelled thyroid hormone (L-3,5,3'-triiodothyronine; T_3 : 1200 μ Ci/ μ g) and [14 C]oleoyl-CoA (40–60 mCi/mmol) were purchased from NEN. Unlabelled T_3 and oleoyl-CoA were obtained from Sigma. Non-fat dry milk was from Snow Brand Milk Products Co. (Sapporo, Japan), Lipidex-1000 was from United Technologies Packard. Anion-exchange resin AG 1X8

(analytical grade; 200–400 mesh; Cl[−] form) was from Bio-Rad Laboratories. Disposable plastic syringes (1 ml) were obtained from Terumo Corp. (Tokyo, Japan).

Stock oleoyl-CoA

Oleoyl-CoA was suspended in propan-1-ol:water (1:1, v/v) at 5 mM and stored at −20 °C under an N₂ atmosphere.

Preparation of Lipidex columns

A small cotton plug was placed at the bottom of a 1 ml disposable syringe, into which a Lipidex-1000 suspension in methanol (0.5 ml) was pipetted. The column (0.3 ml bed volume) was washed with 0.5 ml each of methanol, buffer T [50 mM NaCl/2 mM EDTA/10 % (v/v) glycerol/0.16 mM dithiothreitol/20 mM Tris/HCl, pH 8.2] and buffer T containing 3 % (w/v) non-fat dry milk.

Preparation of unoccupied thyroid-hormone receptor

Nuclear thyroid-hormone receptor (2.4 mg of protein/ml) prepared from rat liver as described previously [8] was incubated in buffer T with oleoyl-CoA (50 μ M) at 0 °C for 20 h. The mixture was put on a Lipidex column, which was then washed with buffer T. The unoccupied receptor was collected in the flow-through fraction. The control sample was treated in the same way, except that oleoyl-CoA was not included.

DEAE-Sephadex A-50 column chromatography

The receptor extract was incubated with [125 I] T_3 , and the resulting [125 I] T_3 -bound receptors were used as the occupied receptors. Unoccupied receptors were prepared with Lipidex as described above. The occupied and unoccupied receptors were eluted with a 50–400 mM NaCl linear gradient in buffer T on parallel columns of DEAE-Sephadex A-50 as described elsewhere [10].

Assay for T_3 and oleoyl-CoA binding

Receptors and [125 I] T_3 (0.1 nM) were incubated at 0 °C in buffer T (0.5 ml) in the presence of various concentrations of oleoyl-CoA. The amount of [125 I] T_3 specifically bound to the receptor was assayed by the AG 1X8 resin method [8] or by the filter-binding method [11]. Non-specific binding was assayed in the presence of a 500–1000-fold molar excess of unlabelled T_3 . Radioactivity of the specific binding was 3.5–8-fold that of the non-specific binding; oleoyl-CoA at 20 μ M increased non-specific binding by 10–25 %. Assays were done in duplicate. Deviations from the means were within 7 %. Oleoyl-CoA-binding activity was measured in a similar way by incubation with [$1\text{-}^{14}\text{C}$]oleoyl-CoA (0.1 μ M) for 12 h at 0 °C, followed by filtration on nitrocellulose membranes [11]. Non-specific binding was assayed in the presence of a 500-fold molar excess of unlabelled oleoyl-CoA.

RESULTS

Accelerated dissociation of T_3 -receptor complex with fatty acyl-CoA

The half-dissociation time ($t_{1/2}$) of the T_3 -receptor complex exceeded 100 h at 0 °C [8]. In the presence of long-chain acyl-CoAs, the dissociation was accelerated (Table 1). From a plotted curve, half and complete dissociations were estimated to be reached in 20 h with 2.5 and 50 μ M oleoyl-CoA respectively (results not shown).

Adsorption of oleoyl-CoA by Lipidex

Lipidex strongly adsorbs hydrophobic molecules. When labelled oleoyl-CoA (10 nmol) was put on a Lipidex column (0.3 ml bed volume), 94 % of the radioactivity was adsorbed. The same value was obtained with 10-fold greater amount of oleoyl-CoA. Thus the flow-through radioactivity was not due to the binding capacity of Lipidex being small, but rather to a contaminant that did not bind to the resin. The results indicate that columns of this size completely remove nanomolar amounts of free oleoyl-CoA from samples. Lipidex also adsorbs free T_3 strongly [12]. Thus, when the nuclear receptor was first incubated with oleoyl-CoA to promote dissociation and then passed through a Lipidex column, the receptor was recovered in its unoccupied form in the flow-through fraction, leaving oleoyl-CoA and the dissociated hormone in the column.

If the unoccupied receptor were recovered in association with oleoyl-CoA from Lipidex, later steps could be affected, particularly in the hormone binding of the receptor [9]. For an examination of this possibility, labelled oleoyl-CoA was incubated with the receptor and put on a Lipidex column. Table 2 shows that the amount of oleoyl-CoA collected with the receptor was negligible (2×10^{-10} – 2×10^{-11} M in the flow-through fraction). Micromolar concentrations of oleoyl-CoA inhibit hormone binding, but concentrations of 10^{-10} to 10^{-11} M do not [9]. The negligible amounts of oleoyl-CoA emerging with the receptor should not affect the receptor.

Effects of non-fat dry milk on the Lipidex column

Passage of the receptor through a Lipidex column (0.3 ml bed volume) reduced the T_3 -binding activity. Treatment of the column with a buffer containing non-fat dry milk prevented this reduction. The effect increased with the milk concentration, reaching a plateau at 3 % (w/v). One-tenth of this concentration gave only 41 % of the maximum yield (results not shown).

When the T_3 receptor was put on untreated Lipidex columns of various sizes (0.3 ml, 0.6 ml and 0.9 ml bed volume), only 54, 27 and 16 % respectively of the T_3 -binding activity was recovered. When columns were washed first with 3 % milk, the activity recovered was the same whatever the column size (results not shown). Without blockage by milk, the receptor protein was apparently adsorbed by the Lipidex matrix. Milk was more effective than albumin (results not shown).

Preparation of the unoccupied receptor

The receptor was incubated with oleoyl-CoA (50 μ M) for 20 h at 0 °C and put on a Lipidex column. The receptor was recovered

Table 1 Dissociation of T_3 -receptor complex accelerated by oleoyl-CoA

The receptor (120 μ g of protein) and [125 I] T_3 (0.1 nM) were incubated in 0.5 ml of buffer T at 0 °C for 12 h. Then oleoyl-CoA at various concentrations and a large excess of unlabelled T_3 (10 μ M) were added. After 20 h at 0 °C, the [125 I] T_3 bound to the receptor was assayed and expressed as percentages of the control value, which was obtained at the start of incubation with oleoyl-CoA. Duplicate determinations were repeated three times and averaged. In other experiments in which oleoyl-CoA was absent, 24, 37 and 43 % of [125 I] T_3 -receptor complexes had dissociated after 20, 50 and 100 h respectively.

[Oleoyl-CoA] (μ M)	[125 I] T_3 bound to receptor (%) (mean $\pm$ S.D., $n = 6$)
0 (Control)	100.0
0	76.4 $\pm$ 2.6
5	42.0 $\pm$ 3.7
10	29.0 $\pm$ 2.4
20	16.0 $\pm$ 2.1
50	0
75	0
100	0

Table 2 Removal of oleoyl-CoA from reaction mixtures by Lipidex

[$1\text{-}^{14}\text{C}$]Oleoyl-CoA (5×10^{-8} M) was incubated in buffer T with thyroid-hormone-receptor extract at 0 °C for 12 h. Non-specific [$1\text{-}^{14}\text{C}$]oleoyl-CoA binding was assayed with a 500-fold molar excess of unlabelled oleoyl-CoA. Portions (0.5 ml; 1500 c.p.m. each) of the incubation mixtures were filtered on nitrocellulose membranes in an assay of the receptor-bound oleoyl-CoA present in the mixtures. The filters were washed and their radioactivity was counted. Other portions of the incubation mixtures were put on columns of Lipidex (0.3 ml bed volume), which were washed with buffer T (1 ml). Flow-through fractions were collected and counted for radioactivity. Values are the means of duplicate measurements.

[$1\text{-}^{14}\text{C}$]Oleoyl-CoA	500-Fold excess of unlabelled oleoyl-CoA	Radioactivity (c.p.m.)	Concn. of [^{14}C]oleoyl-CoA (M)
Oleoyl-CoA per aliquot		1500	5×10^{-8}
Bound oleoyl-CoA collected on membrane filter	— +	85 15	$(2.8 \times 10^{-9})^*$
Bound oleoyl-CoA collected from Lipidex column	— +	2 19	2.2×10^{-11} 2.1×10^{-10}

* Estimated concentration of oleoyl-CoA bound specifically to the receptor during the incubation. The concentration of [$1\text{-}^{14}\text{C}$]oleoyl-CoA collected in the flow-through fractions from Lipidex was only 22–210 pM. These concentrations are not high enough to interfere with the binding of the receptor and hormone [9].

Figure 1 Elution of the unoccupied receptor on DEAE-Sephadex A-50

Unoccupied receptors (▲) prepared by incubation of a nuclear extract with 50 μ M oleoyl-CoA and passage through a Lipidex column were eluted from DEAE-Sephadex A-50 (8 $\times$ 40 mm) with a 50–400 mM NaCl gradient in buffer T. Occupied receptors (●) prepared by incubation of the nuclear extract with [125 I] T_3 were eluted at the same time on a parallel column (10). Fractions of 0.8 ml were collected. Unoccupied receptor was identified by being assayed for T_3 -binding activity as described in the Materials and methods section.

Figure 2 Scatchard analysis of the unoccupied receptor

Unoccupied receptors prepared by incubation of the nuclear extract with 50 μ M oleoyl-CoA and passage through a Lipidex column were incubated with various concentrations (0.1–4.0 nM) of [125 I] T_3 at 0 $^{\circ}$ C for 20 h. The specific T_3 -binding was assayed as described in the Experimental section. Values are means of duplicate determinations. Solid triangles (▲) and circles (●) represent receptors prepared with and without oleoyl-CoA.

in the pass-through fraction in unoccupied form. The complete absence of [125 I] T_3 previously bound to the receptor (results not shown) as well as the results of chromatography on DEAE-Sephadex A-50 (Figure 1) was evidence for the removal of bound hormone from the receptor. As reported previously [13], the

unoccupied receptor was eluted from DEAE-Sephadex at a higher (by 0.03 M) salt concentration than the occupied one (i.e. the peak positions were at 0.18 and 0.15 M NaCl respectively).

T_3 -binding parameters of the unoccupied receptor

The specific T_3 -binding activity of the receptor treated with oleoyl-CoA and passed through a Lipidex column was 1.6-fold that of the untreated sample, indicating that treatment with oleoyl-CoA increased the T_3 binding of the receptor. The Scatchard plots shown in Figure 2 confirm this conclusion. The unoccupied receptor had a dissociation constant (K_d) of 6.1×10^{-11} M, which was close to that of the untreated control (5.8×10^{-11} M). Treatment with oleoyl-CoA changed the maximum binding capacity from 6.2×10^{-14} to 9.2×10^{-14} mol/mg of protein. Three experiments gave a mean $47 \pm 4\%$ increase in the binding capacity.

Estimation of receptor occupancy by the hormone

From the increased T_3 binding after oleoyl-CoA treatment, we estimated a 44% *in vivo* occupancy of the receptor in adult rat livers as follows. The total number (N_t) of receptors (binding sites) in a liver cell extract is expressed as:

$$N_t = N_1 + N_2$$

where N_1 and N_2 are the numbers of free receptors and of receptors occupied by endogenous T_3 respectively. The *in vivo* occupancy by the hormone is thus N_2/N_t . N_1 and N_t can be estimated by Scatchard-plot analysis of the receptors before or after treatment with oleoyl-CoA. In the Scatchard analysis, receptors were incubated with [125 I] T_3 for 20 h at 0 $^{\circ}$ C. Under these conditions, however, 24% of the occupied receptors released the hormone (Table 1 legend). Thus free receptors available for Scatchard [125 I] T_3 binding increased from the initial N_1 to $N_1 + 0.24N_2$. The total number of receptors, N_t , was 1.5 times the number of free receptors, so we obtained the following equation:

$$(N_1 + 0.24N_2):N_t = 1:1.5$$

Substituting for $N_1 = N_t - N_2$, we obtain:

$$[(N_t - N_2) + 0.24N_2]:N_t = 1:1.5$$

Thus $N_2/N_t = 0.44$, and thus 44% of the receptors had been occupied by the hormone *in vivo*.

DISCUSSION

The results showed that treatment with oleoyl-CoA increased hormone binding of the nuclear extract by accelerating dissociation of the T_3 -receptor complex. An inhibitor of the receptor might have been present and removed by the Lipidex, resulting in increased T_3 binding; however, this possibility was ruled out, since the control receptors incubated without oleoyl-CoA were also eluted in the same way on Lipidex columns.

Oleoyl-CoA competes with the hormone for hormone-binding sites [9], and acyl-CoA binds to the receptor with a dissociation constant of 1.2×10^{-7} M [14]. However, almost all of the [14 C]-oleoyl-CoA bound to the receptor was removed during its passage through Lipidex, suggesting that bound oleoyl-CoA was stripped from the receptor by the Lipidex resin.

From the increase in T_3 binding after oleoyl-CoA treatment, we estimated a 44% *in vivo* occupancy for receptors in liver of adult rats. The estimation was based on the numbers of T_3 -binding sites before and after oleoyl-CoA treatment of the

receptors, and correction of the numbers of unoccupied sites was done for the increase due to spontaneous dissociation of occupied sites during incubation (for 20 h in the Scatchard analysis). The estimation could be more accurate if the kinetics of this spontaneous dissociation and the [125 I] T_3 binding to the new sites were more precisely considered; the unoccupied sites increased in a time-dependent way, but [125 I] T_3 binding to these new sites may not have been completed by the end of incubation. Hence, the actual occupancy value would be slightly larger than the estimated one. Oppenheimer et al. [4] reported 47% occupancy. They injected [125 I] T_3 into rats and measured the amounts of plasma and nuclear T_3 at equilibrium, followed by calculation and correction to obtain the nuclear occupancy value. Both their study and ours showed that about 45% of the hepatic receptors are occupied. These high occupancies may be required to maintain the homeostatic level of activities of hormone-responsive genes in adult animals.

Lipidex has been used in assays of thyroid-hormone receptor [12]. There are several other assay procedures. In each, [125 I] T_3 bound to the receptor is separated from unbound [125 I] T_3 . Filtration of incubation mixtures on nitrocellulose membranes [11], vortex-mixing and centrifugation with Dowex AG 1X8 resin [8] and elution on a Lipidex column [12] are typical methods. Filtration gives the most accurate results, but it should be done in a cold room and the procedure takes time; AG 1X8 is the least expensive and the method is the easiest, but deviation of the data is large; the Lipidex method is suitable for a few samples containing much protein, but Lipidex is expensive, and column preparation for many samples would be tedious. Thus although the assay method with Lipidex is our own method [12], we suggest here that Lipidex is more suitable for the preparation of unoccupied receptors than for such an assay.

Unoccupied receptors may be obtained by fractionation of

nuclear extracts on a column of DEAE-Sephadex [13] or Mono-Q [15]. In these methods, however, the column fractions after chromatography should be assayed for the receptor activity to collect unoccupied receptors, and in DEAE-Sephadex chromatography most receptors are inactivated (see the activity scales on the ordinate in Figure 1). Therefore, for its simplicity and rapidity as well as for its provision of intact receptors, the method reported here is useful for the preparation and study of unoccupied receptors.

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