Expression of G-proteins and TSH receptor signalling in hyperfunctioning thyroid nodules with TSH receptor mutations

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Abstract
Objective: Constitutively activating mutations of the thyrotrophin receptor (TSHR) are the main molecular cause of hyperfunctioning thyroid nodules (HTNs). The G protein coupling is an important and critical step in the TSHR signalling which mainly includes Gs, Gi and Gq/11 proteins.
Design: We investigated the in vitro consequences of overexpressing G-proteins on signalling of the wild-type (WT) or mutated TSHR. Moreover, we investigated whether changes in G-protein expression are pathophysiologically relevant in HTNs or cold thyroid nodules (CTNs).
Methods: Wild-type TSH receptor and mutated TSH receptors were coexpressed with Gs, Gi or Gq/11, and cAMP and inositol phosphate (IP) production was measured after stimulation with TSH. The expression of Gs, Gi and Gq/11 proteins was examined by Western blotting in 28 HTNs and 14 CTNs.
Results: Coexpression of Gs with the WT TSH receptor in COS 7 cells significantly increased the basal and TSH-stimulated cAMP accumulation while coexpression of the Gaq or G11 protein significantly increased the production of cAMP and inositol triphosphate (IP3). The coexpression of the TSH receptor mutants (I486F, DEL613–621), known to couple constitutively to Gs and Gaq with Gaq and G11, significantly increased the basal and stimulated cAMP and IP3 accumulation. Coexpression of the TSH receptor mutant V556F with Gs only increased the basal and stimulated cAMP production while its coexpression with Gaq/11 increased the basal and stimulated IP3 signalling. The expression of Gs protein subunits determined by Western blotting was significantly decreased in 14 HTNs with a constitutively activating TSH receptor mutation in comparison with the corresponding surrounding tissue, while in 14 HTNs without TSH receptor or Gs protein mutation and in 14 CTNs the expression of Gs protein was not different compared with the surrounding tissue. The expression of Gi and Gaq/11 proteins in HTNs or CTNs was not significantly different compared with the surrounding tissue.
Conclusions: The reduced expression of Gs protein subunits in HTNs with TSHR mutations could act as a feedback mechanism to desensitise the chronically stimulated cAMP cascade. As Gs protein expression was not significantly increased in the majority of CTNs and HTNs an influence of Gs overexpression on TSH signalling could be excluded in these nodules.

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Introduction
The thyrotrophin (TSH) receptor is known to couple to 11 different G protein α subunits in vitro. TSH receptor signalling mainly involves the G protein α subunits Gs, Gi and Gq/11 leading to an activation of the cAMP and the phospholipase C (PLC) pathways (1, 2). While low concentrations of TSH lead to a sufficient stimulation of cAMP production, up to 100-fold concentrations of TSH are necessary to induce PLC activation (3). The cAMP cascade is the major signal transduction pathway of the TSH receptor (TSHR) in vivo and has been shown to activate mitogenesis and thyroid hormone production (4). Changes in G protein coupling and signal transduction have recently been demonstrated for the TSHR variant Y601H compared with the wild-type (WT) TSH receptor (5, 6). As constitutively activating TSH receptor mutations which frequently occur in hyperfunctioning thyroid nodules (HTNs) cause changes within the intracellular receptor conformation, these mutations could also lead to variations of G protein coupling and signal transduction (5). This hypothesis is also supported by the variable Gαs or Gαi protein expression in HTNs (7–10). However,
it is not known whether the overexpression of the various G protein subunits in HTNs has any effect on the signal transduction of the WT or mutated TSHR.

Constitutively activating TSH receptor mutations and, with a lower prevalence, mutations of the Gα protein have been shown to be the main molecular causes of HTNs (11–16). However, the relative contribution of constitutive TSHR activation and possible variations of G protein expression to the clinical phenotype of HTNs has been debated. Induction of Gα expression increases constitutive as well as stimulated cAMP accumulation. Investigations of G protein expression in HTNs have reported variable results by demonstrating increased, decreased or unchanged Gα protein levels (7–10). However, the molecular aetiology of these HTNs has, for the most part, not been determined. It is therefore not known whether the variable G protein expression reported for HTNs depends on their molecular aetiology.

The aim of our study was, therefore, to investigate the functional consequences for G protein α subunit overexpression on the signal transduction of the WT and mutated TSH receptors in coexpression experiments in COS 7 cells. Moreover, we also determined the expression of Gα subunits in HTNs with different molecular aetiologies and in cold thyroid nodules (CTNs) which also show increased proliferation (8) together with decreased thyroid hormone production.

Materials and methods

Samples

Specimens of 28 HTNs, 14 CTNs and adjacent thyroid tissue of consecutive patients undergoing thyroid resection for treatment of their nodular thyroid growth were obtained at surgery. Prerequisites for inclusion in the study were: the identification of a solitary HTN or a CTN as identified by ultrasound, increased or decreased circumscribed technetium uptake by the nodule with suppression of surrounding thyroid tissue on scintiscan (in the case of HTNs), and macroscopic and microscopic identification of the nodule by a pathologist. All patients with HTNs suffered from overt hyperthyroidism with suppressed TSH and elevated thyroid hormones and negative thyroid antibodies. All patients were euthyroid at the time of surgery. Tissue samples were shock-frozen in liquid nitrogen. Extraction of genomic DNA was performed from the HTNs and the surrounding normal tissue with a Qiagen tissue kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Somatic TSH receptor mutations in HTNs were previously determined by subsequent direct sequencing of the positive bands (12).

Membrane preparations were performed in membrane preparation buffer (40 mmol/l Tris/HCl, 250 mmol/l sucrose, 0.1 mmol/l dithiothreitol, 0.1 mmol/l phenylmethylsulphonylfluoride (pH 7.4). Tissue samples were homogenised in ice-cold membrane preparation buffer and centrifuged (700 g/10 min/4°C). The supernatant was further centrifuged for 45 min at 60000 g at 4°C. The pellet was resuspended in membrane preparation buffer (membrane fraction) and the supernatant was used as the cytosolic fraction. Protein content was determined by the method of Bradford (17).

The study was approved by the local ethics committee. Informed consent was obtained from all patients before surgery.

Plasmid construction

G protein constructs were kindly provided by R K Sunhara (Southwestern Medical Center, University of Texas, Dallas, USA; Gαs), R R Reed (Johns Hopkins University School of Medicine, Baltimore, Maryland, USA; Gαq3), and M Strathmann and M I Simon (California Institute of Technology, Pasadena, USA; Gαq and Gα11). The coding sequence of Gαs was cut by NcoI and HindIII, purified and subsequently subcloned into the EcoRV and HindIII sites of the pcDNA 3.1-Zeo vector (Invitrogen, Groningen, The Netherlands). Gα3 was cut by EcoRI and HindIII and subcloned into corresponding sites of the pcDNA 3.1-Zeo vector. Gαq was cut by NotI and BamHI and also subcloned into pcDNA 3.1-Zeo vector. The coding sequence of Gα11 was cut by Bsu15I and Xhol and subcloned into pcDNA 3.1-Zeo by EcoRV and Xhol. All subcloned fragments were confirmed by sequencing.

Cotransfection of TSHR and G proteins and functional characterisation

Transient expression of the wild-type and mutant TSHR was performed in 12-well plates (1 x 10^5 COS 7 cells) using 1 μg total DNA per well (0.5 μg TSH receptor, 0.5 μg G protein) and FuGene6-reagent (Boehringer, Ingelheim, Germany) according to the manufacturer’s instructions. Twenty-four hours after transfection the cells were prepared for cAMP assays by washing once with PBS buffer and preincubation for 15 min with 1 mmol/l isobutylmethylxanthine (Sigma, Munich, Germany) in serum-free Dulbecco’s modified Eagle’s medium (DMEM). Thereafter, cells were stimulated with bovine TSH for 1 h at 37°C in the same buffer. The reactions were stopped by aspiration of the medium and addition of 0.1 mol/l HCl. Supernatants were collected, dried and resuspended in 200 μl Tris/EDTA buffer (0.05 mol/l Tris, 4 mmol/l EDTA, pH 7.5). cAMP was assayed using a ^3H-cAMP RIA kit (Amersham Co., Amersham, Bucks, UK) according to the manufacturer’s instructions.

For the determination of the TSHR cell surface expression, transfected cells were incubated with antihuman TSHR antibody (2C11, Serotec Ltd, Oxford,
Stimulation of inositol phosphate formation

Inositol phosphates were measured as described (18). Forty hours after transfection 2 × 10⁵ COS 7 cells were incubated with 2 μCi/ml [myo-3H]inositol (18.6 Ci/mmol, Amersham Pharmacia Biotech, Amersham, Bucks, UK) for 8 h. Thereafter, cells were preincubated in serum-free DMEM containing 10 mmol/l LiCl for 20 min. Subsequently, cells were stimulated with bovine TSH for 1 h as described before (18). Stimulation was terminated by aspiration of the medium and addition of 0.5 ml 3% perchloric acid. TSH-induced increases in intracellular inositol phosphate (IP) levels were determined by anion exchange chromatography. IP values are expressed as the percentage of radioactivity incorporated in inositol phosphates and phosphatidylinositols.

Western blot analysis

Equal amounts of protein were added to electrophoresis sample buffer, heated at 95 °C for 5 min and electrophoresed (25 μg protein/per lane for COS 7 cells, 6 μg/lane for thyroid samples) on a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulphate using the discontinuous buffer system described by Davis (19). After electrophoresis the proteins were blotted (Semi dry blotter; BioRad, Hercules, CA, USA) onto nitro-cellulose membranes (Schleicher & Shuell, Dassel, Germany) and after blocking in 5% non-fat dry milk (NFDM) in Tris-buffered saline/Tween (TBS/T; 20 mmol/l Tris/HCl, pH 7.6, 150 mmol/l NaCl, 0.1% Tween 20) (1 h, room temperature (RT)) the membranes were probed overnight with G protein α subunit specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBS/T (20 mmol/l Tris/HCl, pH 7.6, 150 mmol/l NaCl, 0.05 mmol/l Tween 20, 5% BSA, 0.2 μg antibody/ml buffer). After washing in PBS/T (3 times, 5 min, RT) the membranes were probed (1 h, RT) with the secondary horseradish peroxidase-conjugated anti-rabbit antibody (1:2000 dilution in PBS/T and 5% NFDM; New England Biolabs, Beverly, USA) and finally washed 4 times with PBS/T each for 5 min. Following an incubation (5 min, RT) with chemiluminescence reagent (SuperSignal; Pierce, Rockford, IL, USA) the proteins were detected using the Chemi Imager 4000 System (Alpha Innotech Corporation, San Leandro, USA).

Data analysis

G protein expression is given as means ± S.E.M. of duplicate measurements. IP and cAMP values of cotransfection experiments are given as means ± S.E.M. of three independent experiments, each performed in duplicate. Statistical analysis was carried out by Student’s t-test.

Results

Functional characterisation of TSH receptor and G protein interaction

To investigate the functional influence of different overexpressed G protein α subunits on TSH receptor signalling, the TSH receptor and the Gα protein subunits were coexpressed in COS 7 cells and accumulation of cAMP and IP was determined with and without TSH stimulation. A high expression of G proteins in transfected cells could be demonstrated by Western blotting of COS 7 cells transiently transfected with plasmids containing cDNAs of Gα i, q and 11 (Fig. 1). Transfection efficiency of the TSH receptor was 30 to 40% in all experiments as determined by FACS analysis, and surface expression of the receptor was readily detectable at high levels for all transfected TSH receptor constructs. We could not find any difference in the endogenous expression of Gα proteins by transfection of COS 7 cells with the WT TSHR or constitutively activating TSHR mutants. There was no difference in cell surface expression of the constitutively activated TSH receptor mutants Del613–621, 1486F and V656F when coexpressed with Gα or Gα/11 protein and stimulated with TSH compared with the coexpression of the TSH receptor mutants DEL613–621, 1486F and V656F together with the empty expression vector (data not shown).

The overexpression of Gα protein increased the basal and the TSH-stimulated cAMP accumulation in COS 7 cells transiently cotransfected with the WT TSHR or the constitutively activating TSH receptor mutants DEL613–621, 1486F and V656F (Fig. 2). In contrast to Gα protein, the overexpression of Gαi3 protein significantly decreased the stimulated cAMP accumulation of the coexpressed WT and mutated TSH receptors (Fig. 2). Overexpression of Gαs or Goi proteins did not significantly affect the basal and TSH-stimulated IP level of the WT TSH receptor (Fig. 3). Moreover, coexpression of Gαq protein or Gα11 protein with the WT TSH receptor or TSH receptor mutants led to a strong increase in basal and stimulated IP3 (Fig. 3).

Expression of G protein α subunits in HTNs and CTNs

G protein content was decreased to 78% of the corresponding surrounding tissue in HTNs with a somatic
Figure 2 cAMP accumulation in COS 7 cells cotransfected with different TSHR constructs and Gα protein subunits. The basal cAMP accumulation of the WT TSHR was set at 1 and all other data are expressed as fold of the basal cAMP of the WT TSHR. The cells were stimulated with 100 mU/ml bovine TSH for 1 hour as indicated (hatched bars). Data are given as means ± S.E.M. of three independent measurements. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 1 (a) Expression of Gαs in (1) COS 7 cells cotransfected with WT TSHR and Gαs protein, (2) untransfected COS 7 cells and (3) COS 7 cells transfected with WT TSHR and pCDNA3.1-Zeo vector. Cell lysates were electrophoresed on SDS-polyacrylamide gels (25 μg protein per lane), electroblotted and probed with polyclonal rabbit antibodies. (b) Expression of Gαi3 in (1) COS 7 cells cotransfected with WT TSHR and Gαi3, (2) untransfected COS 7 cells and (3) COS 7 cells transfected with WT TSHR and pCDNA3.1-Zeo vector. (c) Expression of Gαq/11 in (1) COS 7 cells cotransfected with WT TSHR and Gαq, (2) COS 7 cells cotransfected with WT TSHR and Gα11 and (3) untransfected COS 7 cells.
constitutively activating TSH mutation (14 cases) compared with their corresponding surrounding tissues, while no significant differences in the expression of Goα protein could be found between CTNs (14 cases) and their corresponding surrounding tissues (Figs. 4 and 5). More than 50% of the HTNs showed a decreased Goα protein expression. In contrast to HTNs with a somatic TSH receptor mutation there was no significant decrease of Goα protein expression in HTNs without a TSH receptor or Goα protein mutation (14 cases) (Fig. 4). Gqi protein expression was not significantly changed in either HTNs or CTNs compared with their surrounding tissues. This was also true for Goq protein expression in CTNs (Fig. 4). Gq/11 protein content was unchanged in CTNs and HTNs compared with their corresponding surrounding tissues (Fig. 4). The data in our patient population followed a Gaussian distribution (normality test passed), and no separate populations could be discerned. Despite the range of standard deviations no further statistical tests were performed.

**Discussion**

Our results showing an increased cAMP accumulation by coexpression of constitutively activating mutations of the TSHR with Goα subunits are in agreement with data showing that an overexpression of Goα protein increases the constitutive as well as the stimulated cAMP accumulation and the transcriptional activation of cAMP-dependent reporter genes (20). The decreased TSH-stimulated accumulation of cAMP by coexpression of Gqi together with the TSHR is most likely due to the known inhibitory potential of the overexpressed Gqi protein subunits on adenylyl cyclase activity (21). TSHR coupling to Gqi subunits has been found in *vitro* experiments and has previously been described as functionally relevant in *vivo* (1, 2). Coexpression of Gqi with mutated TSHR showed an inhibition of stimulated cAMP and IP (in the case of I486F) indicating the possibility for an inhibition of TSHR signalling by Gqi overexpression. Overexpression of Goq protein or Gq11 protein subunits also led to an increase in basal}

![Figure 3](https://example.com)
and stimulated IP accumulation in COS 7 cells when coexpressed with the WT TSH receptor or the TSH receptor mutants. The IP accumulation was not related to the constitutive activity of the TSH receptor mutants. Therefore, increased G\(_a\)q/11 protein expression in vitro can lead to an increased TSHR-PLC signalling. Results from in vitro experiments often differ from in vivo situations. These differences can be caused by the use of artificial promoters, heterologous cell systems and transient transfections in the in vitro experiments. Therefore, the results of our in vitro experiments should be handled with caution when used to analyse the in vivo situation in HTNs.

To investigate the relevance of our in vitro coexpression data in vivo the expression of G\(_a\) proteins in HTNs and CTNs was determined by Western blotting. Reduced expression of G\(_a\)s proteins in HTNs (9), increased G\(_a\)s protein expression (7, 8) or no change in G\(_a\)s protein expression (10) in HTNs have recently been reported. The samples examined in former studies of G\(_a\)s protein expression, except those described by Derwahl et al. (7), were not analysed for mutations in the TSHR or in the G\(_a\) protein (8, 10, 22, 23) or have only been screened for G\(_a\)s protein mutations (22). Some investigators used, in addition to Western blotting, ADP ribosylation experiments to investigate G protein expression (7–9). A further aspect reducing the strength of the former results is the low number of HTNs investigated, 2 (23), 7 (9), 4 (8), 10 (7) and 9 (10) respectively. An increased G\(_a\)s and G\(_a\)i protein content in 5 HTNs with and 5 HTNs without TSHR or G\(_a\) protein mutation has recently been reported (7). Our results of decreased expression of G\(_a\)s in HTNs with TSHR mutations are difficult to reconcile with this report by Derwahl and co-workers. We and others (8) have shown that both G\(_a\) protein and cAMP content in thyroid tumours varies greatly. However, the HTNs included in our study were screened for TSH receptor mutations and mutations in the G\(_a\) protein gene by DNA sequencing and DGGE (12, 16, 24) and were also examined for their clonality (25). In 58% of the HTNs somatic TSHR or G\(_a\)s protein mutations were identified, making a constitutive activation of the cAMP pathway in these HTNs very likely (12). However, 42% of the HTNs were negative for TSHR or G\(_a\)s protein mutations even with the sensitive DGGE method (12, 16, 24), which indicates that in HTNs without TSHR mutation different growth stimulating mechanisms bypassing the TSHR are likely to exist. Therefore, the molecular cause and the activated signalling cascade in these HTNs are currently not known, which makes them unsuitable candidates for studies trying to elucidate the in vivo consequences of chronic cAMP activation on the pattern of G\(_a\) protein subunit expression. Therefore, our clinical and molecular characterisation allows us to relate the expression of G\(_a\) subunits to defined clinical entities such as HTNs with TSHR mutations compared with HTNs without TSHR or G\(_a\)s mutations and with CTNs.

**Figure 5** Expression of G\(_a\)s proteins in two representative hyperfunctioning thyroid nodules with TSH receptor mutation (HTN1 and 2) and the corresponding adjacent thyroid tissue (ATT1 and 2). Cell lysates were electrophoresed on SDS-polyacrylamide gels (25\(\mu\)g protein per lane), electroblotted and probed with a polyclonal rabbit antibody. ST, standard protein marker.
Gas protein degradation is increased after stimulation of the cAMP cascade (26) and the activity of the Gαs protein correlates well with the amount of protein (20). Therefore, the chronically activated cAMP pathway in HTNs harbouring constitutively activating TSHR mutations (11) could induce a higher degradation of Gαs proteins leading very probably to a reduced activity of the Gαs protein pathway in these HTNs. The decreased Gas protein content in TSHR mutation positive samples is most likely a cellular feedback mechanism to the chronic stimulation of the cAMP cascade. A feedback mechanism by a specific phosphodiesterase activation reducing the increased cAMP production in HTNs with TSH and Gαq/11 protein mutations has recently been described (27). Both mechanisms, which are not sufficient to prevent tumour development, may exist in parallel and may reduce the constitutively produced cAMP in HTNs with TSHR or Gas protein mutations.

However, Gas protein content has been shown to be increased after 18 h of TSH stimulation of thyroid follicular cells in vitro (28) while an increased degradation of Gas protein has been shown after stimulation of the cAMP cascade (26). Differences between expression data from in vivo and in vitro conditions demonstrated by primary thyroid cells and HTNs (29, 30) most likely result from unknown adaptation processes during chronic stimulation in an intact cell system in vivo (28). A decreased Gas protein content in lung tissue of rats chronically treated with albuterol has recently been described (31) as the molecular aetiology for the heterologous desensitisation of pulmonary Gas-coupled receptors. Therefore, the chronic stimulation of the cAMP system caused by constitutively activating mutations of TSHR in most of the HTNs could similarly desensitise the cAMP pathway by decreasing the Gas protein expression.

An increased Gαi protein expression in HTNs not investigated for TSHR mutations (10) and in both HTNs with and without TSHR or Gas mutations (7) has been reported. We only found an overexpression of Gαi proteins in 5 out of 28 thyroid HTNs compared with their corresponding surrounding tissue that could have increased the inhibition of the adenylyl cyclase activity in these HTNs. The differences between our study, the study by Selzer et al. (10) and the study of Derwahl and co-workers (7) may result from different detection systems, the use of different antibodies, and the different selection and characterisation of the samples. However, the mean Gαi and Gαq/11 protein expression in our HTNs and CTNs as well as the Gas protein expression in our CTNs was not different compared with their surrounding tissue. Similar results have previously been reported for CTNs (8). Therefore, overexpression of Gas, Gαi and Gαq/11 subunits is unlikely to mediate the increased mitogenic signalling in HTNs or CTNs.

In conclusion, the reduced Gas protein expression in HTNs with a TSH receptor mutation is most likely induced by a feedback mechanism which desensitises the constitutively activated cAMP cascade in HTNs harbouring a constitutively activating TSH receptor mutation. In vitro overexpression of Gas protein and Gαq/11 protein results in an increase of the basal and activated cAMP and IP3 signalling of the WT TSH receptor or TSH receptor mutants. While the in vivo expression of Gαi and Gαq/11 subunits in CTNs or HTNs varies greatly the influence of Gαq/11 or Gαi subunit overexpression on TSH receptor signalling could contribute to nodular growth in some HTNs or CTNs.

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