EXPERIMENTAL STUDY

TSH receptor antibodies do not alter the function of gonadotropin receptors stably expressed in eukaryotic cells

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Abstract

Objective: TSH receptor (TSHr) mediates the activating action of TSH on the thyroid gland resulting in the growth and proliferation of thyrocytes and thyroid hormone production. TSHr is a major autoantigen in Graves' disease (GD) and is the target for TSHr antibodies. In GD, thyroid-stimulating antibodies (TSAb) are competitive agonists of TSH. In atrophic thyroiditis (AT), thyroid-stimulating blocking antibodies (TSHBAb) are TSH antagonists. The TSHr together with the LH receptor (LHr) and FSH receptor (FSHr) are G-protein-coupled receptors with considerable amino acid homologies in the extracellular domain. We studied the cross-reactivity of the antibodies measured in sera from patients with GD or AT on the LHr and FSHr function.

Methods: We tested the activity of TSAb and TSHBAb in cell lines expressing the LHr and the FSHr. To this purpose a pSVL-FSHr construct was transfected in CHO cells and one clone was used.

Results: Twenty-eight sera from patients with GD and four from patients with AT, known to contain TSHr antibodies measured with a radioreceptor assay, were selected. TSAb and TSHBAb activities were measured in CHO cells expressing the TSHr (CHO-TSHr). TSAb and TSHBAb were then tested with the cell lines expressing the LHr and the FSHr for their ability to elicit cAMP accumulation or inhibit FSH/LH-induced cAMP production. None of the TSAb identified was able to stimulate cAMP increase in CHO-LHr or CHO-FSHr. Similarly, none of the TSHBAb was able to block the cAMP response induced by FSH or LH in the respective cell lines.

Conclusions: Our results confirm the notion of the organ-specific nature of the TSHr antibodies.

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Introduction

The thyrotropin (TSH) receptor (TSHr) mediates the activating action of TSH on the thyroid gland resulting in the growth and proliferation of thyrocytes and thyroid hormone production. TSHr is a major autoantigen in Graves' disease (GD) (1) and is the primary target for TSHr antibodies (TRAb) (2). In GD, thyroid-stimulating antibodies (TSAb) are competitive agonists of TSH (2, 3). TSAb, after the interaction with the receptor, increase cAMP accumulation causing goiter and hyperthyroidism (2, 3). In atrophic thyroiditis (AT), thyroid-stimulating blocking antibodies (TSHBAb) are TSH antagonists, inhibiting TSH binding or TSH-increased cAMP levels causing thyroid atrophy and hypothyroidism (2, 4–6). The different activities of TSAb and TSHBAb suggest they might interact with different epitopes on the TSHr (2). It has been demonstrated that the extracellular domain of the receptor is responsible for recognition

and binding of TSH (7, 8). Some TSAb and TSHBAb are conformation-dependent and may span the entire extracellular domain of the TSHr with multiple discontinuous contact sites (2, 9). The TSHr, together with the luteinizing hormone (LH) receptor (LHr) and follicle-stimulating hormone (FSH) receptor (FSHr), are members of a subfamily of G-protein-coupled receptors with large extracellular domains (8). Considerable amino acid homologies along with a number of potential N-linked glycosylation sites in the extracellular domain of these receptors suggest a similar three-dimensional structure (10). However, TSHr has an additional 64 residues that make up two regions in the N- and C-terminus of the extracellular domain that are non-homologous with gonadotropin receptors (10). Although hormone–receptor interaction is usually highly specific, cross-reactivity between glycoprotein hormones and their receptors has been suspected because of the structural similarities of the individual receptors.

In order to elucidate the cross-reactivity of the antibodies measured in sera from patients with autoimmune thyroid diseases on the LHr and FSHr function we tested the activity of TSAAb and TSHBAb in cell lines stably expressing the LHr (11) and the FSHr (prepared in our laboratory). To this purpose the pSVL-FSHr was stably transfected in CHO cells and one clone was used.

Twenty-eight sera from patients with GD and four sera from patients with AT, known to contain TRAb measured with a radioreceptor assay, were selected. TSAAb and TSHBAb activities were measured in CHO cells stably expressing the TSHr (CHO-TSHr). TSAAb and TSHBAb were then tested with the cell line stably expressing the LHr and the cell line stably expressing the FSHr for their ability to elicit cAMP accumulation or inhibit FSH- or LH-induced cAMP production.

None of the TSAAb identified in an assay using CHO-TSHr was able to elicit any cAMP increase in the cell lines stably expressing LHr or FSHr. Similarly none of the TSHBAb was able to block the cAMP response induced by FSH or LH in the respective cell lines stably expressing the receptors.

Our results confirm that TSAAb and TSHBAb do not alter the function of FSHr and LHr.

Patients, materials and methods

Patients

Twenty-eight patients with GD, four with AT and ten normal subjects were included in this study. Mean ages were 49.3 ± 11.6 years (mean $\pm$ S.D.) in GD, 45 ± 6.7 years in AT and 47.2 ± 7.9 in normal subjects. There were 21 female patients and seven male patients with GD, three females and one male patient with AT and seven female and three male normal subjects.

Diagnosis of GD was based on the evidence of clinical hyperthyroidism associated with elevated serum free thyroxine (FT4) and free triiodothyronine (FT3) levels and suppressed serum TSH concentrations. A typical diffuse goiter with increased vascularization was detected by ultrasound. All the patients showed an increased thyroïdal iodine uptake in thyroid scintiscans. All of them had high titers of thyroperoxidase antibodies (TPOAb), thyroglobulin antibodies (TgAb) and TRAb, measured by a radioreceptor assay.

Diagnosis of AT was based on the evidence of clinical hypothyroidism with elevated serum TSH levels and low FT4 and FT3 associated with a non-palpable gland and high titers of circulating TgAb and TPOAb. A small hypoechogenic gland was detected by ultrasonography in these patients. In this group of patients TRAb were measured in all the patients by a radioreceptor assay.

Ten normal subjects were specifically selected as a control group. In these subjects there was no evidence of thyroid disease.

Establishment of the CHO-K1 cell line expressing the FSHr

The FSHr gene vector (pSVL-FSHr) and a neomycin resistance gene vector (pSV2NEO) were co-transfected into CHO-K1 cells using a modified calcium phosphate method (12). Cells were plated out in 60 mm culture dishes and after 24 h were transfected with the two vectors. After 6 h incubation, cells were washed twice with Dulbecco's Modified Eagle's Medium (DMEM) and fresh medium containing 10% fetal calf serum was added. Selection was started 48 h after transfection by addition of medium containing 400 μ g/ml geneticin (G418; Gibco, Paisley, Strathclyde, UK). Medium was changed every day for about 3 weeks. When non-transfected cells in control dishes had all died, cells were trypsinized and subjected to screening, using the cAMP activity assay. The mixed CHO-K1 cell population was subjected to limiting dilution to produce individual clonal cell lines. Briefly, serial dilutions of the transfection product were seeded into 96-well microtiter plates. Wells were screened the following day by inspection under the microscope and those containing only one cell were retained. Cells in these wells were allowed to grow to confluence, trypsinized and further amplified in 90 mm culture dishes.

IgG preparation

IgGs were prepared from sera with a commercial kit according to the manufacturer's instructions (Mab Trap G II; Pharmacia Biotech).

TSH-binding inhibiting antibody (TBIAb)

TBIAb was detected by a second generation radioreceptor assay (13) based on the inhibition of the binding of 125 I-labeled bovine TSH (bTSH) to human recombinant TSHr (DYNO test, TRAK human, BRAHMS, Berlin, Germany). The TBIAb concentration in the patients' sample can be calculated from a standard curve of samples with a known TBIAb concentration. TBIAb values < 1 IU/I are regarded as negative, values > 1.5 IU/I as positive.

Adenylyl cyclase activity assay in the three cell lines

The CHO-TSHr (JPO9), CHO-LHr (FC11) and CHO-FSHr (AE11) cell lines were cultured in Petri dishes in RPMI, F12 and DMEM respectively. Cells were seeded at the concentration of 25 000 cells/well into 96-well plates with medium and used for the assay the following day. Cells were washed with Hanks' balanced salt solution and incubated for 1 h at 37°C in hypotonic buffer. The cell lines were stimulated with various concentrations of human recombinant FSH (hrFSH) (Sigma-Aldrich,

St Louis, MO, USA), human recombinant chorionic gonadotropin (hrCG) (Sigma-Aldrich), bTSH (Sigma-Aldrich), or IgGs (TSAb and TSHBAb) at a concentration of 0.5–1 mg/ml. Assays were performed in triplicate for each experiment. Extracellular cAMP accumulation in the medium was determined by a in-house RIA (3), using a commercial anti-cAMP antibody.

TSAb activity in CHO-TSHr

TSAb activity in the three cell lines was measured with respect to the cAMP production obtained in the presence of IgGs from 20 normal subjects (102 ± 18 for CHO-TSHr; 93 ± 2 for CHO-FSHr; 94 ± 2 for CHO-LHr). Results were expressed as the percentage of cAMP released in the extracellular medium with respect to the basal value. A value superior to 138% (for CHO-TSHr), 97% (for CHO-FSHr) and 98% (for CHO-LHr) was considered positive.

TSHB Ab activity in CHO-TSHr

Cell cultures were incubated with IgG alone, TSH (1 mU/ml) alone, or IgG plus TSH in a total volume of 100 μ l of hypotonic buffer–BSA–3-isobutyl-1-methylxanthine (IBMX). Hypotonic buffer–BSA–IBMX alone was added to some cultures in each experiment to measure basal cAMP production. After 1 h of incubation at 37°C in a 5% CO₂–95% air atmosphere, cAMP was measured in the extracellular medium by RIA. All experiments were performed in triplicate, and the results (in pmoles/well) were expressed as the average of these. To evaluate the effect of IgGs, an index of inhibition of TSH-dependent cAMP production was calculated according to the following formula:

$$1 - \frac{[\text{cAMP (TSH + IgG)} - \text{cAMP (IgG)}]}{[\text{cAMP (TSH)} - \text{cAMP (basal)}]} \times 100$$

All IgGs producing an inhibition greater than 2 s.d. from the mean of controls (>25%) were considered positive for TSHBAb.

TSHBAb activity in CHO-FSHr and CHO-LHr

Cell cultures were incubated with IgG alone, FSH (100 mU/ml) (or LH (1 mU/ml)) alone, or IgG plus FSH (or LH) in a total volume of 100 μ l hypotonic buffer–BSA–IBMX. Hypotonic buffer–BSA–IBMX alone was added to some cultures in each experiment to measure basal cAMP production. After 1 h of incubation at 37°C in a 5% CO₂–95% air atmosphere, cAMP was measured in the extracellular medium by RIA. All experiments were performed in triplicate, and the results (in pmoles/well) were expressed as the average of these. To evaluate the effect of IgGs, an index of inhibition of FSH- (or LH-) dependent cAMP production was calculated according to the same formula used for CHO-TSHr.

All IgGs producing an inhibition greater than 2 s.d. from the mean of controls (>46 for both CHO-FSHr and CHO-LHr) were considered positive for blocking activity for FSH or LH action.

Results

Isolation of CHO-K1 clones expressing the FSHr

To check FSHr function, adenylyl cyclase activity assay studies were carried out on both the transfected CHO-K1 mixed cell population and the control (wild-type CHO-K1 cells).

The production of cAMP in the mixed cells was present after stimulation with 100 mU/ml of hrFSH suggesting that a functioning FSHr was present. Different cell clones were obtained and screened for cAMP production (Fig. 1). A cell line termed AE11 that

Figure 1 Basal cAMP production (white bars) and after stimulation with 100 mU/ml hrFSH (black bars) in different cell clones. The cell line AE11, showing the highest levels of cAMP production, was selected.

Figure 2 cAMP production in different cell lines. CHO-FSHr were treated with increasing concentrations of hrFSH (A), CHO-LHr were treated with increasing concentrations of hrCG (B), and CHO-TSHr were treated with increasing concentrations of bTSH (C). Results are expressed in pmoles/well. The data are the means $\pm$ s.d. of triplicate determinations from one representative experiment.

showed the highest level of cAMP production among all screened colonies was characterized and then used for further experiments.

Effects of hrFSH on CHO-FSHr

The incubation of the cell line AE11 with increasing doses of hrFSH determined a dose-dependent response curve of cAMP as shown in Fig. 2A. The minimal amount of hrFSH able to produce a significant increase of cAMP was 10 mU/ml and reached a plateau at 5000 mU/ml. The EC_{50} was 150 ± 8 mU/ml.

Effects of hrCG on CHO-LHr

The cell line stably expressing the LHr was described in (11) and was prepared by one of the present authors (F C). The incubation of cell line EC11 with different doses of hrCG determined a dose-dependent response curve of cAMP as shown in Fig. 2B. The minimal amount of hrCG able to produce a significant increase of cAMP was 0.05 mU/ml and reached a plateau at 10 mU/ml. The EC_{50} was 1.96 ± 0.3 mU/ml.

Effects of bTSH on CHO-TSHr

The incubation of the cell line JPO9 with increasing doses of bTSH determined a dose-dependent response curve of cAMP as shown in Fig. 2C. The minimal amount of bTSH able to produce a significant increase of cAMP was 0.1 mU/ml and reached a plateau at 25 mU/ml. The EC_{50} was 2.00 ± 0.6 mU/ml.

Effects TSAb on the three cell lines

CHO-TSHr (JPO9) We studied the effect of 28 IgGs from patients with GD with measurable TRAb in the radioreceptor assay. IgGs produced a cAMP production in JPO9 cells from 140 to 1800% with respect to cells incubated with buffer only or with normal IgGs from subjects not affected by thyroid diseases (Fig. 3).

CHO-FSHr and CHO-LHr None of the IgGs with thyroid-stimulating activity was able to produce a significant increase of cAMP in both of the cells stably expressing the FSHr and the LHr (Fig. 3).

Effects of TSHBAb on the three cell lines

CHO-TSHr (JPO9) We studied the effect of four IgGs from patients with AT with measurable TRAb in the radioreceptor assay. The four IgGs incubated with submaximal doses of bTSH in JPO9 cells showed a inhibition of production cAMP between 30 and 80% with respect to JPO9 cells tested with only buffer or with normal IgGs (Fig. 4).

Figure 3 Results obtained in the assay for TSAb using IgGs from patients with GD and CHO-TSHr, CHO-FSHr and CHO-LHr. Results are expressed as the percentage of cAMP released in the extracellular medium with respect to the basal value. The shaded areas indicate the mean ± 2 s.d. (138% for CHO-TSHr, 97% for CHO-FSHr, 98% for CHO-LHr) of results obtained with normal IgGs.

CHO-FSHr and CHO-LHr None of the four IgGs was able to stimulate cAMP production in FSHr and LHr cell lines and none of these TSHBAb was able to inhibit cAMP production in FSHr cell line and in LHr cell line incubated with hrFSH and hrCG respectively (data not shown).

Discussion

The glycoprotein hormones TSH, LH and hCG, have a common alpha-subunit and a unique beta-subunit associated non-covalently (10). The receptors for these glycoprotein hormones are also members of a subfamily of G-protein-coupled receptors and are structurally and functionally related with relatively high degrees of sequence similarity (10). Although hormone-receptor interaction is usually highly specific, cross-reactivity between glycoprotein hormones and their receptors has been suspected because of the structural similarities of the individual receptors (14). Neonatal hypothyroidism in rats can cause an increase in adult reproductive organ size, despite suppressed levels of FSH and LH throughout adulthood (15). In addition, in humans severe juvenile hypothyroidism can cause a distinct form of isosexual precocity, characterized by breast development, uterine bleeding multicystic ovaries in girls and macroorchidism in boys (16). Therefore, the possibility that TSH stimulation of LHr/hCG receptor (hCGr) or FSHr might play a role in the development of this kind of precocious puberty has been suggested. Pathologically high concentrations of hCG in hydatiform moles and choriocarcinomas have been shown to cause hyperthyroidism by activating the TSHr (17).

In autoimmune thyroid diseases, autoantibodies directed against the TSHr have a clear pathogenic role. In GD, TSAb are competitive agonists of TSH.

Figure 4 Results obtained in the cAMP production inhibition assay of CHO-TSHr with four IgGs (IgG1, IgG2, IgG3, IgG4) from patients with autoimmune hypothyroidism that were incubated at 1 mg/ml with bTSH (1 mU/ml). IgG1, IgG2, IgG3 and IgG4 were all able to inhibit TSH-induced cAMP production in CHO-TSHr cells. An IgG from a subject not affected by thyroid disease was used as control.

TSAb, after the interaction with the receptor, increase cAMP accumulation causing goiter and hyperthyroidism. In AT, TSHBAb are TSH antagonists, inhibiting TSH binding or TSH-increased cAMP levels causing thyroid atrophy and hypothyroidism. Theoretically TSAbs and TSHBAb might interact with the FSHr or LHr as a potential pathogenetic mechanism of impairment of gonadal function. In this paper a new cell line stably expressing the FSHr (prepared in our laboratory) and a cell line stably expressing the LHr were used to search for autoantibodies able to stimulate the function of CHO-FSHr or CHO-LHr or inhibiting the action of FSH and LH on their own receptors. hrFSH and hrCG produced a dose-dependent increase of cAMP in CHO-FSHr and CHO-LHr respectively. Antibodies directed against the TSHr and able to stimulate cAMP production in CHO-TSHr were incubated with CHO-FSHr and CHO-LHr. Our results clearly show that none of the TSAbs are able to elicit cAMP production. Antibodies from sera from patients with AT and containing TSHBAb were incubated with CHO-FSHr and CHO-LHr. Similarly none of the TSHBAb are able to inhibit FSH or LH stimulation of their own receptors.

These data support the notion of the organ-specific nature of TRAb in autoimmune thyroid diseases and

show that TRAb do not stimulate LHr/hCGr or FSHr, nor interfere with their respective hormones. Contrasting with their high homology at the mRNA level, the mature structure of TSH and gonadotropin receptors is different (2). While the LHr/hCGr and FSHr are expressed in target cells as glycosylated monomers the TSHr is a heterodimer with an A extracellular subunit and a broad B transmembrane and intracellular subunit (2). The intramolecular cleavage of TSHr and the shedding process may be at the origin of circulating antigenically active TSHr ectodomain detected in human blood. This process may be at the origin of TRAb.

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