Yamanashi Med. J. **11**(3), 53~58, 1996

Thyrotropin Regulates the Expression of mRNA for G-Protein α-Subunits (αs and αi2) in FRTL-5 Cells

Xiao-ding PENG, Kazutaka HARAGUCHI, Toyoshi ENDO, and Toshimasa ONAYA

The Third Department of Internal Medicine, Yamanashi Medical University, Tamaho, Yamanashi 409–38, Japan

Abstract: In order to determine the molecular mechanism of regulation of the GTP-binding protein (G-protein)-adenylyl cyclase system by thyrotropin (TSH), we examined the expression of mRNA for G-protein stimulatory α subunit (αs) and inhibitory α subunit (αi2) by the reverse transcription-polymerase chain reaction (RT-PCR) method. TSH (10 mU/ml) stimulated the expression of αs mRNA in FRTL-5 cells by 40% after 6 hr, which was gradually reduced to a normal level by 24 hr. Expression of β-actin mRNA was unchanged. In contrast, expression of αi2 mRNA was reduced by 25% after 6 hr and returned to the normal level by 24 hr. This concomitant change increased the relative abundance of αs to αi2. The effect of TSH on the expression of αs mRNA was mimicked by dibutyryl cAMP and forskolin. These findings show that the expression of G-protein subunits is regulated by TSH via a cAMP-dependent pathway on the mRNA level.

Key words: Thyrotropin, G-protein, αs, αi, FRTL-5 cell

INTRODUCTION

It is known that guanine nucleotide-binding proteins (G-proteins) play an important role in signal transduction in all eukaryotic cells[1]. In thyroid cells, thyrotropin (TSH) receptor and its effector, adenylyl cyclase, are coupled by G-protein α-subunits that stimulate (αs) or inhibit (αi2) adenylyl cyclase activity. Thus the regulation of G-proteins[2,3] and the TSH receptor[4] in thyroid cells has been of interest in physiological or pathological states. Saunier *et al.*[5] found that αs protein was increased when primary porcine thyroid follicles were cultured in the presence of TSH or forskolin. In order to examine the mechanisms of requlation of the TSH receptor-G-protein-adenylyl cyclase system from the molecular and biological perspective, we used FRTL-5 cells: a continuous, well-differentiated cell line of the rat thyroid[6,7].

The present study was designed to determine whether mRNA expression levels of αs and αi2 in FRTL-5 cells are regulated by TSH and, if so, whether such regulation would be mediated by a cAMP-dependent mechanism.

Address for correspondence and reprints: Toshimasa Onaya, MD. Chairman and Professor, The Third Department of Internal Medicine, Yamanashi Medical University, Tamaho, Yamanashi 409–38, Japan
Received May 11, 1996
Accepted July 24, 1996

MATERIALS AND METHODS

Cell culture

FRTL-5 cells were obtained from Flow Laboratories (Mclean, VA, U.S.A.) and cultured as previously described[8]. Cells were cultured in Ham's F-12 supplemented with 5% calf serum and a six-hormone mixture (6H medium) that included TSH (10 mU/ml), insulin (10 μg/ml), hydrocortisone (1 nM), transferrin (5 μg/ml), somatostatin (10 ng/ml), and glycyl-L-lysine acetate (10 ng/ml). TSH was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). When the cells reached

about 90% of confluency, the medium was changed to 5H medium (6H medium without TSH). After 5 days, the cells were used for the experiments.

RNA isolation and reverse-transcription

Total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method[9]. mRNA was reverse-transcribed into cDNA as described previously[10].

Primer preparation and PCR

Primers specific for αs and $\alpha i2$ were designed based on the nucleic acid sequence reported[11] as follows: primer A: 5'-TTCTATGAGCATGCCAAGGC, primer B: 5'-CTCAATCTTCGATTTCCCAG, primer C: 5'-CAAGGCATGCTTCCGGAAGA, primer D: 5'-TACTCAGGGAAACAGATGGT.

Primers A and B corresponded to peptide residues (A: 146–152 and B: 302–308) of αs, and primer C and D corresponded to peptide residues (C: 117–123 and D: 285–291) of $\alpha i2$. Primers were labeled with 10 μCi of ^{32}P-γATP by 10 units of T4 kinase and purified by Nick columns. The PCR reaction mixture contained a cDNA template derived from 70 ng of total RNA, 2.5 U of AmpliTaq DNA polymerase (Takara Shuzo Co.), 66 ng of A and B primers for αs or 87 ng of C and D primers for $\alpha i2$. Quantitative analysis was performed by adding labeled primer A for αs or primer C for $\alpha i2$. The PCR reaction was performed for 33 cycles: 1 min at 93°C, 1.5 min at 55°C, and 1 min at 72°C with 30 sec of ramping time. The samples were applied to 2% agarose gels. Triplicate determination of the amount of PCR products was performed as follows. PCR products (n=3) were obtained from three separate PCR samples from different tubes and applied on three separate gels. Corresponding parts of the gels were cut out from each lane to measure radioactivity. Radioactivity from a sample that lacked a template in the reaction mixture was used as the background. In some experiments, to avoid the possible inheritant cause for the intra-assay variations which occur during the course of RT-PCR procedure, radioactivities of αs and $\alpha i2$ bands were corrected by that of β actin. The significance of differences between experimental values was determined by Student's t-test.

Results

Effects of TSH on αs and $\alpha i2$ mRNA levels

To determine the effect of TSH on mRNA expression of αs as well as of $\alpha i2$, FRTL-5 cells were exposed to 6H or 5H medium for 0, 6, 12 and 24 hr. When RT-PCR products from mRNA obtained from the cells were run on an agarose gel, bands for αs (486 bp) and $\alpha i2$ (522 bp) of expected sizes were detected. Bands for αs obtained from cells treated with 6H medium were more intense than those from cells treated with 5H medium at 6 and 12 hr (Fig. 1).

Treatment of cells with 6H medium decreased with an expression of $\alpha i2$ after 6 and 12 hr of incubation. Radioactivity obtained from the bands for $\alpha i2$ showed the corresponding results (954.7±91.7 cpm cf 722.7±46.8 cpm at 6 hr and 1056.1±41 cpm cf 894.3±60.3 cpm at 12 hr, 5H medium cf 6H medium, respectively). At 48 hr and 72 hr of incubation, expression of αs and αi did not differ between 5H-treated and 6H-treated cells (data not shown).

During the period of treatment, the expression of β-actin mRNA remained unchanged.

Effects of TSH based on the radioactivity of bands for αs and $\alpha i2$ are summarized in Fig. 2.

Effects of cAMP on αs mRNA expression levels

To determine the contribution of cAMP to the increase in expression of αs, FRTL-5 cells were treated for 12 hr with 0.1 mM dibutyryl cAMP (DBC), 1 mM DBC, or 1 μM forskolin. Each of these additives increased intensity of the bands (Fig. 3). Radioactivity obtained from

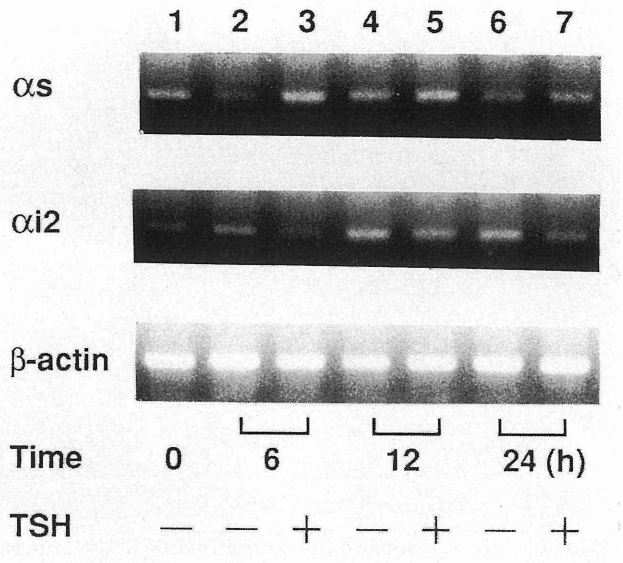

Fig. 1. Effects of TSH on expression of mRNA for αs, αi2 and β-actin. RT-PCR products were obtained from cells treated with 5H medium for 0 hr (lane 1), 6 h (lane 2), 12 hr (lane 4), 24 hr (lane 6) or with 6H medium for 6 hr (lane 3), 12 hr (lane 5), 24 hr (lane 7). A representative gel among three similar ones is shown. Only the corresponding portions of the gels are shown. Representative data from three similar experiments are shown.

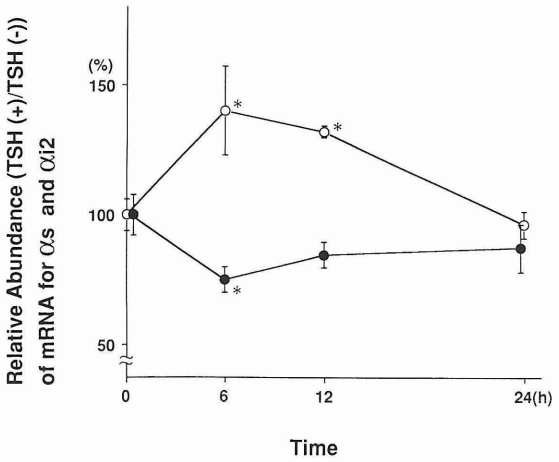

Fig. 2. Effect of TSH on radioactivity of bands for αs and αi2. Effects of TSH on αs (-○-) and αi2 (-●-) were expressed as the ratio of the radioactivity obtained from the cells treated with 6H to that from cells treated with 5H. Representative data from three similar experiments are shown. Values are the means±SEM (n=3).
*P<0.01 vs 0 hr

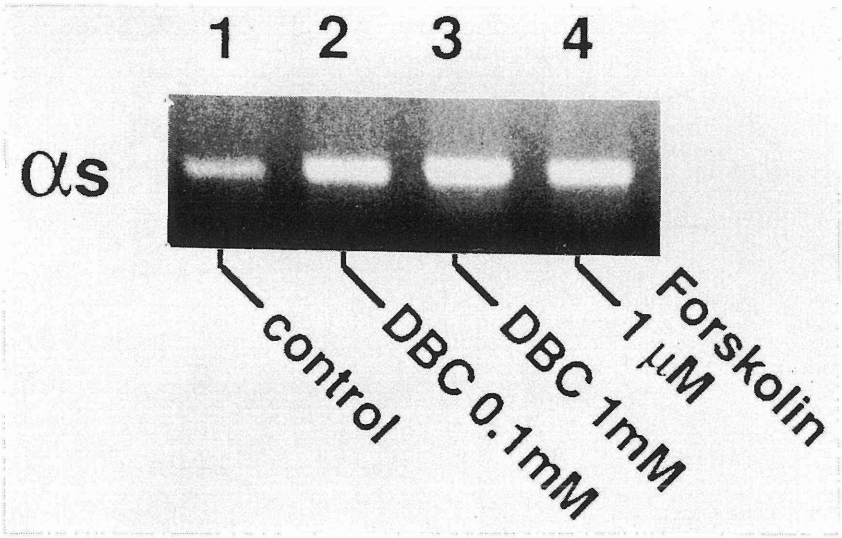

Fig. 3. Effects of DBC and forskolin on expression of mRNA for αs. FRTL-5 cells were incubated with no additives (lane 1), or in the presence of 0.1 mM DBC (lane 2), 1 mM DBC (lane 3) or 1 μM forskolin (lane 4).

the bands agreed with the intensities of the bands (5H medium; 1077±76cpm, 0.1 mM DBC; 2248±127cpm, 1 mM DBC; 3626±215 cpm, 1 μM forskolin; 3006±86 cpm,).

The DNA fragments of αs and αi2 generated by PCR were cut out of the gel and sequenced. Sequencing revealed that the bands were cDNA for αs and αi2 (data not shown).

Discussion

TSH binds to the TSH receptor and stimulates thyroid metabolism by a cAMP-dependent pathway activated through αs[12] and an IP3-Ca-dependent pathway activated through αq[13]. In the former, αs is activated by the third intracellular loop of the TSH receptor[14]. The TSH receptor is thought to be coupled with αi, but the mechanism of activation of αi is not known.

Although TSH initiates the trip down of these metabolic pathways, prolonged exposure to TSH causes a lack of responsiveness, or desensitization[15]. For example, TSH increases cAMP in FRTL-5 cells to the maximal level in 15 to 30 min, but in 12 hr, cAMP is decreased to less than 10% of the maximal level (data not shown). This series of activations and deactivations of signals involves various aspects of signal transduction[16,17]. As for the TSH receptor, Akamizu *et al.*[4] reported that TSH down-regulated TSH receptor mRNA.

Little is known regarding the effect of TSH on the expression of G-proteins except that αs protein is positively regulated in porcine thyroid follicles[5]. However, it should be recalled that (a) cultured porcine thyroid follicles and rat cloned thyroid FRTL-5 cells differ in some properties[18]. Therefore, it is of interest to know whether TSH-induced increase of αs protein happens in FRTL-5 cells; a cell line most often used in the field of thyroidology. However, (b) it is difficult to evaluate the quantity of G-protein subunits accurately on the plasma membrane[19,20]. Besides, no study has focused on the measurement of α proteins which exist in cytosol; cytosolic α proteins comprize a huge portion of whole cell (data not shown). Cytosolic α proteins are separated from β and γ subunits and are poor substrates

for ADP-ribosylation by toxins. This makes the measurement of the amount of cellular G protein more difficult. Apart from such complexed problems as mentioned, it would be interesting to determine wheter mRNA expression of G-proteins is regulated by TSH in FRTL-5 cells. Our data showed that, once the TSH receptor-G-protein-adenylyl cyclase system is switched on, there is an up-regulation of αs and a down regulation of αi at the mRNA level. Our findings are in agreement with the increased amount of Gs protein reported in porcine thyroid cell[5]. The overall effect of alteration in the ratio of αs to αi2 is to strengthen the up-regulation of αs. DBC and forskolin increased the expression of αs to a greater extent than TSH. This could be explained by the continuous stimulation of a cAMP-dependent pathway which cannot be achieved with TSH due to desensitization. The thyroid is not the only organ whose expression of αs is regulated by cAMP[21]. Whether the decreased expression of αi is regulated by increased cAMP level or other TSH-induced signal such as elevated intracellular calcium level remains to be solved.

In summary, TSH down-regulated TSH receptor and up-regulated αs mRNA in a cAMP-dependent manner. It is reasonable to assume that this phenomenon helps to maintain the TSH receptor-G-protein-adenylyl cyclase system in a homeostatic state, while the overall regulation is to decrease responsiveness as indicated by the occurrence of desensitization. The explanation for these complicated homeostatic mechanisms awaits further experiments including studies on βγ-subunits[22], NAD kinase[23], and adenylyl cyclases[24] in the thyroid.

References

1) Simon MI, Strathmann MP, Gautam N. Diversity of G proteins in signal transduction. Science 1991; **252**: 802–808.
2) Delemer B, Dib K, Patey M, Jacquemin C, Correze C. Modification of the amounts of G proteins and of the activity of adenylyl cyclase in human benign thyroid tumors. J Endocrinol 1992; **132**: 477–485.
3) Lyond J, Landis CA, Harsh G, Vallar L, Grunewald K, Feichtinger H, Duh QY, Clark OH, Kawasaki E, Bourne HR, McComick F. Two G protein oncogenes in human endocrine tumors. Science 1990; **249**: 655–659.
4) Akamizu T, Ikuyama S, Saji M, Kosugi S, Kozak C, McBride OW, Kohn LD. Cloning, chromosomal assignment, and regulation of the rat thyrotropin receptor: expression of the gene is regulated by thyrotropin, agents that increase cAMP levels, and thyroid autoantibodies. Proc Natl Acad Sci USA 1990; **87**: 5677–5681.
5) Saunier B, Dib K, Delmer B, Jacquemin C, Correze C. Cyclic AMP regulation of Gs protein: thyrotropin and forskolin increase the quantity of stimulatory guanine nucleotide-binding proteins in cultured thyroid follicles. J Biol Chem 1990; **265**: 19942–19946.
6) Bidey SP, Lambert A, Robertson WR. Thyroid cell differentiation and function in the FRTL-5 cell line: a survey. J Endocrinol 1988; **119**: 365–376.
7) Ambesi-Impiombato FS. The FRTL-5 cells, an in vitro thyroid system: introduction and development. In: Ambesi-Impiombato FS, Perrild H, eds Elsevier Science Publishers. FRTL-5 today. Excerpt Med Int Cong Ser 1989; 3–7.
8) Endo T, Shimura H, Saito T, Onaya T. Cloning of malignantly transformed rat thyroid (FRTL) cells with thyrotropin receptors and their inhibition by 3', 5'-cyclic adenosine monophosphate. Endocrinology 1990; **126**: 1492–1497.
9) Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; **162**: 1492–1497.
10) Ling L, Haraguchi K, Ohta K, Endo T, Onaya T. β-adrenergic receptor mRNA is overexpressed in neoplastic human thyroid tissues. Endocrinology 1993; **130**: 547–549.
11) Jones DT, Reed RR. Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium. J Biol Chem 1987; **262**: 14241–14249.
12) Van Sande J, lejeune C, Ludgate M, Munro DS, Vassart G, Dumont JE. Thyroid stimulating immunoglobulins, like thyrotropin activate

both cyclic AMP and PIP2 CHO cells expressing the TSH receptor. Mol Cell Endocrinol 1992; **88**: R1-R5.

13) Taylor SJ, Chae HZ, Rhee SG, Exton JH. Activation of the b1 isozyme of phospholipase C by a subunits of the Gq class of G proteins. Nature 1991; **250**: 516–518.

14) Chazenbalk GD, Nagayama Y, Russo D, Wadsworth HL, Rapoport B. Functional analysis of the cytoplasmic domains of the human thyrotropin receptor by site directed mutagenesis. J Biol Chem 1991; **265**: 20970–20975.

15) Foti D, Caltalfamo R, Russo D, Costante G, Filetti S. Lack of relationship between 3', 5'-cyclic adenosine monophosphate desensitization and thyrotropin receptor down regulation in the rat thyroid cell line FRTL5. J Endocrinol Inv 1991; **14**; 213–218.

16) Haraguchi K, Peng X, Kaneshige M, Anzai F, Endo T, Onaya T. Thyrotropin-dependent desensitization by Chinese hamster ovary cells that express the recombinant human thyrotropin receptor. J Endocrinol 1993; **139**: 425–429.

17) Saji M, Ikuyama S, Akamizu T, Kohn LD. Increases in cytosolic Ca^{++} down regulate thyrotropin receptor gene expression by a mechanism different from the cAMP signal. Biochem Biophys Res Commun 1991; **176**: 94–101.

18) Bjorkman U, Ekholm R. Hydrogen peroxide generation and its regulation in FRTL-5 and porcine thyroid cells. Endocrinology 1992; **130**: 393–399.

19) Haraguchi K, Rodbell M. isoproterenol stimulates shift of G proteins from plasma membrane to pinocytotic vesicles in rat adipocytes: a possible way of signal transduction. Proc Natl Acad Sci USA 1990; **87**: 1208–1212.

20) Haraguchi K, Rodbell M. Carbachol-activated muscarinic (M1 and M3) receptors transfected into Chinese hamster ovary cells inhibit trafficking of endosomes. Proc Natl Acad Sci USA 1991; **88**: 5964–5968.

21) Hadcock JR. Ross M, Watkins DC, Malbon CC. Cross regulation of between G-protein-mediated pathways. Stimulation of adenylyl cyclase increases expression of the inhibitory G-protein, Gi α 2. J Biol Chem 1990; **265**: 14784–14790.

22) Hildebrandt JD, Kohnken RE. Hormone inhibition of adenylyl cyclase. Differences in the mechanisms for inhibition by hormones and G-protein $\beta\gamma$. J Biol Chem 1990; **265**: 9825–9830.

23) Kimura N, Shimada N. Evidence for complex formation between GTP-binding protein (Gs) and membrane associated nucleotide diphosphate kinase. Biochem Biophys Res Commun 1990; **168**; 99–106.

24) Tang WJ, Krupinski J, Gilman AG. Expression and characterization of calmodulin-activated (type 1) adenylyl cyclase. J Biol Chem 1991; **266**: 8595–8603.