Tri-iodothyronine-Induced Increase in Rat Liver Nuclear Thyroid-Hormone Receptors Associated with Increased Mitochondrial α -Glycerophosphate Dehydrogenase Activity

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The effect of tri-iodothyronine injection on the nuclear tri-iodothyronine receptor (putative thyroid-hormone receptor) was examined in rat liver. Nuclear receptors were extracted from isolated nuclei with 0.4M-KCl, and their association constants (K_a) and maximal binding capacities (C_{max}) were determined by Scatchard analyses with and without correction for the endogenous hormone. The amount of endogenous tri-iodothyronine bound to non-histone protein was estimated on the basis of the specific radioactivity of [125 I]tri-iodothyronine injected 2h before the rats were killed. It was demonstrated that C_{max} of the nuclear receptors was 2.5-fold higher in severely hyperthyroid than in hypothyroid rats. However, irrespective of the thyroid status, the K_a of the receptors remained unchanged when corrected for endogenous tri-iodothyronine bound to non-histone protein. The validity of the correction was supported by experiments *in vitro* in which nuclear receptors were preincubated with unlabelled tri-iodothyronine. The increase in C_{max} of nuclear receptors was directly related to mitochondrial α -glycerophosphate dehydrogenase activity. These results suggest a hormonal modulation of the nuclear receptors which is associated with hormonal action.

Early studies of Tata & Widnell (1966) indicated that nuclear RNA synthesis is an initial and essential step for thyroid-hormone action. However, no nuclear binding sites related to hormone action were known until Oppenheimer *et al.* (1972) found high-affinity low-capacity sites for tri-iodothyronine in the nuclei of rat liver and kidney. Subsequent studies by these investigators indicated that the nuclear tri-iodothyronine-binding sites are probably thyroid-hormone receptors closely linked to the expression of hormonal effect. The maximal binding capacity (C_{max}) of nuclear receptors is larger in hormone-responsive organs, such as the liver, than in non-responsive organs, such as the spleen (Oppenheimer *et al.*, 1974). Furthermore, a degree of hormone saturation of the receptors is closely related to the hormonal effect (Oppenheimer *et al.*, 1976, 1977). These findings suggest that the binding capacity and occupied number of nuclear receptors may be associated with the hormone effect.

However, it is not known whether the C_{max} or K_a values (or both) of the nuclear tri-iodothyronine receptors can be changed by thyroid hormones. It has been reported that, in rat liver, nuclear-receptor capacities remain unchanged under various thyroid

conditions (Oppenheimer *et al.*, 1975; Spindler *et al.*, 1975; DeGroot *et al.*, 1976), and that in cultured GH1 cells they are depleted when the incubation medium is enriched with tri-iodothyronine (Samuels *et al.*, 1976, 1977). On the other hand, the possibility was suggested (Bernal *et al.*, 1976) that the K_a value for the hormone may be changed in human lymphocytes when tri-iodothyronine is administered *in vivo*. In the present studies, we have demonstrated a significant increase in the C_{max} of liver nuclear receptors in severely hyperthyroid rats, which is associated with a significant increase in α -glycerophosphate dehydrogenase activity.

Materials and Methods

Male Wistar-strain rats (140–160g) were thyroidectomized and fed on a laboratory rat diet together with 0.5% CaCl₂ as drinking water. The animals were used for experiments after 1 week, when the body weights were nearly stabilized. The thyroidectomized animals were segregated into three groups: group I, injected subcutaneously with 40 μ g of tri-iodothyronine/100g body wt. per day for 3 days (two-thirds of the dose in the morning and one-third in the evening); group II, injected subcutaneously with 230ng of tri-iodothyronine/100g

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body wt. per day for 3 days; group III, raised without administration of the hormone for the same period of time. Tri-iodothyronine (Sigma) to be injected was dissolved in 0.01M-NaOH/0.03% bovine serum albumin.

On the 4th day all groups of animals were given an intraperitoneal injection of 0.7 μ Ci of [125 I]tri-iodothyronine (500–600 μ Ci/ μ g; Dainabott Laboratories, Tokyo, Japan)/100g body wt., and exsanguinated 2h later. The livers were perfused *in situ* with 20ml of cold 0.9% NaCl, and then removed and homogenized in 4vol. of 0.25M-sucrose/3mM-MgCl₂ with a Teflon-pestle homogenizer. The homogenate was centrifuged at 700g for 10min, and the sediment obtained was resuspended in 2.4M-sucrose/3mM-MgCl₂ and centrifuged at 80000g (r_{max}) for 45min. The purified nuclear pellet was washed successively in 0.25M-sucrose/3mM-MgCl₂ with and without 0.5% Triton X-100. The washed pellet was suspended in the extraction buffer (0.4M-KCl/5mM-MgCl₂/0.05M-Tris/glycine buffer, pH8.5) at a ratio of 1g-equiv. of liver nuclei/ml, and stirred at 4°C for 1h. After centrifugation at 106800g (r_{max}) for 30min, the KCl extract was enriched with 1mM-dithiothreitol, and stored at -20°C until use. The preparations were stable for 3 weeks under these conditions. Recoveries of radioactivity in the extracts did not differ significantly in group I (76.8 $\pm$ 7.8%, mean $\pm$ S.D.), group II (77.8 $\pm$ 9.4%) and group III (82.9 $\pm$ 5.6%). DNA contents per g of liver remained nearly the same in the three groups.

Scatchard analyses

Nuclear extract (0.1 ml) was enriched with 50 μ l of 25mM-dithiothreitol and increasing amounts of [125 I]tri-iodothyronine (0.01–0.1 pmol/tube) with or without unlabelled tri-iodothyronine (0.1–0.2 pmol/tube), and incubated at 20°C for 3h. The stability of nuclear receptors in the assay system was verified by preliminary experiments. After the incubation, unbound hormone was separated by including 1 ml of 2% (w/v) Dowex 1 (Cl⁻ form; 1X8, 200–400 mesh; Dow Chemical Co., Midland, MI, U.S.A.) anion-exchange resin in the extraction buffer, followed by centrifugation at 3000 rev./min for 20min. The supernatant and resin pellet were counted for 125 I in an automatic well-type scintillation counter. The amount of endogenous hormone in the nuclear extract was estimated on the basis of the specific radioactivity of [125 I]tri-iodothyronine in the nuclear extract and serum. Scatchard (1949) plots were calculated with and without correction for the endogenous hormone, and the lines for high-affinity low-capacity sites were drawn including four points close to the ordinate. The values for K_d and C_{max} in each preparation were means of at least two determinations.

Serum concentrations of tri-iodothyronine were determined by the radioimmunoassay method of Takeda (1975). Tri-iodothyronine contents in the nuclear extracts were also measured by the radioimmunoassay technique of Surks *et al.* (1972) with a minor modification described in detail in the following paper (Nakamura *et al.*, 1979). Mitochondrial α -glycerophosphate dehydrogenase activity was measured by the method of Lee & Lardy (1965), and protein concentration by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. DNA was measured by Burton's (1956) method after extraction from the liver by the method of Schmidt-Thannhauser-Schneider (Schneider, 1946).

Results

Effects of injected tri-iodothyronine on serum and liver nuclear tri-iodothyronine concentration, α -glycerophosphate dehydrogenase activity and non-histone protein

The results obtained in the three groups of rats are summarized in Table 1. The tri-iodothyronine concentrations in serum and in liver non-histone protein were increased markedly in group I and slightly in group II, compared with those in group III. α -Glycerophosphate dehydrogenase activity in group I was 5-fold higher than that in the other two groups. The concentration of non-histone protein was increased slightly but significantly ($P < 0.05$) in group I.

Fig. 1. Effect of preincubating liver nuclear receptors with unlabelled tri-iodothyronine on the Scatchard plot of their binding

Non-histone proteins were extracted from liver nuclei of thyroidectomized rats, and K_d and C_{max} of the nuclear tri-iodothyronine receptors were determined by Scatchard analyses before and after preincubation with 200pg of tri-iodothyronine/ml of the extracts. For further details see the text. $\square$, Before incubation; $\circ$ and $\bullet$, Scatchard plots obtained after incubation, without and with correction for added hormone respectively.

Table 1. Effects of tri-iodothyronine (T₃) injection on serum tri-iodothyronine concentration, mitochondrial α -glycerophosphate dehydrogenase activity, liver non-histone protein concentration, endogenous tri-iodothyronine bound to non-histone protein assessed by the tracer method and radioimmunoassay, and C_{max} and K_a of nuclear receptors from livers of rats in various thyroid states

All the data given are means $\pm$ s.d.

T ₃ injected	No.	Serum T ₃ (ng/100ml)	α -Glycero- phosphate dehydrogenase (ΔA /min per mg)	$10^6 \times K_a$ (M ⁻¹)		C _{max} (pg of T ₃ /g of liver)		Non-histone protein (mg/g of liver)		Endogenous T ₃ bound to non-histone protein (pg/ml)	
				Un- corrected	Corrected	Un- corrected	Corrected			Tracer	Radio- immunoassay
Group I (40 μ g, 3 days)	5	816.4 $\pm$ 380.8	163.1 $\pm$ 26.2	4.8* $\pm$ 1.3	10.3 $\pm$ 4.1	490* $\pm$ 66	573* $\pm$ 143	2.47† $\pm$ 0.58	2.47† $\pm$ 0.58	518 $\pm$ 257	869 $\pm$ 369
Group II (230ng, 3 days)	6	44.6 $\pm$ 28.5	27.8 $\pm$ 9.5	12.2 $\pm$ 3.6	14.8 $\pm$ 3.3	248 $\pm$ 84	267 $\pm$ 87	1.56 $\pm$ 0.30	1.56 $\pm$ 0.30	92 $\pm$ 49	88 $\pm$ 77
Group III (non- injected)	5	<20	30.8 $\pm$ 10.5	12.7 $\pm$ 2.3	12.7 $\pm$ 2.3	233 $\pm$ 94	233 $\pm$ 94	1.63 $\pm$ 0.24	1.63 $\pm$ 0.24	<20	<20

* Significance of difference from the corresponding values of group III, $P < 0.005$.
† Significance of difference, $P < 0.05$.

Fig. 2. Effect on Scatchard plot of correction for endogenous tri-iodothyronine

Non-histone proteins were prepared from hyperthyroid rats of group I (see the Materials and Methods section), and K_a and C_{max} of the nuclear receptors were determined from Scatchard plots calculated without (○) and with correction (●) for endogenous tri-iodothyronine bound to the proteins.

Fig. 3. Scatchard plots corrected for endogenous tri-iodothyronine of the nuclear receptors in rats given graded doses of tri-iodothyronine

Non-histone proteins were prepared from three groups of rats given (a) 40 μ g (group I), (b) 230 ng (group II) and (c) no tri-iodothyronine (group III) (as described in the Materials and Methods section), and Scatchard plots were drawn with correction for endogenous tri-iodothyronine bound to the proteins.

Scatchard plot of tri-iodothyronine binding by the nuclear receptor with or without preincubation with hormone

To evaluate the effect of endogenous tri-iodothyronine on apparent K_a and C_{max}, non-histone proteins obtained from thyroidectomized rats were preincubated with stable tri-iodothyronine at a

concentration equivalent to hyperthyroidism, and then analysed by Scatchard plot with or without correction for the hormone added in advance (Fig. 1).

Without the correction, the values were substantially decreased for K_a and slightly decreased for $C_{max.}$. With the correction, however, the Scatchard plot obtained was almost superimposed on that of the original preparation, and hence the values for K_a and $C_{max.}$ remained the same, irrespective of the preincubation.

Effect of administered tri-iodothyronine on K_a and $C_{max.}$ of nuclear receptors

The effect of the correction for endogenous tri-iodothyronine should be most pronounced in group I, because the amount of endogenous hormone in non-histone proteins was much increased in this group. Fig. 2 illustrates a typical example of a Scatchard plot for the group-I results with or without correction for endogenous tri-iodothyronine. The correction for endogenous hormone had the same effect on the Scatchard plot as observed for results of the experiment *in vitro* (Fig. 1).

Table 1 summarizes the results obtained in the three groups, and Fig. 3 shows their representative Scatchard plots corrected for endogenous tri-iodothyronine. Although the uncorrected value for K_a was markedly decreased in group I, the corrected value did not differ significantly from that of the other groups (Table 1). By contrast, the $C_{max.}$ value was significantly ($P < 0.005$) increased in group I

compared with the other groups, and the corrected value was even greater than the uncorrected one.

Relation between $C_{max.}$ and mitochondrial α -glycerophosphate dehydrogenase activity

As shown in Fig. 4, α -glycerophosphate dehydrogenase activity was significantly ($r = +0.821$, $P < 0.001$) correlated with the $C_{max.}$ of liver nuclear receptors.

Discussion

In previous studies with rat liver (Oppenheimer *et al.*, 1975; DeGroot *et al.*, 1976) and human lymphocytes (Bernal *et al.*, 1976; Lemarchand-Béraud *et al.*, 1977), no special attention was directed towards endogenous tri-iodothyronine bound to the receptors. Endogenous tri-iodothyronine occupies about half the $C_{max.}$ in euthyroidism and almost all the $C_{max.}$ in hyperthyroidism (Oppenheimer *et al.*, 1976), and hence, the values for K_a and $C_{max.}$ are falsely low if the amount of endogenous hormone is not taken into account. This notion was verified by the present studies *in vitro* and *in vivo*. The results obtained indicate that correction for endogenous tri-iodothyronine is essential for accurate determination of K_a and $C_{max.}$, especially in hyperthyroidism. The corrected K_a value remained the same in all the three groups, whereas the corrected $C_{max.}$ value per g of liver was 2.5 times higher for group-I than group-III rats. Because DNA contents per g of liver and also the yields of tracer tri-iodothyronine from the nuclei did not differ significantly in these groups, it was concluded that the $C_{max.}$ value per nucleus is increased in the severely hyperthyroid rats.

The increase in the $C_{max.}$ value was significantly correlated ($r = +0.821$, $P < 0.001$) with an increase in α -glycerophosphate dehydrogenase activity. It has been reported that the magnitude of $C_{max.}$ of the nuclear responsiveness of certain tissues (Oppenheimer *et al.*, 1974), and further, that induction of α -glycerophosphate dehydrogenase depends on both the degree of hormone saturation and the duration of time occupied by hormone (Oppenheimer *et al.*, 1976, 1977). Therefore it seems reasonable to infer that the increased $C_{max.}$ of the nuclear receptors may be responsible for the induction of this enzyme together with the hormone occupancy. The tri-iodothyronine-binding sites found on mitochondrial membranes (Tata, 1975; Sterling *et al.*, 1977) appear to differ in their mechanism of hormone action from the nuclear receptors, because oxidative phosphorylation increases a few minutes after incubation with hormone *in vitro* (Sterling *et al.*, 1977).

The nuclear-receptor capacities of rat liver were reported not to be affected in hyperthyroid and hypo-

Fig. 4. Relationship between mitochondrial α -glycerophosphate dehydrogenase activity and $C_{max.}$ of the nuclear tri-iodothyronine receptors

$C_{max.}$ values of the nuclear receptors in rats given graded doses of tri-iodothyronine were determined from the corrected Scatchard plots, and were compared with the induced enzyme activity. ○, Group I; ●, group II; △, group III.

thyroid rats. However, DeGroot *et al.* (1976) gave a single injection of at most 750 ng of tri-iodothyronine 2 h before the rats were killed. On the other hand, in rats given a single injection of a few mg of tri-iodothyronine before 3 days, Oppenheimer *et al.* (1975) indirectly estimated $C_{\max}$ from the plasma/nuclei ratio of tracer hormone, which was assumed to be proportional to an [intracellular free tri-iodothyronine]/ $C_{\max}$ ratio. This assumption, however, might not be accurate enough, since with a huge amount of administered tri-iodothyronine, the nuclear binding sites may be totally saturated, and hence, an equilibrium may not be established between nuclear and serum binding sites.

$C_{\max}$ was not increased in group-II rats, which were found to still be hypothyroid at the α -glycerophosphate dehydrogenase activity and serum tri-iodothyronine concentrations. Therefore it is not clear whether $C_{\max}$ increases in euthyroid or slightly hyperthyroid states. Nevertheless, the 2.5-fold increase in $C_{\max}$ of severely hyperthyroid rats indicates a possibility that $C_{\max}$ of liver nuclear receptors is increased in association with the hormonal effect.

Samuels *et al.* (1976, 1977) showed, on the basis of kinetic studies, that concentrations of nuclear receptors in cultured GH1 cells, a rat pituitary tumour cell line, were decreased during incubation with tri-iodothyronine. The system employed and the time of exposure to the hormone were quite different from ours, and hence no comparison is possible between the two results. However, O_2 consumption of the pituitary is known to decrease after the administration of thyroid hormone. Therefore the inconsistent results might be ascribed to the difference in the tissues employed.

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