Evaluation of activation of G proteins in response to thyroid stimulating hormone in thyroid gland cells from euthyroid and hyperthyroid cats

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Objective—To evaluate alterations in ligand-stimulated activity of G proteins in thyroid gland cells of hyperthyroid cats.

Sample Population—Membranes of thyroid gland cells isolated from 5 hyperthyroid cats and 3 age-matched euthyroid (control) cats immediately after the cats were euthanatized.

Procedures—Isolated thyroid cell membranes were treated with thyroid-stimulating hormone (TSH), and activation of G protein was quantified by measurement of the binding of guanosine triphosphate γ-labeled with sulfur 35 (GTPγ35S). The separate effects of G-protein inhibitory (G) and G-protein stimulatory (Gs) proteins were determined by the use of pertussis toxin and cholera toxin, respectively.

Results—Thyroid cell membranes from hyperthyroid cats had higher basal GTPγ35S binding than did thyroid cell membranes from euthyroid cats. Thyroid cell membranes from hyperthyroid and euthyroid cats had a concentration-dependent increase in TSH-stimulated GTPγ35S binding over the TSH range of 0 to 100 mU/mL, with maximal activity at 1 to 100 mU/mL for both. The percentage increase in GTPγ35S binding stimulated by TSH was similar in magnitude between the membranes from hyperthyroid and euthyroid cats. The TSH-stimulated activation of Gs and Gi was not different between euthyroid and hyperthyroid cats.

Conclusions and Clinical Relevance—Ligand-stimulated activation of G proteins was the same in thyroid cell membranes obtained from hyperthyroid and euthyroid cats. Therefore, alterations in inherent Gs or Gi activities did not appear to be part of the pathogenesis of hyperthyroidism in cats. (Am J Vet Res 2010;71:643–648)

Hyperthyroidism is the most common endocrinopathy in cats, and it affects a large percentage of middle-aged to older animals. Since its recognition as a clinical entity approximately 30 years ago, it has been diagnosed with increasing frequency; however, the cause of the disease remains unknown. Studies have indicated that the disease is at the level of the thyroid gland, with cells from hyperthyroid cats functioning autonomously to result in unregulated cell growth and hormone secretion. Although nutritional and environmental causes have been implicated in the etiology of the disease, no discrete factor has been identified that causes thyroid gland cells to become autonomic.

Synthesis and secretion of thyroid hormone is directly regulated by TSH that is released by the pituitary gland. The interaction of TSH with its receptor on the surface of thyroid cells results in activation of receptor-coupled heterotrimeric G proteins that control cAMP concentrations in the thyroid gland cells. Activation of this signal transduction system and subsequent elevation of intracellular cAMP concentrations result in growth and differentiation of thyroid gland cells as well as secretion of thyroid hormone. Thus, abnormalities of any part of the receptor-G protein-cAMP signal transduction system could be postulated to result in the unregulated growth and hormone production seen in cats with hyperthyroidism.

The Gi and Gs families of G proteins control intracellular cAMP concentrations, with Gi proteins causing activation of adenylyl cyclase and elevation of cAMP concentrations and Gs proteins inhibiting adenylyl cy-
class and decreasing intracellular cAMP concentrations. Binding of TSH to its receptor causes a conformational change of the TSH receptor that results in replacement of GTP for GDP on the α subunit of the coupled heterotrimeric G protein. This results in activation of the G protein such that α-GTP separates from the βγ dimer. Both the α subunit and βγ dimer interact with various effector molecules, including adenylyl cyclase, to cause cellular activation. The G, has inherent GTPase activity and catalyzes the dephosphorylation of GTP, which results in reformation of the inactive GDP-bound βγ heterotrimer. By taking advantage of this activation-deactivation cycle, heterotrimeric G protein activity can be measured by use of a nonhydrolyzable analogue of GTP, GTPγS labeled with sulfur 35 (35S). This analogue binds to active G and provides a stable radiolabel. The radioactivity then can be measured to quantify G protein activity.

The TSH receptor is coupled to both G, and G proteins. Therefore, in thyroid gland cells, the relative expression or activation of G, and G proteins in a cell ultimately determines the intracellular cAMP concentration that directly stimulates cell growth, proliferation, and hormone production. For instance, if the ratio of G, expression to G expression is higher for a disease state than for healthy tissue, the cAMP concentrations may be artificially increased. Specific bacterial toxins can affect the activities of G, and G proteins through a process called ADP-ribosylation. These toxins catalyze the transfer of an ADP ribose group onto G, and cause permanent changes in G protein activity. Cholera toxin is specific for G, proteins and results in constitutive activation of G, protein. Similarly, PTX catalyzes a reaction specific to G, proteins and results in an inactivation of G, protein. These toxins are important for the pathogenesis of their particular diseases, and they can also be used experimentally to define the separate roles of G, and G proteins in overall cellular activation of G proteins.

Hyperthyroidism in cats is clinically and histopathologically similar to a hyperthyroid disease in humans (ie, toxic nodular goiter). Abnormalities of the G protein-cAMP signaling pathway have been implicated in the pathogenesis of toxic nodular goiter. Gain-of-function mutations of the α subunit of G proteins and the TSH receptor that result in unregulated growth and function of the affected thyocytes have been described. Altered expression of the α subunits of G, and G proteins have also been detected in humans with toxic nodular goiter. Similar mechanisms appear to be involved in the pathogenesis of hyperthyroidism in cats. Previous studies conducted by our laboratory group have indicated a specific decrease of the expression of G, protein (more specifically, the G, subset) in membranes isolated from hyperthyroid cells. We postulate that a decrease in G, expression leads to a relative increase of G expression and the unregulated hyperfunction of the thyroid cells seen in cats with hyperthyroidism.

Although the amount of expression appears to be affected, the activity of the TSH receptor-coupled G proteins in cats with hyperthyroidism has not been investigated. The study reported here was conducted to examine activation of G, and G proteins in response to TSH in thyroid cell membranes isolated from hyperthyroid cats to determine whether abnormal activation of G proteins is part of the molecular pathogenesis of hyperthyroidism in cats.

Materials and Methods

Sample population—Thyroid gland tissue was surgically removed from 5 hyperthyroid cats (3 females and 2 males; 8 to 13 years old) and 3 age-matched euthyroid cats (2 males and 1 female; 8 to 14 years old) that were euthanatized at our hospital because of traumatic injuries. Cats were euthanatized by an IV injection of sodium pentobarbital. Thyroid glands were removed immediately after cats were euthanatized.

Review of the medical record for each cat was performed by a veterinarian board-certified by the American College of Veterinary Internal Medicine (CRW), and a diagnosis of hyperthyroidism was determined on the basis of clinical signs and an increase in serum thyroxine concentration. Histologic examination of the thyroid gland tissue confirmed the diagnosis of hyperthyroidism. All hyperthyroid cats in this study had disease in both lobes of the thyroid gland. Euthyroid cats did not have clinical signs of hyperthyroidism; had serum thyroxine concentrations within the reference range, had thyroid gland tissue that was judged to be histologically normal, and did not have evidence of systemic disease, as determined on the basis of the medical history and results of physical examination and point-of-care biochemical analyses.

Tissue preparation—Thyroid glands were snap-frozen immediately after harvest in liquid nitrogen and stored at -80°C until use. Minced thyroid tissue (0.175 to 0.435 g) was placed in 2 mL of ice-cold buffer solution containing 50 mM Tris, 1 mM EDTA, and 250 mM sucrose (pH 7.4) and then was homogenized. Samples were centrifuged at 2,500 × g for 10 minutes at 4°C, and the resultant supernatant was collected and centrifuged at 30,000 × g for 30 minutes at 4°C. The supernatant from the second centrifugation was discarded, and the membrane-enriched pellet was resuspended in Tris-EDTA buffer, divided into aliquots, and stored at -80°C. Protein concentration of the membranes was determined by use of a bicinchoninic acid protein assay with bovine serum albumin used as the standard.

GTPγS binding analysis—The membrane-enriched pellets from the hyperthyroid and euthyroid cats were diluted in ice-cold buffer solution (50 mM Tris-1 mM EDTA, pH 7.4) to achieve equivalent amounts of protein in each sample to be assayed (25 to 35 μg). They were then added to a binding buffer solution containing 100 mM Tris-2 mM EDTA (pH 7.4), 100 mM MgCl2, 100 mM dithiothreitol, 500 μM GDP, and 1 mM GTPγS. To obtain the concentration-response curve, various concentrations of bovine TSH (0 to 100 mU/mL) were added. These concentrations were selected on the basis of TSH concentrations used to stimulate cultured feline thyroid cells and Wistar rat thyroid cells. In some experiments, 50 μM mastoparan, a wasp venom peptide that nonspecifically activates all means.

Table 1. Characteristics of Thyroid Glands

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Weight (g)</th>
<th>Conditions</th>
</tr>
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<tbody>
<tr>
<td>Hyperthyroid</td>
<td>0.25</td>
<td>Euthyroid</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>0.30</td>
<td>Normal</td>
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<table>
<thead>
<tr>
<th>Condition</th>
<th>TSH Concentration (mU/mL)</th>
<th>CAMP Concentration (pmol/mL)</th>
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</thead>
<tbody>
<tr>
<td>Hyperthyroid</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>5</td>
<td>50</td>
</tr>
</tbody>
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classes of G proteins was added as a positive control group (data not shown). For each data point, a separate set of duplicate tubes containing a 1,000-fold excess of unlabeled GTP\(\gamma\)S was included to quanititate non-specific GTP\(\gamma\)S\(^{35}\)S binding. The experimental samples were placed in a 30°C water bath for 15 minutes and then filtered through a vacuum-controlled 2-μm filter. Ice-cold buffer solution (50mM Tris–5mM MgCl\(_2\) [pH 7.5]) was used to stop the reaction. Filters were allowed to dry for 30 minutes and then placed into scintillation vials containing 1 mL of scintillation fluid. Samples were analyzed for GTP\(\gamma\)S\(^{35}\)S binding by use of a scintillation machine. Specific GTP\(\gamma\)S binding was determined by subtracting the nonspecific binding obtained with the 1,000-fold excess of GTP\(\gamma\)S from the total GTP\(\gamma\)S binding. Triplicate determinations were made for each condition tested in each experiment. To examine the activation of G\(_i\) and G\(_s\) proteins, thyroid cell membranes were subjected to CTX- or PTX-catalyzed ADP-ribosylation. Thyroid cell membranes were resuspended in ice-cold buffer solution (50mM Tris-HCl, 1mM EDTA, 20mM β-glycerophosphate, 10 μg of leupeptin/mL, and 10 μg of aprotinin/mL [pH, 7.5]) to achieve a protein concentration of 100 μg/mL. Activated toxin (CTX or PTX) and nicotinamide adenine dinucleotide were added to the membranes to achieve a final concentration of 20 μg/mL and 5.3μM for CTX and PTX, respectively. Membranes were incubated at 30°C for 60 minutes to allow the CTX- or PTX-catalyzed ADP-ribosylation to proceed. The treated membranes were then assayed for TSH-induced GTP\(\gamma\)S\(^{35}\)S binding as described previously.

Statistical analysis—Statistical analysis was performed by use of a commercial statistical program. The dependent variable, GTP\(\gamma\)S binding, was analyzed via a 2-way ANOVA by use of a general linear models procedure to assess effects of membrane type and concentration of TSH. The percentage change in GTP\(\gamma\)S\(^{35}\)S binding was also analyzed by use of a 2-way ANOVA by use of a general linear models procedure to assess effects of membrane type and treatment (TSH, CTX, or PTX). Differences between means were determined by use of the Duncan multiple range test. For all analyses, values of P < 0.05 were considered significant.

Results

Isolated thyroid cell membranes from euthyroid and hyperthyroid cats had a significant (P = 0.01) quadratic relationship between GTP\(\gamma\)S binding when stimulated by TSH at concentrations ranging from 0 to 100 μU of TSH/mL (Figure 1). At a TSH concentration of 0 μU/mL (unstimulated), membranes from hyperthyroid cats had significantly higher basal GTP\(\gamma\)S binding than did membranes from euthyroid cats: Maximal response of 4.0 and 4.9 fmol of GTP\(\gamma\)S binding/μg of protein was detected at a TSH concentration of 1 μU/mL for membranes from euthyroid and hyperthyroid cats, respectively. This response was significantly higher than the basal GTP\(\gamma\)S binding for the unstimulated mem-

branes. Although basal and maximal GTP\(\gamma\)S binding was higher in membranes from the hyperthyroid cats, the percentage increase of GTP\(\gamma\)S binding did not differ significantly between membranes from euthyroid and hyperthyroid cats (Table 1). The GTP\(\gamma\)S binding did not increase significantly as the concentration of TSH increased from 1 to 100μU/mL.

In an effort to examine the separate effects of G\(_i\) and G\(_s\) proteins to the total GTP\(\gamma\)S binding, membranes were incubated with PTX (an inactivator of all subgroups of G\(_i\) proteins) or CTX (a specific activator of G\(_i\) proteins). Thus, the separate contributions of G\(_i\) and G\(_s\) proteins could be quantified. Effects of toxin treatment on the percentage of TSH-stimulated GTP\(\gamma\)S binding were determined (Table 1). The percentage of TSH-stimulated GTP\(\gamma\)S binding was not significantly (P = 0.68) different for the membrane type-by-toxin treatment interaction. However, there was a significant (P < 0.001) effect of toxin treatment. In PTX-treated membranes, TSH-stimulated GTP\(\gamma\)S binding was significantly inhibited, compared with binding in non-PTX-treated membranes, regardless of the membrane type.
type. In membranes incubated with CTX, there was also a significant decrease in TSH-stimulated GTPyS binding, compared with binding in non-CTX-treated membranes. However, the inhibition of GTPyS binding did not differ significantly between the PTX and CTX treatments. Inhibition of GTPyS binding attributable to membrane type (euthyroid or hyperthyroid cats) did not differ significantly (P = 0.06).

**Discussion**

Analysis of results for the study reported here revealed that isolated feline thyroid cell membranes underwent measurable activation of G proteins when stimulated with their natural ligand, TSH. This was a concentration-dependent effect, which indicated a specific effect of the ligand-receptor interaction. Maximal ligand stimulation was seen at a TSH concentration of 1 mU/mL, with a plateau effect for concentrations up to 100 mU/mL. These data agree with those in another study conducted by our laboratory group in which cultured feline thyroid cells were treated with TSH to induce the cellular responses of mitogenesis and hormone production. In the experiments in that study, stimulation was detected at a TSH concentration of 1 mU/mL, although the maximal effect was detected at a TSH concentration of 50 mU/mL. It is not surprising that the maximal effect on GTPyS binding was detected at a lower concentration in the present study because we used the activation of G proteins as the endpoint, rather than a total cellular response such as mitogenesis or hormone production. Additionally, in the experiments in that previous study, were performed on cultured cells, compared with the experiments in the present study that were conducted by use of isolated membranes, which would be expected to respond to lower concentrations of ligand. Therefore, the ligand concentration that yielded optimal results in the study reported here appeared to be consistent with previous data. We believe that this is a physiologically appropriate system in which to quantify ligand-stimulated G protein responses in feline thyroid membranes.

Separate experiments were not conducted to examine TSH binding to the membranes from euthyroid or hyperthyroid cats. In another study, researchers investigated mutations in the feline TSH receptor but were not able to identify any such mutations, and binding characteristics of cloned feline TSH receptors were identical to those of human TSH receptors. In addition, TSH specifically stimulates cultured thyrocytes from hyperthyroid cats and also stimulates a hormonal response in hyperthyroid cats. In thyroid membranes incubated with PTX, G proteins are not able to be stimulated by TSH. Although there was a significant decrease in TSH-stimulated GTPyS binding in membranes treated with CTX and PTX, analysis of our data did not reveal a significant difference in activation of G proteins between thyroid membranes from euthyroid and hyperthyroid cats. This provides further evidence that G and G can be activated by the TSH ligand in thyroid cells from hyperthyroid cats.

Multiple environmental factors, including exposure to flame retardants, canned foods, and flavonoids, have been implicated as a cause for hyperthyroidism in cats. The evidence for involvement of these factors in the disease is largely epidemiological, however, all 3 studies implicated commercially available cat food as a risk factor for development of hyperthyroidism in cats. In another study, investigators examined blood samples from cats with hyperthyroidism and from age-matched euthyroid (control) cats and found increased concentrations of some metabolites of flame retardants in some of the hyperthyroid cats. In a collaborative study conducted by our laboratory group, we detected direct effects of a variety of flavonoid compounds on
the ability to stimulate cultured feline thyocytes. The role these factors may have on the changes in G proteins seen in cells from hyperthyroid cats is not clear, but it remains a fertile area for future research.

Analysis of results from the study reported here indicated that TSH-stimulated activity of G proteins was the same in cells from euthyroid and hyperthyroid cats. This supports the hypothesis that it is a specific decrease in expression in G, and not a change in G protein activity, that is part of the molecular pathogenesis of hyperthyroidism in cats. It is postulated that a decrease in expression of G, proteins causes cAMP to be increased in an unregulated manner. Reasons for this decrease in expression are unknown; however, the relationship with identified environmental factors implicated in causing hyperthyroidism needs to be investigated further. Also of interest is the effect of cAMP production in these cells. Future research efforts into this area need to be completed.

References

34. Ward CR, Achenbach SE, Holt D, et al. Thyrotropin-stimulated DNA synthesis and thyroglobulin expression in normal and
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