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LIGAND (T_3) DEPENDENT AND INDEPENDENT EFFECTS OF THYROID HORMONE RECEPTORS UPON HUMAN TRH GENE TRANSCRIPTION IN NEUROBLASTOMA CELLS

Pei Feng, Qiao-Ling Li, Teturou Satoh, and John F. Wilber

Division of Endocrinology, Department of Medicine, University of Maryland, School of Medicine, Baltimore, Maryland 21201

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Summary: Thyrotropin releasing hormone (TRH) gene is regulated negatively at the transcriptional level by thyroid hormone (T3) in rat anterior hypothalamus. The actions of T_3 upon other target genes are known to be mediated through the thyroid hormone receptors (TR), TRα and TRβ. To explore whether the inhibitory regulation of human (h) TRH gene transcription by T_3 is TR isoform specific and whether TRH gene transcription can be modulated as well by unliganded TR isoforms, transient gene expression studies have been carried out using hTRH-luciferase (TRH-Luc) chimeric constructs and TR expression constructs, co-transfected into a human neuroblastoma cell line (HTB-11). Data herein demonstrate T_3-dependent inhibitory regulation of the hTRH gene promoter by TR-T_3 complexes. Moreover, significant inhibition (39%-60%) could be achieved by T_3 bound to either hTRα$_1$, hTRβ$_1$, or rTRβ$_1$, β$_2$ and was comparable quantitatively, indicating an absence of TR isoform specificity for T_3 inhibition. Conversely, basal promoter activity of the hTRH gene could be activated significantly by unliganded hTRα$_1$, β$_1$, rTRβ$_1$, and β$_2$ (150% to 334%), but not by hTRα$_2$. Thus, TRs appear to exert opposite effects on hTRH gene transcription, depending on the presence or absence of ligand (T_3). These dual effects of TR suggest that the addition of the T_3 ligand effects conformational changes that can abrogate the initiation of transcription.

TRH from the hypothalamic paraventricular nucleus (PVN) stimulates the synthesis and secretion of thyroid stimulating hormone (TSH) from the anterior pituitary (1). We have demonstrated previously that the expression of the TRH gene in PVN is regulated negatively by thyroid hormones in vivo (2). However, the molecular mechanisms underlying inhibition of TRH gene transcription by T_3 have not been elucidated. Recently, a study of the regulation of the rat TRH gene by T_3 has been carried out in transiently transfected primary cultures of embryonic chick hypothalamic neurons, which have revealed a T_3-dependent and isoform-specific inhibition of transcription of the rat TRH gene by chick (c) TRβ, but not cTRα (3).

Thyroid hormones exert their biological effects through nuclear receptor proteins (TR) (4,5). Splicing variant TR isoforms are derived from two distinct TR genes (α and β) which

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have been identified in several species (6). TRβ_1, TRβ_2, and TRα_1 bind T_3 and mediate its actions by binding to cis-acting thyroid hormone response elements (TRE) of target genes. In contrast, TR-α_2, a splicing variant of TRα which lacks a functional ligand-binding domain, does not exert T_3 actions (7-9). Unliganded TRs are located in the cell nucleus and, unlike glucocorticoid receptors, can bind to their TREs on target genes, even in the absence of T_3 (6,10,11). In general, basal transcription of genes that are activated by T_3-TR complexes is repressed by TRE-bound unliganded TR (12,13). Whereas cell-type and isoform-specific ligand-independent activation by unliganded TR have also reported. Such as in GH_4C_1 and GH_1 cells, gene transcriptional effects of unliganded TRs have been in the same direction as those produced by TR-T_3 complexes (14). However, effects of unliganded TR isoforms on basal promoter activity of genes regulated negatively by T_3 have not been studied extensively.

Results herein will demonstrate that T_3 can significantly inhibit hTRH gene promoter activity in the presence of transfected TR, and that T_3 inhibition of the TRH gene transcription does not exhibit TR isoform specificity. Moreover, basal promoter activity of the hTRH gene can be significantly enhanced by unliganded hTRα_1, β_1, rat TRβ_1, and rβ_2, but by hTRα_2.

Materials and Methods

Cell culture: Human neuroblastoma cells (HTB-11) purchased from the ATCC were cultured in Eagle's minimum essential medium with 10% fetal bovine serum (Sigma), using 165 cm^2 flasks. On the day prior to transfections, cells were treated with trypsin and aliquoted into 100 mm culture dishes (10^6 cells /plate). Serum free medium, supplemented with transferrin (Sigma, 2.5 mg/500 ml) and insulin (Sigma, 5 mg/500 ml), was utilized when transfected cells were treated with L-T_3 (10^{-8}M, Sigma).

Plasmids: TRH-Luc constructs with progressively shortened 5' flanking sequences of the hTRH gene (15) from -785, -603, -242, and -140 to +54 bp, respectively, were cloned up-stream of the firefly luciferase-coding region in a $pSVA_3$Luc-plasmid vector, as described previously (16). Human TRβ_1 was cloned into the expression vector $pSVA3_3$Luc (17). Human TRα_1, α_2, rTRβ_1, and rβ_2 were cloned into the expression vector pCDM (18,19).

Transfections and luciferase assays: HTB-11 cells were transfected with TRH-Luc construct plasmid DNA (15μg) and co-transfected with TR plasmid DNA (1μg) by the calcium-phosphate method (20). Transfection efficiency was monitored by co-transfection of rat β-galactosidase plasmid DNA (5μg). β galactosidase activity was measured by a specific enzymatic assay (20,21). Luciferase activity was detected with the Promega Luciferase assay system (Madison, WI). Light units were measured for 10 seconds with a standard luminometer. Transfections were performed in triplicate plates, and each experiment was repeated for three times.

Statistical analysis: Statistical analysis was performed using Duncan's multiple test for determining the differences between multiple groups under the same experimental conditions. For paired groups, Student's t test was employed .

Results and Discussion

Inhibition of human TRH promoter activity by T_3 in the presence of hTRβ_1.

Transient gene expression assays were carried out in HTB-11 cells transfected with pTRHLuc plasmids containing progressive deletions of the 5′ promoter region of the hTRH gene and were co-transfected with hTRβ_1. L-T_3 (10^{-8}M) alone, in the absence of co-transfection of TRβ_1, did not inhibit TRH promoter activity (data not shown). In contrast, striking and significant inhibition of hTRH promoter activity by L-T_3 (10^{-8}M) was observed when human TRβ_1 (1 μg/plate) was co-transfected with four progressively shortened hTRH gene constructs: -785, -603, -242, and -140 to +54 bp, respectively (Fig. 1). Inhibition of TRH promoter activity ranged from -45% to -59% of control. Deletion of 5′ flanking sequences between -785 and -140 bp did not diminish T_3 (10^{-8}M) inhibitory action significantly. However, this progressive truncation did cause progressive reductions in basal promoter activity. Two candidate TRE 1/2 sites, GCCAGTGC and GGTCCCCAC, are located in the sequence of the hTRH gene at -191/-184 bp and -166/-158 bp, respectively, and are homologous to the inhibitory TRE in the 5′ flanking region of human TSH β gene (15). When these sequences were removed, e.g. in the shortest construct (-140/+54 bp), the degree of suppression by L-T_3 still appeared significantly compared

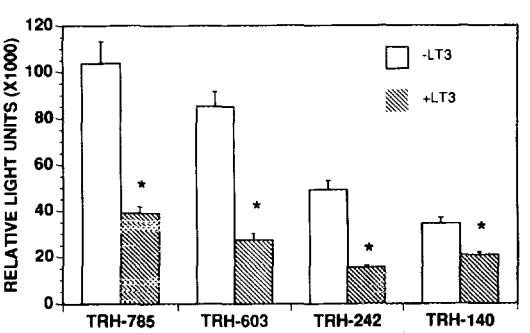

Fig. 1. Inhibitory effect of L-T_3 with co-transfected human TR-β_1 on promoter activity of hTRH deletion constructs in human HTB-11 cell.

HTB-11 cells were transfected with pTRHLuc plasmids (15μg/plate) containing varying deletions of the 5'-promoter region of hTRH gene. Cells were co-transfected with hTR-β_1 (1μg) and β-galactosidase plasmids (5μg). Following transfection, the cells were cultured with (hatched bars) or without (open bars) L-T_3 (10^{-8}M) for 24 h. Cell lysates were assayed for luciferase activity, normalized by β-galactosidase activity. Statistical significance was determined by the Student t test (* $p<0.05$).

to its control (-45%). Thus, despite elimination of upstream regulatory elements, including two candidate TRE sequences, significant promoter inhibition by T_3 was not attenuated (Fig. 1). These data suggest that the human TRH gene promoter can be inhibited by L-T_3 in the presence of sufficient TR, and that the functional TRE(s) for this T_3 inhibitory effect appears to be localized downstream of -140 bp of TRH gene sequence. In this regard, a consensus inhibitory TRE half site, TGACCT, has been identified by us in the hTRH promoter region between -60 to -55 bp. This TRE site assumed to be separated by a three bp spacer with an inverted palindrome sequence, TCGAGC, located at -51 to -46 bp.

Inhibitory effects of T_3 upon human TRH gene transcription do not exhibit TR isoform specificity.

Promoter activity of the human TRH gene could be inhibited significantly by L-T_3 (10^{-8}M) when the hTRH-Luc construct (-242/+54 bp) plasmid was co-transfected with equal masses (1μg) of either human TRα_1, β_1, rTRβ_1, or rβ_2. The degree of inhibition of hTRH promoter activity ranged from 39% to 60% of control (Fig.2). In contrast, no significant inhibitory effect of L-T_3 on human TRH gene promoter activity was observed when human TRα_2 was co-transfected with the hTRH-Luc construct (Fig.3). It has been reported recently that co-transfection of chick TRα

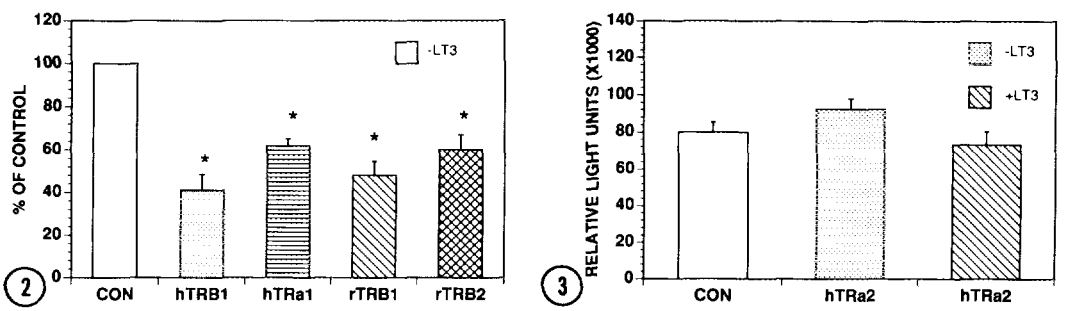

Fig. 2. Inhibitory effects of L-T_3 on human TRH gene promoter activity (-242/+54) co-transfected with four different TR isoforms.

Fifteen μg of hTRHLuc plasmid containing -242/+54 bp of the 5'-promoter region was transfected into human HTB-11 cells. Four groups were co-transfected with TR plasmids (1μg): hTRβ_1, hTRα_1, rTRβ_1, and rTRβ_2, respectively. In each group, the control is represented by the cells without L-T_3 treatment (open bar). Inhibitory effects of L-T_3 (10^{-8}M) upon hTRH gene promoter activity after co-transfection with TR plasmids are expressed as a percentage of the luciferase activity of each TR isoform group in the presence of L-T_3 over the counterpart control. Statistical significance of L-T_3 treatment was determined by Duncan's multiple test (* $p<0.05$).

Fig. 3. Human TRH gene promoter activity in the co-transfection of hTRα_2 is not affected by L-T_3.

HTB-11 cells transfected with hTRHLuc plasmid (15μg) containing the -242/+54 bp of the 5'-promoter sequence were co-transfected with hTRα_2 plasmid DNA (1μg). Cells were cultured in the presence (hatched bar) or absence (dotted bar) of L-T_3 (10^{-8}M) for 24 h. Cells unexposed to hTRα_2, or L-T_3, served as controls (open bar). Luciferase activities were normalized by β-galactosidase activities. No significant difference was observed within the groups by Duncan's multiple test ($p>0.05$).

or cTRβ could activate the basal level of rat TRH promoter, but the T_3-dependent inhibitory regulation of rat TRH promoter activity was caused selectively by cTRβ and not by cTRα in a primary embryonic chick hypothalamic neuron system (3). Our data, in contrast, indicates clearly that the inhibitory effect of L-T_3 on the human TRH gene promoter with human or rat TRs is not characterized by any TR isoform specificity. Why T_3 inhibitory effects were not observed with TRα in the earlier study is not clear (3). Possible explanations would include the possibility that cTRα co-transfected into chick hypothalamic cells was bound competitively by other binding protein(s), which thereby could interfere DNA binding of cTRα. There also could be species' differences in the transfected cells or species specificity of the TR isoforms utilized. Since no $TR\alpha_2$ analog, the splicing variant of TRα, has been found in chicken (6), possible qualitative differences may exist between chick TRα and the $TR\alpha_1$ found in rat, mice, and man.

Unliganded TR isoforms activate human TRH gene promoter activity.

Previous studies using transient transfection assays have demonstrated that unliganded TRs can act as a constitutive repressor of mammalian genes whose transcription can be activated by T_3 (6,22). However, the effect of unliganded TR on transcription of genes that are negatively regulated by T_3 has not been investigated extensively (6). The data herein demonstrate that co-transfection of unliganded (1μg) human $TR\alpha_1$, β_1, rat $TR\beta_1$, or $r\beta_2$ can stimulate hTRH (-242/+54 construct) promoter activity significantly. The degree of enhancement ranged from 150% to 334% above control (Fig.4). However, it was noted that the stimulatory effect of $rTR\beta_2$ upon basal promoter activity of the hTRH gene was significantly lower than that of $hTR\alpha_1$, $TR\beta_1$, and $rTR\beta_1$. Of great interest was that human $TR\alpha_2$, which lacks T_3 binding capacity, did not cause stimulation of the human TRH gene promoter (Fig. 3). This may be due to the absence, also,

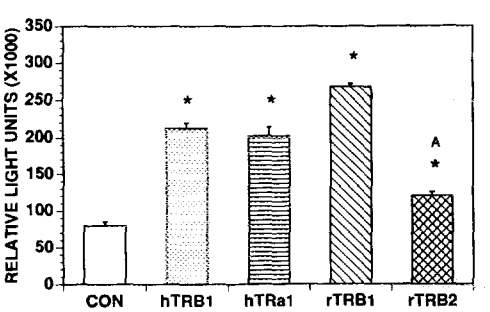

Fig. 4. Stimulatory effects of TR isoforms on human TRH gene promoter activity. HTB-11 cells were transfected with hTRHLuc plasmid (15μg) containing the -242/+54 bp fragment of the 5'-promoter region. Cells were co-transfected with plasmid DNA of $hTR\beta_1$, $hTR\alpha_1$ and $rTR\beta_1$, $rTR\beta_2$ (1μg), respectively. Cells untransfected with TR plasmids served as control (open bar). After 48 h, cells were harvested, and luciferase activities were normalized by β-galactosidase activities. Statistical analyses were performed by Duncan's multiple test. Significant differences were determined between each TR and the control (*, A, $p<0.05$).

of TR dimerization capacity of this TR splicing variant (23). In any event, it is clear that TR, in the absence of T_3, can exert effects upon the TRH promoter opposite those elicited when T_3 is bound to TR. The mechanism of these ligand-dependent and independent effects of TR has to be elucidated. A recent study on paradoxical activation of DNA transcription by unliganded TRα has ascribed to two qualitatively unique TRE 1/2 sites in an RSV construct expressed in HeLa cells (24). Such a mechanism cannot be operative in the human TRH promoter, however, since TR effects did not exhibit isoform specificity for TRα. Moreover, these novel TRE sequences were not identified by us between -242 to +54 bp of the hTRH 5′ flanking domain. The addition of T_3 may effect TR conformational changes that enable possible TR-T_3 complex interference with pre-initiation protein-DNA interactions, thereby abrogating the transcriptional activation effected by unliganded TRs (25).

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