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Induction of Cytosolic Triiodo-L-Thyronine (T_3) Binding Protein (CTBP) by T_3 in Primary Cultured Rat Hepatocytes

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Abstract. Cytosolic 3, 5, 3'-triiodo-L-thyronine (T_3)-binding protein (CTBP) plays an important role in the regulation of intracellular T_3 translocation from cytoplasm to the nuclear T_3 receptor. We examined whether the CTBP activity could be induced by T_3 or not in cultured hepatocytes prepared from thyroidectomized rats. CTBP activity was not detected in primary cultured hepatocytes from thyroidectomized rats. However, the protein was induced by the addition of T_3 to the culture medium. The increase in the activity of CTBP was time dependent and the maximal level was obtained by 48 h in the presence of 300 nM T_3. CTBP activity was also increased by retinol (35 μM) or by 1,25-$(OH)_2$-vitamin D_3 (10 nM). On the other hand, the activity of malic enzyme (ME) was induced by the addition of T_3 to the culture medium. The maximal activity of ME was obtained by 48 h in the presence of 300 nM T_3. The increase in ME activity was also induced by retinol or 1,25-$(OH)_2$-vitamin D_3. These results suggested that not only ME activity but also CTBP activity is induced by T_3. Further, retinol and vitamin D_3 have similar effects on the induction of CTBP activity and ME activity.

Key words: Rat hepatocytes, Triiodothyronine (T_3), Cytosolic T_3 Binding Protein (CTBP), Malic enzyme.
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WE DEMONSTRATED that 4.7S NADPH-dependent cytosolic 3,5,3'-triiodo-L-thyronine (T_3)-binding protein (CTBP) is present in rat kidney. The mol wt of the protein was 58,000, calculated from the Stokes radius (Rs; 32.5 A) and the sedimentation coefficient [1]. The protein was activated by NADPH [2, 3] or NADP plus dithiothreitol (DTT) [4]. The former active form inhibited the nuclear transport of T_3, whereas the latter active form accelerated the transport of T_3 from cytoplasm to nuclear T_3 receptor *in vitro* [4, 5]. These observations suggested that the NADPH-dependent CTBP plays an important role in the regulation of intracellular T_3 translocation [6].

Previously we observed that the level of CTBP was decreased by thyroidectomy in rat kidney. The level was restored by the administration of T_4 without changes in the affinity constant for T_3 binding [7]. These results indicated that CTBP is one of the thyroid hormone responsive proteins.

Malic enzyme which is known to be induced by thyroid hormone plays an important role in NADPH production in cytosol. In this study, we examined whether the CTBP and malic enzyme could be induced by T_3 or not in cultured hepatocytes prepared from thyroidectomized rat. Further we examined the effect of retinol (Vitamin A) and 1,25-$(OH)_2$-dihydroxycholecalciferol (1,25-$(OH)_2$-D_3) on CTBP.

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Materials and Methods

Preparation of experimental animals and cell culture

Male Wister rats (100g) were surgically thyroidectomized 4 weeks prior to primary culture and they were given 0.025% methimazole (MMI) and 0.9% $CaCl_2$ as drinking water. Control rats were sham-operated 4 weeks prior to primary culture. Serum levels of T_3 and T_4 (0.4 nmol/*l* and 6.4 nmol/*l*, respectively) were markedly lower in the thyroidectomized animals than those (1.5 nmol/*l* and 70.8 nmol/*l*, respectively) in control sham-operated rats. Hepatocytes were isolated by the method of Seglen [8]. Cells were inoculated at an initial density of 1.5×10^5 cells/cm^2 in 10 cm diameter dishes with culture medium (William's medium E) (Flow Laboratories, Irvine, Scotland) supplemented with 10% new born calf serum, penicillin (50 units/m*l*), streptomycin (50 μg/m*l*) and 10^{-7} M insulin. Cells were cultured at 37°C in a humid atmosphere of 5% CO_2 in air, and culture media were changed every 24 h. After incubation, cells were detached with 0.02% EDTA and 0.25% trypsin (Flow Laboratories), and a cytosol fraction was prepared. Concentrations of T_3 and T_4 in the culture medium were undetectable.

Preparation of cytosol fraction

Cells were washed with phosphate-buffered saline (PBS), pH 7.4, twice and homogenized in 1.5 m*l* of 10 mM Tris-HCl, pH 7.4, containing 0.32 M sucrose. The cytosol fraction was obtained by centrifuging the homogenate at 100,000 × g for 30 min. This fraction was used for the determination of malic enzyme activity. For CTBP assay the cytosol fraction was further incubated with 10% charcoal (Sargent-Welch Scientific Co., Skokie, IL) at 0°C for 30 min in order to remove pyridine nucleotides and T_3.

[^{125}I]T_3 binding assay

The charcoal-treated cytosol fraction was incubated with 27.6 fmol [^{125}I]T_3 (3000 μCi/μg) (New England Nuclear, Boston, MA) in the presence or absence of 50 μM NADPH (tetra sodium salt) (Sigma Chemical Co., St. Louis, MO) for 30 min at 0°C. In studies of Scatchard analysis, incubation for T_3 binding assay was performed in the presence of various concentrations (0–10^{-6}M) of unlabeled T_3 (Sigma). After incubation, [^{125}I]T_3 bound to CTBP was determined by the dextran-coated charcoal method.

Miscellaneous

Malic enzyme activity was measured by the method of Ochoa [9]. The protein concentration was determined by the method of Lowry with bovine serum albumin as the standard [10].

Results

Effect of T_3 on the levels of CTBP and activity of malic enzyme in cultured hepatocytes prepared from thyroidectomized rats

Hepatocytes were prepared from thyroidectomized rats as described in the Materials and Methods. The cells were cultured for 5 days in the absence of T_3. Five days after the beginning of the primary culture, T_3 (300 nM) or control buffer was added to the medium, and the culture was continued. As shown in Fig. 1, the maximal binding capacity (MBC) for NADPH-dependent

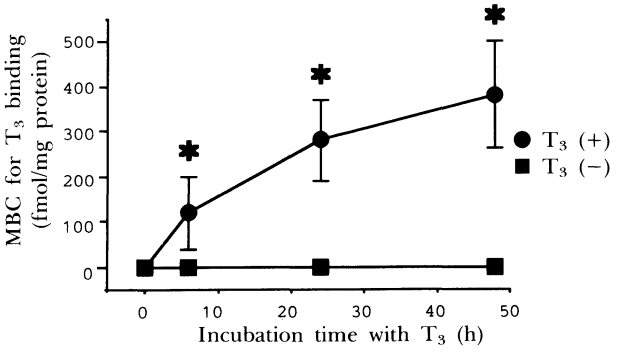

Fig. 1. Changes in the levels of CTBP during culture in the presence or absence of T_3 in hepatocytes prepared from a thyroidectomized rat. Hepatocytes were prepared from thyroidectomized rats. Five days after the beginning of the primary culture, 300 nM of T_3 (closed circle) or buffer (closed square) was added to the culture medium. At 0, 6 h, 24 h, and 48 h after the addition of T_3 or buffer, hepatocytes were harvested and a cytosol fraction was prepared. The maximal binding capacity (MBC) of each cytosol fraction was calculated from Scatchard analysis. Each point indicates the mean ± SD of three determinations. Similar results were obtained in another experiment. *, Different from control, $P<0.05$.

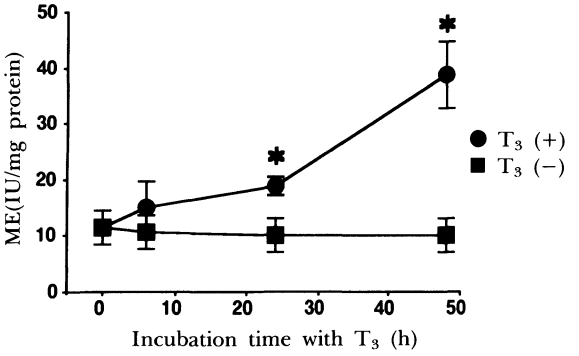

Fig. 2. Change in the malic enzyme activity during the culture in the absence or presence of T_3 in hepatocytes prepared from a thyroidectomized rat. Hepatocytes were prepared from thyroidectomized rats. Five days after the beginning of the primary culture, 300 nM of T_3 (closed circle) or buffer (closed square) was added to the culture medium. At 0, 6 h, 24 h and 48 h after the addition of T_3 or buffer, hepatocytes were harvested and a cytosol fraction was prepared to measure malic enzyme activity. Each point indicates the mean ± SD of 3 determinations. Similar results were obtained in another experiment. *, Different from control, $P<0.05$.

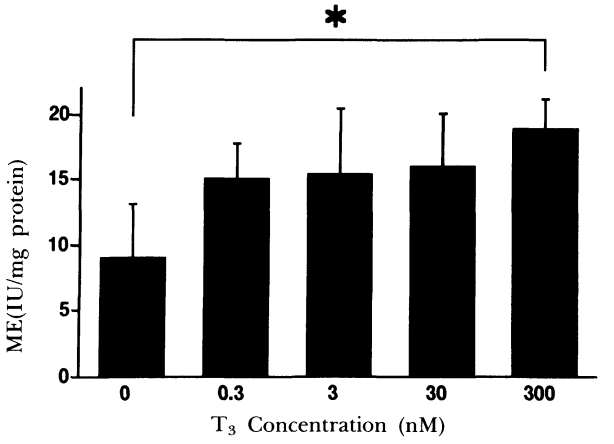

Fig. 3. Effect of various concentrations of T_3 on malic enzyme in hepatocytes prepared from a thyroidectomized rat. Hepatocytes were prepared from thyroidectomized rats. Five days after the beginning of the primary culture, various concentrations of unlabeled T_3 were added to the culture medium. Hepatocytes were cultured for a further 24 h and for each cytosol was prepared. Each point indicates the mean ± SD of three determinations. *, Different from control (0 nM), $P<0.05$.

cytosolic T_3 binding was not detected at the beginning. The level was not increased in the cells cultured in the absence of T_3. In contrast to this result, the level of MBC increased in the cells

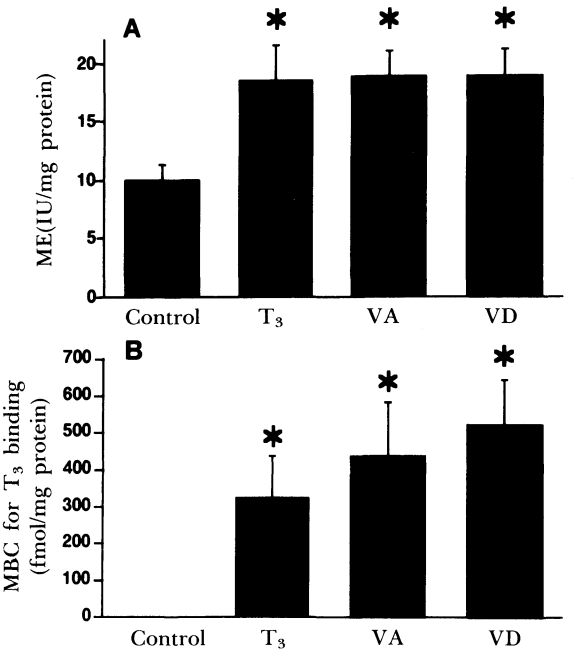

Fig. 4. Effects of retinol and 1, 25 $(OH)_2D_3$ on CTBP and malic enzyme. Hepatocytes were prepared from thyroidectomized rats. Five days after starting the primary culture, 0.5% ethanol, as a control, and 300 nM T_3, 35 μM retinol (VA) or 10 nM 1,25$(OH)_2$ D_3 (VA) were added to the culture medium, and 24 h after the addition of each hormone cytosol was prepared. The levels of malic enzyme activity (ME) (Fig. A) and MBCs for T_3 binding (Fig. B) are shown. Each value represents the mean ± SD. Similar results were obtained in another experiment. *, Different from control, $P<0.05$.

cultured in the presence of T_3. The increase was time dependent until 48 h in culture. The activity of malic enzyme also increased in the cells cultured in the presence of T_3. However, the level did not increase in the cells cultured in the absence of T_3 (Fig. 2). As shown in Fig. 3, the activity of malic enzyme in the presence of 300 nM T_3 was statistically higher than that of the control (0 nM T_3).

Effects of retinol and 1,25-$(OH)_2D_3$ on the CTBP and malic enzyme in hepatocytes prepared from thyroidectomized rats

In order to compare the effects of T_3 with retinol and vitamin D_3, the cells were cultured in the presence of retinol (35 μM) or 1,25-$(OH)_2D_3$ (10 nM). As shown in Fig. 4A, the activity of malic enzyme was increased by retinol or 1,25-$(OH)_2D_3$. Further, the level of MBC for cytosolic T_3 binding

was also increased by retinol or 1,25-$(OH)_2D_3$ (Fig. 4B).

Effects of T_3 on the CTBP and malic enzyme in hepatocytes prepared from control rats

NADPH-dependent cytosolic T_3 binding protein was detected in cells prepared from control rats. As shown in Fig. 5, the MBC for T_3 binding was 4 pmol/mg protein in the cells 5 days after the beginning of the primary culture. The level of MBC for T_3 binding was ten times as high as that obtained in T_3 (300 nM)-stimulated hepatocytes which were prepared from thyroidectomized rats.

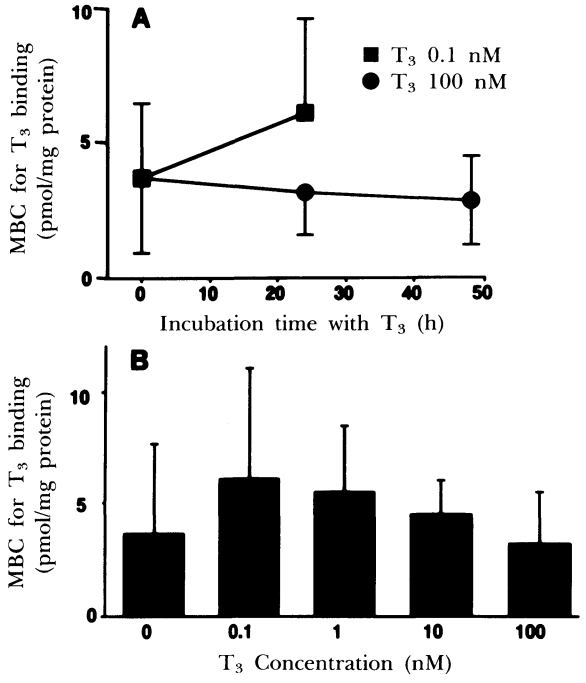

Fig. 5. Effect of T_3 on CTBP in hepatocytes prepared from a control rat. Hepatocytes were prepared from normal rats. Five days after the beginning of the primary culture, 100 nM T_3 was added to the culture medium. At 0, 24 and 48 h after the addition of T_3, T_3 binding to CTBP was measured. The maximal binding capacity (MBC) of each CTBP fraction was calculated from Scatchard analysis. Closed circles and closed squares indicate the MBCs after the culture in the presence of 100 nM T_3 and 0.1 nM T_3, respectively. Each point indicates the mean ± SD. Similar results were obtained in another experiment (Fig. A). Fig. B shows the MBCs for T_3 binding in CTBP prepared from cells cultured for 24 h in the presence of various concentrations of T_3. Each value indicates the mean ± SD. Similar results were obtained in another experiment.

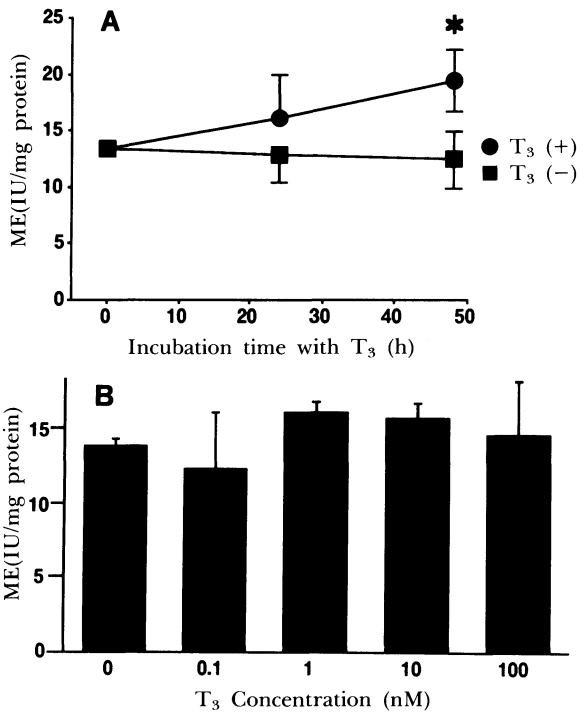

Fig. 6. Effect of T_3 on malic enzyme activity in hepatocytes prepared from a control rat. Hepatocytes were prepared from a control rat. Malic enzyme was prepared as shown in Fig. 4. In Fig. A the time course of the effect of T_3 on malic enzyme (ME) activity is shown. Closed circles and closed squares indicate the level of ME activity in the presence of 100 nM T_3 and that in the absence of T_3, respectively. Fig. B shows the effect of various concentrations of T_3 on malic enzyme activity. Each result is the mean ± SD. Similar results were obtained in another experiment. *, Different from control, $P<0.05$.

In these cells the levels of MBCs in the presence of 100 nM for 24 h and 48 h were not significantly different from that of the control (0 h) (Fig. 5A). When hepatocytes were incubated with various concentrations of T_3, the levels of MBCs were not significantly different from that in the absence of T_3 (Fig. 5B). Under the same conditions, the levels of ME activity were not significantly different from that in the absence of T_3 (Fig. 6B). The activity of ME was significantly increased 48 h after the addition of 100 nM T_3 (Fig. 6A), but the degree of increase was small.

Discussion

Previously we demonstrated that thyroid hor-

mone increased the level of NADPH-dependent cytosolic T_3 binding in rat liver and kidney *in vivo*. However, we could not evaluate whether the mechanism of the action of thyroid hormone is direct or indirect. In this study, we demonstrated that thyroid hormone increased the MBC for NADPH-dependent T_3 binding in isolated hepatocytes prepared from thyroidectomized rats, suggesting that thyroid hormone directly influences the amount of NADPH-dependent CTBP. As shown in this study, the action of thyroid hormone in increasing the level of cytosolic T_3 binding was not observed in cells prepared from control rats. The precise reason why we failed to observe the action of thyroid hormone is not clear. The level of CTBP was very low in hepatocytes prepared from thyroidectomized rats. On the other hand, the level was higher in cells prepared from control rats than in T_3-stimulated hepatocytes derived from thyroidectomized rats. These results suggested that the CTBP activity which is maximally induced by endogenous T_3 *in vivo* might be maintained in the cells even 5 days after the beginning of the primary culture, resulting in the lower responsiveness to T_3 in these cells. But this could not explain why responsiveness to T_3 was also attenuated in malic enzyme activity. Recently it was reported that hepatocytes cultured with proteoglycan fraction or in an uncoated plastic dish with a positively charged surface formed floating multicellular spheroids [11, 12]. The hepatocytes had lower growth activity and maintained greater ability to produce albumin than those in monolayer cells. The responsiveness to T_3 was attenuated in proliferating hepatocytes or in regenerating liver but not in spheroid cells or non proliferating liver [13, 14]. These observations mean that the proliferation rate may be inversely proportional to T_3 responsiveness. Because we could not evaluate the proliferation rate, whether lower responsiveness to T_3 in hepatocytes from control rats is related to the proliferation rate is not known.

We observed that retinol and vitamin D_3 also increased the level of CTBP in the hepatocytes prepared from thyroidectomized rats. This suggested that not only T_3 but also these agents may have a similar action to that of T_3 in increasing the CTBP activity. However, the mechanism of these agents in increasing the CTBP was not clarified. Some proteins are known to be regulated by thyroid hormone, retinoic acid and vitamin D_3. Like T_3, retinoic acid increased the growth hormone gene expression in GH3 as well as in the GH1 cell [15–17]. Retinoic acid as well as vitamin D_3 was able to induce the osteocalcin gene [18]. Not only thyroid hormone but also retinoic acid and vitamin D_3 increased Apo B mRNA concentrations with Caro-2 cell differentiation [19]. Although further examination is necessary to elucidate the mechanism of the CTBP increase, it is possible that these agents may regulate gene expression.

As mentioned in our previous reports, NADPH-dependent CTBP may play a key role in the regulation of intracellular T_3 translocation. In particular, the movement of thyroid hormone from cytoplasm to nuclear thyroid hormone receptor may be regulated by this protein [4]. The T_3 regulation of the CTBP activity may therefore directly influence the intracellular T_3 translocation. It is known that the malic enzyme which is one of the thyroid hormone responsive proteins is important in producing intracellular NADPH. In this study, we confirmed that thyroid hormone increased the level of activity of malic enzyme in the isolated hepatocytes prepared from thyroidectomized rats. This finding suggested that thyroid hormone regulates intracellular T_3 translocation not only by modifying CTBP but also by changing the concentration of intracellular NADPH. The precise mechanism of thyroid hormone-dependent intracellular T_3 translocation, however, remains to be elucidated.

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