Increase in Liver Nuclear Tri-iodothyronine Receptors associated with Increased Deoxyribonucleic Acid by Long-Term Administration of Tri-iodothyronine in Thyroidectomized Rats

By Hirotoshi NAKAMURA,* Satoshi HAMADA†† and Hiroo IMURA*

*Department of Internal Medicine II, Kyoto University School of Medicine, Kyoto 606, and

†Department of Endocrinology and Nuclear Medicine, Tenri General Hospital, Tenri 632, Japan

(Received 22 May 1979)

The effect of long-term administration of small amounts of tri-iodothyronine was examined on the nuclear tri-iodothyronine receptors in rat liver. The maximal binding capacity ($C_{\max}$) and association constant (K_a) of the receptors were determined in thyroidectomized rats given vehicle alone (group A), 2 μ g of tri-iodothyronine/100g body wt. (group B) or 7 μ g of tri-iodothyronine/100g body wt. (group C) for 2 weeks. Scatchard analyses with correction for the amount of endogenous tri-iodothyronine revealed that $C_{\max}$ values per g of liver were increased to 1.5 and 2.7 times the control value in groups B and C respectively. Since concentrations of DNA per g of liver were significantly increased in the two groups of hormone-treated rats, $C_{\max}$ values per mg of DNA were nearly the same in group B, but still increased significantly in group C compared with group A. K_a values remained unchanged in all three groups of animals. Mitochondrial α -glycerophosphate dehydrogenase activity was 9.6 and 28.7 times as high in groups B and C, respectively, as in group A. Concentrations of endogenous tri-iodothyronine bound to non-histone protein were substantially increased in groups B and C, although concentrations of serum tri-iodothyronine remained rather low. The results obtained indicate that the long-term administration of tri-iodothyronine in small doses induces an increase in the nuclear receptors associated with increased DNA with and without accompanying a relative increase in the receptor concentration in thyroidectomized rats. Also the hormonal effect is closely related to the total number of the nuclear receptors and the concentrations of nuclear tri-iodothyronine bound to the receptors rather than the serum tri-iodothyronine concentrations.

Accumulating evidence strongly suggests that the high-affinity, low-capacity binding sites for tri-iodothyronine in nuclei are thyroid-hormone receptors relevant to an initiation of hormone actions, and that occupancy by the hormone of these sites is closely related to the hormonal effects (Oppenheimer *et al.*, 1976). Concentrations of the nuclear receptors were previously thought to be unchanged under various thyroid conditions, although they are much higher in hormone-responsive organs such as the liver than in non-responsive organs such as the spleen (Oppenheimer *et al.*, 1974). More recently, however, we have found that concentrations of the rat liver nuclear receptors are increased 2.5-fold in hyperthyroid rats given 40 μ g of tri-iodothyronine for 3 days (Hamada *et al.*, 1979), and that a single injection of tri-iodothyronine at a dose of 1250 μ g induces sequential increases in the concentration of nuclear receptors and mitochondrial α -glycerophosphate dehydrogenase activity (Nakamura *et al.*, 1979). In these studies the administered doses of tri-iodothyronine were very large, and hence a question might be raised as to whether these hormonal

effects were pharmacological rather than physiological.

The present studies indicate that concentrations of the rat liver nuclear receptors are increased by smaller amounts of tri-iodothyronine injected for a longer period of time. The relationships between concentrations of the nuclear receptors, DNA and tri-iodothyronine in serum and in nuclear non-histone protein were also examined with regard to the hormone action.

Materials and Methods

Preparation of nuclear extracts

Wistar-strain male rats (body wt. 150g) were obtained from Shimizu Laboratory Animal Co., Kyoto, Japan. They were housed throughout the studies in a constant-environment apparatus kept at 25°C and a humidity of 55% and were fed on a laboratory rat diet (Oriental Yeast Industrial Co., Chiba, Japan). After a few days of housing, they were all thyroidectomized and given 0.5% CaCl_2 solution as drinking water.

After 2 weeks the thyroidectomized animals, weighing approx. 200g, were divided into three

† To whom reprint requests should be addressed.

groups and treated as follows: group A, injected subcutaneously with a vehicle only (0.01 M-NaOH/0.03% bovine serum albumin) for 2 weeks; group B, injected daily subcutaneously with 2 μ g of tri-iodothyronine/100 g body wt. for 2 weeks (two-thirds of the dose in the morning and one-third in the evening); and group C, injected daily subcutaneously with 7 μ g of tri-iodothyronine/100 g body wt. for 2 weeks. All the groups of animals weighed about 240–250 g at the time of death, and the liver weight was approx. 10 g. There were no significant differences in the body weight and the liver weight among the three groups.

The animals were killed by exsanguination through the abdominal aorta under light ether anaesthesia, and the livers were perfused with 20 ml of cold 0.9% NaCl until completely blanched. After the excised liver was homogenized, the nuclear fraction was obtained by centrifugation at 600g for 10 min, and then hepatic nuclei were purified by centrifugation through 2.4 M-sucrose followed by washing with 0.5% Triton X-100, as previously described (Hamada *et al.*, 1979). From the purified nuclei, non-histone proteins were extracted with 0.4 M-KCl/5 mM-MgCl₂/0.05 M-Tris/glycine buffer (pH 8.5) at a concentration of 1 g tissue equivalent/ml of nuclear extracts. The yields of proteins in the extraction did not differ significantly in group A ($46.5 \pm 4.7\%$; mean $\pm$ S.D.), group B ($51.8 \pm 3.2\%$) or group C ($51.7 \pm 4.8\%$). The nuclear extracts were supplemented with 1 mM-dithiothreitol and stored at -20°C . Scatchard analyses were performed within 1 week of preparation, although the binding activity of nuclear proteins for tri-iodothyronine remained unchanged for about 3 weeks.

Scatchard analyses

Binding properties of the nuclear receptors were estimated by Scatchard analyses with and without correction for the amount of tri-iodothyronine endogenously bound to the receptors (Hamada *et al.*, 1979). The amount of tri-iodothyronine in serum and non-histone protein was determined by the radioimmunoassay used previously (Nakamura *et al.*, 1979). The values for K_d and C_{max} were estimated by a regression line by using at least four points close to the ordinate, and were the means of at least two determinations.

Other assays

Mitochondrial α -glycerophosphate dehydrogenase activity was measured by the method of Lee & Lardy (1965). Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. DNA was extracted by the Schmidt-Thannhauser method (Schneider, 1946)

Table 1. Concentrations of serum tri-iodothyronine (T3), endogenous tri-iodothyronine bound to non-histone protein, DNA, nuclear proteins and non-histone proteins and α -glycerophosphate dehydrogenase activity after the injection of tri-iodothyronine
All data given are means $\pm$ S.D. All the values in groups B and C are significantly different from the corresponding values in group A ($P < 0.01$).

Group	No. of animals	Serum T3 (ng/100 ml)	Endogenous T3 bound to non- histone protein (ng/100 ml)	Nuclear protein (mg/g of liver)	Non-histone proteins (mg/g of liver)	DNA (mg in nuclei isolated from 1 g of liver)	$10^3 \times \alpha$ -Glycero- phosphate dehydro- genase activity ($\Delta A/\text{min}$ per mg of protein)
A: Vehicle, 2 weeks	7	<12.5	8.3 ± 3.1	3.25 ± 2.50	1.71 ± 0.19	1.54 ± 0.18	19.9 ± 14.0
B: T3 (2 μ g, 2 weeks)	6	18.9 ± 9.2	32.0 ± 10.6	4.91 ± 4.20	2.68 ± 0.17	2.40 ± 0.12	190.8 ± 89.3
C: T3 (7 μ g, 2 weeks)	6	77.8 ± 29.9	63.6 ± 16.9	5.06 ± 5.59	2.90 ± 0.28	2.43 ± 0.53	570.3 ± 47.0

and measured by Burton's (1956) method. The yields of DNA in preparation of the purified nuclei from the liver were not significantly different between the hormone-treated ($61.9 \pm 6.2\%$, mean $\pm$ s.d.) and non-treated rats ($65.8 \pm 11.6\%$).

Results

Concentrations of serum tri-iodothyronine, endogenous tri-iodothyronine, DNA, nuclear proteins and non-histone proteins, and mitochondrial α -glycerophosphate dehydrogenase activity

The results obtained are summarized in Table 1. Serum tri-iodothyronine was undetectable (less than 12.5 ng/100 ml) in group A of rats given vehicle alone for 2 weeks. The hormone concentrations were well below the normal limit in group-B rats, given 2 μ g of tri-iodothyronine for 2 weeks, but within the normal range in group-C rats, given 7 μ g of tri-iodothyronine for 2 weeks (Fig. 1).

On the other hand, concentrations of tri-iodothyronine bound to non-histone protein in the liver were 8.3 ± 3.1 ng (mean $\pm$ s.d.)/100 ml in group A, 32.0 ± 10.6 ng/100 ml in group B and 63.6 ± 16.9 ng/100 ml in group C. These concentrations were high as compared with serum tri-iodothyronine concentrations in separate groups.

Mitochondrial α -glycerophosphate dehydrogenase activity of the liver was 19.9 ± 14.0 Δ /min per mg of protein in group A. The values were increased to 190.8 ± 89.3 in group B, and further to 570.3 ± 47.0 in group C.

DNA contents in nuclei isolated from the liver were 1.54 ± 0.18 mg/g of liver in group A, 2.40 ± 0.12 mg/g in group B and 2.43 ± 0.53 mg/g in group C. The DNA contents in the hormone-treated groups were significantly ($P < 0.01$) higher than those

Fig. 1. Concentrations of tri-iodothyronine in serum and in non-histone protein, mitochondrial α -glycerophosphate dehydrogenase activity and DNA among groups A, B and C. Thyroidectomized rats were divided into three groups: group A, injected subcutaneously with vehicle only, group B, injected with 2 μ g of tri-iodothyronine/100g body wt., and group C, injected with 7 μ g of tri-iodothyronine/100g body wt., for 2 weeks respectively. The amount of tri-iodothyronine in serum (S-T3) and non-histone protein (N-T3) was measured by radioimmunoassay. Mitochondrial α -glycerophosphate dehydrogenase activity (α -GPD) was measured by the method of Lee & Lardy (1965). DNA was measured by Burton's (1956) method. Each bar indicates mean $\pm$ s.d., and closed circles and the broken line in the S-T3 columns show individual values for serum tri-iodothyronine and the undetectable range respectively.

in group A of non-treated rats. No significant difference in the recovery of DNA extraction and in the weight of livers was detected between the tri-iodothyronine-treated and non-treated rats.

Fig. 2. Scatchard plot with and without correction for endogenous tri-iodothyronine. Maximal binding capacity and association constant of the nuclear tri-iodothyronine (T3) receptors were determined by Scatchard plot with (b) and without (a) correction for endogenous tri-iodothyronine bound to non-histone protein; $\bullet$, group A; $\circ$, group B; Δ , group C. The broken line shows the value for non-specific binding.

Table 2. Association constant (K_a) and maximal binding capacity (C_{max}) of nuclear tri-iodothyronine (T3) receptor with and without correction for endogenous hormone after the injection of tri-iodothyronine

All data given are means $\pm$ s.d. Significance of difference from the corresponding values of group A: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Group	No. of animals	Uncorrected			Corrected		
		$10^9 \times K_a$ (M^{-1})	C_{max} (pg of T3/g of liver)	C_{max} (pg of T3/mg of DNA)	$10^9 \times K_a$ (M^{-1})	C_{max} (pg of T3/g of liver)	C_{max} (pg of T3/mg of DNA)
A: Vehicle, 2 weeks	7	13.6 $\pm$ 2.2	210.0 $\pm$ 52.1	135.6 $\pm$ 30.9	15.5 $\pm$ 3.0	231.1 $\pm$ 61.8	149.6 $\pm$ 37.9
B: T3 (2 μ g, 2 weeks)	6	8.0*** $\pm$ 1.0	279.9* $\pm$ 48.8	116.9 $\pm$ 21.8	14.8 $\pm$ 2.7	354.8** $\pm$ 71.6	147.6 $\pm$ 30.1
C: T3 (7 μ g, 2 weeks)	6	6.6*** $\pm$ 1.1	437.8*** $\pm$ 104.2	189.6 $\pm$ 63.8	14.7 $\pm$ 2.6	629.8*** $\pm$ 97.7	268.5** $\pm$ 63.5

Protein concentrations of total nuclei and non-histone protein extracted from the livers in group A were 3.23 ± 2.50 and 1.71 ± 0.19 mg/g wet wt. of tissue respectively. Both of them were significantly ($P < 0.01$) increased after the injection of tri-iodothyronine at the doses of 2 μ g (group B) and 7 μ g (group C) for 2 weeks. Since DNA contents were increased to a similar extent, the ratio of the protein concentrations and DNA contents remained unchanged after the injection of hormone.

K_a and C_{max} of nuclear receptors

In Scatchard plots without correction for endogenous tri-iodothyronine bound to the nuclear receptors, the slopes of the regression lines were quite different among the three groups, and the apparent values for K_a were significantly ($P < 0.001$) decreased in group B, and even more decreased in group C (Fig. 2a). With correction for the endogenous hormone, however, the slopes of the lines in Scatchard plots became nearly the same (Fig. 2b), and the corrected values for K_a were virtually equal in all the three groups (Table 2).

C_{max} values per g of liver were significantly increased in both hormone-treated groups even in

Fig. 3. Maximal binding capacity and association constant of the rat liver nuclear tri-iodothyronine receptors

Corrected values of maximal binding capacity (C_{max}) and association constant (K_a) of the nuclear tri-iodothyronine (T3) receptors were obtained in the three groups of animals. C/liver represents the C_{max} value per g of liver, and C/DNA the C_{max} value per mg of DNA. Each bar indicates mean $\pm$ s.d. * $P < 0.01$, ** $P < 0.001$, as compared with the corresponding values for group A.

uncorrected Scatchard plots (Fig. 2a). After correction for the endogenous hormone, the values were much greater than the control (Fig. 2b). The corrected values were 1.5 and 2.7 times as large in groups B and C, respectively, as the control (Fig. 3). As DNA contents per g of the liver in groups B and C were significantly increased, corrected values per mg of DNA remained the same in group B, but increased 1.8-fold in group C as compared with the control (Fig. 3).

Discussion

It was shown in the present study that the liver nuclear tri-iodothyronine receptors were increased by the long-term administration of smaller amounts of tri-iodothyronine than used in the previous studies (Hamada *et al.*, 1979; Nakamura *et al.*, 1979). The C_{max} values per g of liver were increased to 1.5 times the control value in rats given 2 μ g of tri-iodothyronine for 2 weeks, and to 2.7 times the control value in rats given 7 μ g of hormone for 2 weeks. It was confirmed further that the K_a values were unchanged after administration of the hormone when the amount of endogenous tri-iodothyronine bound to the receptors was taken into account in the Scatchard analysis.

In contrast with the previous studies, DNA contents per g of liver were elevated in the two groups of animals injected with tri-iodothyronine for 2 weeks. This increase in DNA does not appear to be due either to dehydration or to any physicochemical conditions to decrease the size of liver cells, because the whole liver weights together with the body weights remained the same irrespective of the injection of tri-iodothyronine. Two possibilities may be considered as the reason for the increase in DNA. First, the long-term administration of tri-iodothyronine may induce a shift of cell ploidy pattern. It is known that cell ploidy pattern in rat liver cells is changed as the animal grows older: mononucleated diploid cells predominate in newborn-rat livers, whereas tetraploid cells begin to increase 4–5 weeks after birth and are in the majority in the adult liver (Alfert & Geschwind, 1958; Nadal & Zajdela, 1966). Furthermore, the ploidy pattern is influenced by various treatments such as hypophysectomy, adrenalectomy, partial hepatectomy and administration of steroid hormones (Geschwind *et al.*, 1958; James *et al.*, 1966; Swartz, 1967; Wiest, 1974). Therefore it is possible that the change in cell ploidy pattern may be stimulated by the tri-iodothyronine injection into thyroidectomized rats at the age of 6 weeks. Another possibility for the increment in DNA is that the livers from rats given tri-iodothyronine may contain larger numbers of hepatic cells per g of the liver than do those from hypothyroid rats. It is probable that thyroid hormones possessing pronounced effects on growth of animals may stimulate

DNA synthesis and cell proliferation, whereas glucocorticoids inhibit (Henderson *et al.*, 1971; Henderson & Loeb, 1974; Wiest 1974). Studies in our laboratory (H. Nakamura, T. Ashiwaru, S. Hamada & H. Imura, unpublished work) have revealed that there is no apparent difference in the cell ploidy patterns in the livers between the hormone-treated and non-treated rats, indicating the latter possibility.

Since DNA contents per g of liver were increased after the injection of tri-iodothyronine, concentrations of the receptors per mg of DNA remained unchanged in rats given 2 μ g of tri-iodothyronine, but still increased in rats given 7 μ g of tri-iodothyronine. The results obtained indicate that the long-term administration of tri-iodothyronine induces the increase in the nuclear receptors associated with increased DNA, and that a relative increment in nuclear receptors compared with DNA occurs when relatively large amounts of tri-iodothyronine are administered, but not when small amounts of the hormone are administered. The relative increment in the nuclear receptors appears to indicate a selective induction of the receptors by administered tri-iodothyronine, as shown in our previous studies (Hamada *et al.*, 1979; Nakamura *et al.*, 1979). It is possible, however, that the receptor increase parallel with DNA contents may be inherent in the nucleus proliferation, since it was accompanied by corresponding increases in nuclear proteins and non-histone proteins. It is noteworthy that receptor concentrations on the basis of DNA do not necessarily represent a change in the total number of the receptors.

Mitochondrial α -glycerophosphate activity was increased 9.6- and 28.7-fold by injection of 2 μ g and 7 μ g of tri-iodothyronine for 2 weeks respectively. In rats given 2 μ g of tri-iodothyronine, the nuclear receptors were increased 1.5-fold per g of liver, and endogenous tri-iodothyronine bound to non-histone protein was increased to 3.9 times the control value. As a result, nuclear occupancy of the hormone was much increased in this group of rats. It seems reasonable to infer that this elevation of the enzyme activity results from the increased tri-iodothyronine occupancy on the increased numbers of receptor sites through a certain amplification mechanism.

Despite the remarkable elevation of mitochondrial α -glycerophosphate dehydrogenase activity, tri-iodothyronine concentrations in serum were well below the normal limit in rats given 2 μ g of tri-iodothyronine, and remained within the normal range in rats given 7 μ g of hormone. Since injected tri-iodothyronine is rapidly transferred from the blood to peripheral cells (Sterling, 1970), the blood specimens obtained 12–13 h after the last injection of hormone should represent the lowest values for serum tri-iodothyronine. Nevertheless, the marked difference noted in serum rather than in nuclear tri-

iodothyronine concentrations appears to indicate that a hormonal effect of tri-iodothyronine, either injected or probably secreted from the thyroid, cannot be estimated correctly from the serum concentrations.

We thank Miss Yoshimi Ikebe for her secretarial work, and the Dinabott Laboratories, Tokyo, for generously giving the radioactive material.

References

- Alfert, M. & Geschwind, I. I. (1958) *Exp. Cell Res.* **15**, 230-232
- Burton, K. (1956) *Biochem. J.* **62**, 315-323
- Geschwind, I. I., Alfert, M. & Schooley, C. (1958) *Exp. Cell Res.* **15**, 232-235
- Hamada, S., Nakamura, H., Nanno, M. & Imura, H. (1979) *Biochem. J.* **182**, 371-375
- Henderson, I. C. & Loeb, J. N. (1974) *Endocrinology* **94**, 1637-1643
- Henderson, I. C., Fischel, R. E. & Loeb, J. N. (1971) *Endocrinology* **88**, 1471-1476
- James, J., Schopman, M. & Delfgaauw, P. (1966) *Exp. Cell Res.* **42**, 375-379
- Lee, Y. P. & Lardy, H. A. (1965) *J. Biol. Chem.* **240**, 1427-1436
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Nadal, C. & Zajdela, F. (1966) *Exp. Cell Res.* **42**, 99-116
- Nakamura, H., Hamada, S. & Imura, H. (1979) *Biochem. J.* **182**, 377-382
- Oppenheimer, J. H., Schwartz, H. L. & Surks, M. I. (1974) *Endocrinology* **95**, 897-903
- Oppenheimer, J. H., Schwartz, H. L., Surks, M. I., Koerner, D. & Dillmann, W. H. (1976) *Recent Prog. Horm. Res.* **32**, 529-565
- Schneider, W. C. (1946) *J. Biol. Chem.* **164**, 747-751
- Sterling, K. (1970) *Recent Prog. Horm. Res.* **26**, 249-274
- Swartz, F. J. (1967) *Exp. Cell Res.* **48**, 557-568
- Wiest, L. (1974) *J. Endocrinol.* **60**, 315-319