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DIFFERENTIAL REGULATION OF THYROTROPIN-RELEASING HORMONE RECEPTOR mRNA LEVELS BY THYROID HORMONE *IN VIVO* AND *IN VITRO* (GH3 CELLS)

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SUMMARY: We studied the effect of thyroid status on thyrotropin-releasing hormone receptor (TRH-R) mRNA levels both *in vivo* and *in vitro* (GH3 cells) using a cloned rat TRH-R cDNA by RT-PCR. Experimental hypothyroid rats were produced by total thyroidectomy and were then killed 7 days after the operation. TRH receptor binding in the anterior pituitary and serum TSH level were elevated approximately 2-fold and 8-fold, respectively, in 7 day thyroidectomized rats. TRH-R mRNA levels in hypothyroid rats were also increased significantly compared with those of normal rats. In GH3 cells, however, no significant change of TRH-R mRNA level was observed between cultures treated with triiodothyronine (T3, 10^{-9} and 10^{-7} M) and the untreated group. The present data indicate that 1) the *in vivo* effects of thyroid status on TRH-R mRNA levels differ from the *in vitro* one, and that 2) the down regulation of TRH-R binding by thyroid hormone in GH3 cells may be mediated by translational or post-translational mechanisms.

Thyrotropin-releasing hormone (TRH) is a major stimulator of TSH secretion and synthesis in the anterior pituitary. These effects follow the interaction of TRH with its receptor (TRH-R) in the anterior pituitary, and the majority of the effects are mediated by the activation of the inositol phospholipid-calcium protein kinase C transduction pathway (1). The cDNA encoding the mouse TRH-R has been recently cloned and characterized (2). The primary structure deduced from the mouse TRH-R cDNA has the structure common to G-protein coupled receptors, seven transmembrane domains. We and others have recently reported the down regulation of TRH-R binding by thyroid hormone both *in vivo* and *in vitro* as a feedback mechanism of the hypothalamo-pituitary-thyroid axis (3-8). However, it is still unknown whether thyroid hormone regulates the level of TRH-R transcription, thereby regulating the number and affinity of these recep-

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tors. Therefore, we conducted the present experiment to examine the effect of thyroid status on the TRH-R mRNA levels *in vivo* and *in vitro*.

MATERIALS AND METHODS

Cloning of the rat anterior pituitary cDNA encoding TRH-R

A) Preparation of RNA: Total RNA was isolated from the anterior pituitaries of thyroidectomized rats by the Guanidine/CsCl centrifugation method described by Chirgwin et al. (9).

B) Polymerase Chain Reaction(PCR): The first strand of cDNA was synthesized from 8 μg of total RNA of the rat anterior pituitary and 200 ng oligo$(dT)_{12-18}$ with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim). The second strand was extended at 72°C for 40 min in 100 μl of the standard mixture containing a sense primer, TR1, an antisense primer, TR2, and 2.5 Units of Thermus aquatics DNA polymerase (Perkin-Elmer-Cetus). The TR1 and TR2 primers encoded the regions unique for the mouse TRH-R, when compared with those of other G-protein coupled receptors (TR1, 5'-GCCCTCTGAACCAGGTGGTTACCATC-3'; TR2, 5'-AGTAGGAATTCC TGGAGATCTTGTAGCCA-3'). These primers were designed to contain an EcoR I or Xba I restriction enzyme site by exchanging 2 nucleotides to facilitate the subsequent subcloning. The amplified fragment (TRHR-1) encompassed the region between the first and fourth transmembrane domain of the rat TRH-R. Amplification was performed with 35 cycles of a step program (94°C for 1 min, 45°C for 2 min, 72°C for 2 min, and final extension at 72°C for 15 min) utilizing a DNA Thermal Cycler (Perkin-Elmer-Cetus). Another amplification (TRHR-2) was performed with primers, TR3 and TR4 (TR3, 5'-ACCACTGCAGG-CATCTTGGTGACCTG-3';TR4, 5'-CCATCTAGAGGTACATAGCAATCTGC-3'), which encompassed the region between the fourth and fifth transmembrane domain of the rat TRH-R. The amplified fragments were subcloned into the vector pGEM3Z (Promega) and sequenced by the dideoxynucleotide-termination method. The sequence of the mouse TRH-R cDNA was compared with that of the mouse TRH-R cDNA by using a computer program (GENETYX, Software development Co.Ltd).

Animals: The male Wister rats employed in the experiments weighed 200-250 g and were allowed ad libitum access to water and standard laboratory chow. An ambient temperature of 25°C and 12-hr altering light-dark cycles were controlled automatically. Experimental hypothyroidism was produced by surgical thyroidectomy following supplementation with drinking water containing 0.1 % $CaCl_2$. Animals were decapitated 7 days after thyroidectomy, and blood samples were collected for the measurement of serum TSH. Poly(A)$^+$ RNA was prepared from twenty anterior pituitaries by standard methods using an oligo(dT) cellulose column.

TRH receptor assay: Each anterior pituitary was homogenized in 0.45 ml of cold buffer (50 mM Tris) and then used for the measurement of TRH binding as described previously (8). In brief, 200 μl of each pituitary homogenate and 10 μl of [^{3}H]TRH (2 pmol) were incubated in the presence or absence of a 500-fold-excess of unlabeled TRH. After incubation at 0°C for 40 min, the incubated mixture was passed through a GF/B filter. The radioactivity on each filter was determined. Specific binding was assessed by subtracting the binding in the presence of unlabeled TRH from the total binding in the absence of unlabeled TRH. Protein concentration was determined by the Lowery method (10).

Cell culture: GH3 cells were maintained in Dulbecco'modified Eagles Medium (DMEM) supplemented with 10 % (vol/vol) fetal bovine serum. Cells were grown at 37°C in a humidified atmosphere of 5 % CO_2, 95 % air. Experiments were performed using replicate 100-mm dishes. The cells were permitted to adhere to the dishes and grow for 48 hrs in this medium. Cells were cultured for 24 hrs in DMEM containing 10 % (vol/vol) charcoal-stripped fetal calf serum in the presence or absence of 100 nM T3 or 1 nM T3. After the cells were harvested, Poly(A)$^+$ RNAs were extracted as described above.

Northern blot analysis: Three micrograms of poly(A)$^+$ RNA of the rat anterior pituitary or GH3 cells were separated by electrophoresis on a 1.2 % formaldehyde-agarose gel and transferred to a nylon membrane. Hybridization was performed using a digoxigenin-UTP labeled cRNA probe (TRHR-1) at high stringency. As an internal control, β-actin mRNA was also hybridized with a digoxigenin-labeled mouse β-actin cRNA. Chemiluminescent detection was used for autoradiography with AMPPD according to the manufacturer's instructions (Boehringer Mann-

heim). The density of hybridized bands was estimated with a densitometer (Pharmacia), and TRH-R mRNA levels were normalized by those of β-actin.

RESULTS

The extent of hypothyroidism by thyroidectomy was evaluated by measuring blood TSH concentrations. Blood TSH concentration was increased progressively 7 days after thyroidectomy (control, 108.4±28.6 vs. 7days, 848.7±74.7 μU/ml; n=5, P<0.01).

Figure 1 shows the nucleotide and amino acid sequence of the cloned rat TRH-R cDNAs. These cDNAs encompassed the region between the first and fifth transmembrane domain of the rat TRH-R. The homology of the rat TRH-R in the cloned region with the mouse counterpart is 93.5 % at the nucleic acid level and 95.7 % at the amino acid level. The major difference be-

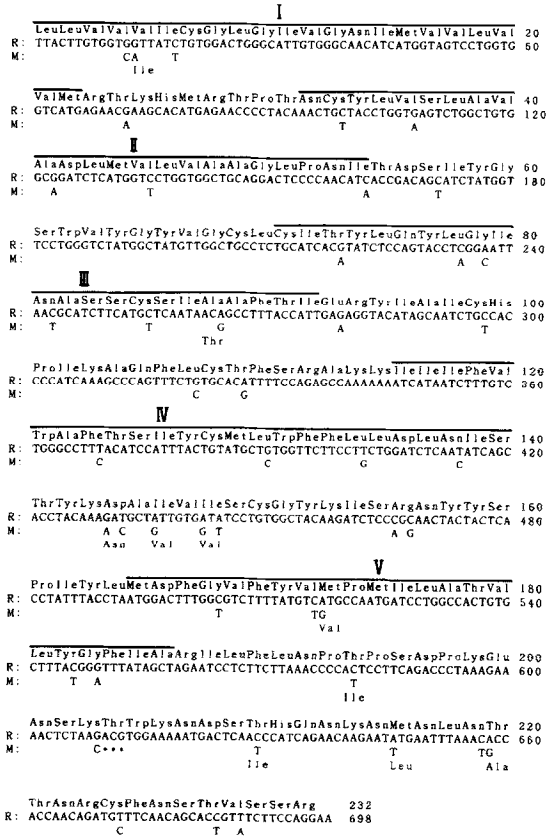

Fig. 1. Nucleotide and amino acid sequence of the cloned rat TRH-R cDNA.
Amino acid and nucleotide numbering begin at the first residue of the cloned cDNA. The putative transmembrane domains are indicated by continuous lines. The major difference between the rat and mouse TRH-R is the two amino acid deletion in the rat TRH-R indicated by asterisks. The residues in the mouse TRH-R (M:) that differ from those in the rat TRH-R (R:) are shown on the sequence of the mouse TRH-R.

tween the rat and mouse TRH-R is a two amino acid deletion in the region between the 5th and 6th transmembrane domain of the rat TRH-R.

Figure 2 shows TRH-R bindings and Northern blot analysis of the TRH-R mRNA and β-actin mRNA in the anterior pituitary of normal and thyroidectomized rats. Thyroidectomy increased TRH-R binding approximately 2-fold after 7 days (control, 18.8±4.8 vs. 7 days, 43.5±6.17 fmol/mg protein; N=5, P<0.01). Northern blot analysis revealed that a TRH-R mRNA band of approximately 3.8 kb was found in both normal and thyroidectomized rats, and this was identical in size to that reported in the mouse anterior pituitary (2). While β-actin mRNA levels showed no significant changes, TRH-R mRNA levels in hypothyroid rats were significantly increased as compared to those of normal rats, which were undetectable even with 5 μg poly(A)$^+$ RNA and the cRNA probe.

Figure 3 depicts the Northern blot analysis of TRH-R mRNA and β-actin mRNA in GH3 cells incubated in the absence or presence of 10^{-7} or 10^{-9} M triiodothyronine. GH3 cells exhibited a single band of TRH-R mRNA that was the same length as that of intact rats. GH3 cells treated

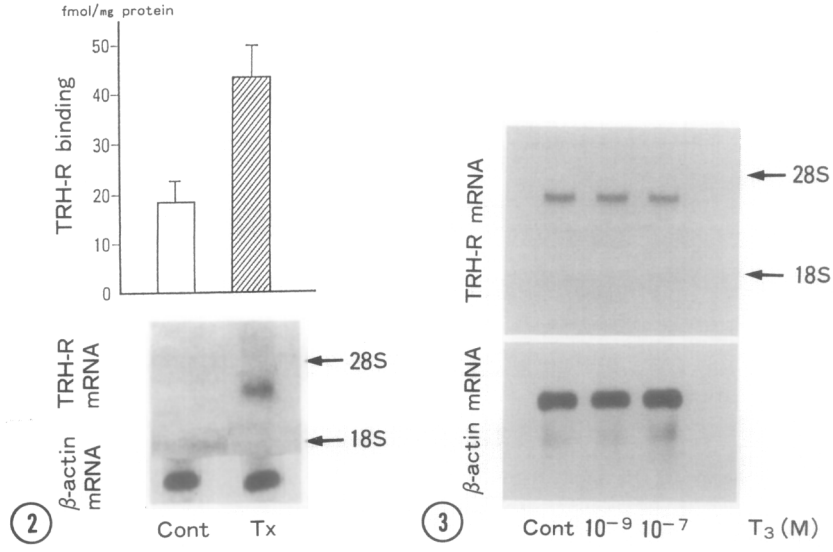

Fig. 2. Effect of thyroidectomy on TRH-R binding and TRH-R mRNA level in the anterior pituitary. TRH-R binding and TRH-R mRNA level were increased 7 days after thyroidectomy (TX) compared with those of control rats (Cont). No significant changes were observed in β-actin mRNA.

Fig. 3. Effect of varying dose of triiodothyronine (T3) on TRH-R mRNA level in GH3 cells. GH3 cells were treated with 10^{-9} or 10^{-7} M T3 for 24 hrs. The TRH-R mRNA and β-actin mRNA levels of the T3-treated groups were not significantly different from the respective levels in the control group.

with 10^{-9} or 10^{-7} M T3 showed no significant changes in either TRH-R mRNA level or β-actin mRNA level.

DISCUSSION

The present study demonstrated that the sequence of the rat TRH-R exhibits strong homology to that of mouse TRH-R, and the influences of thyroid status on TRH-R mRNA levels *in vivo* are different from those observed in the *in vitro* GH3 cells. The homology of the rat TRH-R in the cloned region is high, which is consistent with the results published by Straub et al. who reported that the injection of the antisense mRNA of mouse TRH-R into the *Xenopus* oocytes inhibited the expression of the rat TRH-R mRNA (2).

We and others have reported the down regulation of TRH-R binding by thyroid hormone *in vivo* and *in vitro* (3-8). While the maximum binding of TRH-R was increased following thyroidectomy from 0 to 28 days, concomitantly with the elevation of serum TSH levels, the affinity constants of the TRH-R were unchanged during the same period (3,8). Moreover, the same effects have been reported using GH3 cells by Perrone et al. who reported that the maximal TRH binding was reduced by 50% in the culture treated with 10 nM triiodothyronine (4). On the basis of these findings, it is anticipated that thyroid hormone would down-regulate TRH-R transcription levels both *in vivo* and *in vitro*.

In the present experiments, thyroidectomy, experimental hypothyroidism, increased TRH-R bindings and TRH-R mRNA levels concomitantly. TRH-R mRNA level was increased from an undetectable level to a well-detectable level on the Northern blot analysis. With 5 μg of poly(A)$^+$ RNA extracted from 40 intact rats and the cRNA probe, Northern blotting failed to detect TRH-R mRNA, indicating that the expression of the TRH-R mRNA is extremely low in the anterior pituitary of normal rats as compared to that of β-actin mRNA. We have confirmed this by the RT-PCR technique, which showed that after 40 cycles of PCR-amplification of the TRH-R mRNA from intact anterior pituitaries, only a faint band of TRH-R mRNA on the agarose gel was detected; This means that it may be impossible to detect TRH-R mRNA in normal rats by conventional methods such as Northern blot analysis and the RNase protection assay (unpublished data). Therefore, the mRNA levels of TRH-R in thyroidectomized rats were increased at least several-fold compared to those of normal rats. The binding studies revealed that thyroidectomy increased TRH-R binding approximately 2-fold. This discrepancy between the binding and mRNA levels may mean that the increase of the TRH-R mRNA levels in hypo-

thyroid rats was due to not only the increase of transcription levels but also the decrease of TRH-R mRNA degradation.

Northern blot analysis of the GH3 cells revealed that GH3 cells have a single species of TRH-R mRNA with a length of 3.8 kb, being identical in size to that of normal rats. Furthermore, TRH-R mRNA levels were not affected by the treatment of triiodothyronine, indicating that the TRH-R of GH3 cells is probably identical to the normal TRH-R, and thyroid hormone does not regulate the TRH-R mRNA level in GH3 cells. GH3 cells have been well-known to possess T3 receptors. Moreover, the down regulation of TRH-R binding by thyroid hormone has been reported in GH3 cells (4). Therefore, it is likely that the decrease of TRH-R binding in GH3 cells after incubation with T3 may be mediated by translational or post-translational mechanisms.

In summary, the results of this investigation show that the *in vivo* effects of thyroid status on TRH-R mRNA levels are different from the effects observed in an *in vitro* system (GH3 cells) and raise possibilities that 1) *In vivo* thyroid hormone mediated TRH-R regulation is different from the *in vitro* one, 2) factors other than thyroid hormone are required for the regulation of the TRH-R gene by thyroid hormone, or 3) characteristics of TRH-R in GH3 cells have been changed. These results also indicate that the down regulation of TRH-R binding by thyroid hormone in GH3 cells may be mediated by translational or post-translational mechanisms.

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