Altered expression of G proteins in thyroid gland adenomas obtained from hyperthyroid cats

Kimberly B. Hammer, BS; David E. Holt, BVSc; Cynthia R. Ward, VMD, PhD

Objective—To determine whether expression of G proteins (G_i and G_s) is altered in thyroid gland adenomas obtained from hyperthyroid cats.

Sample Population—Adenomatous thyroid glands obtained from 8 hyperthyroid cats and thyroid glands obtained from 4 age-matched euthyroid cats.

Procedure—Expression of G_i and G_s was quantified in enriched membrane preparations of thyroid gland tissue, using immunoblotting with G_i and G_s antibodies and toxin-catalyzed ADP-riboseylation.

Results—Expression of G_i was significantly reduced in thyroid gland adenomas from hyperthyroid cats, compared with normal thyroid gland tissue from euthyroid cats. Expression of G_s was similar between the 2 groups.

Conclusions and Clinical Relevance—A decrease in expression of G_i in adenomatous thyroid glands of cats may reduce the negative inhibition of the cAMP cascade in thyroid cells, leading to autonomous growth and hypersecretion of thyroxine. Understanding the molecular mechanisms for hyperthyroidism in cats may lead to better treatment or, ultimately, prevention of the disease. (Am J Vet Res 2000;61:874–879)

Hyperthyroidism is a common endocrinopathy in cats. Diagnosis of the disease and treatment of affected animals have become routine; however, its cause is unknown. The disease usually results from benign adenomatous hyperplasia or adenoma of the thyroid gland, although thyroid gland adenocarcinoma is rare. Adenomatous thyroid gland tissue functions autonomously, and the result is increased secretion of thyroid hormones causing clinical onset of disease. Most hyperthyroid cats have multiple hyperplastic nodules that are surrounded by inactive paranodular tissue in both lobes of the thyroid gland.

Thyroid hormone is a critical regulator of metabolism in mammalian systems. Its synthesis and secretion are regulated by thyroid-stimulating hormone (TSH) that is released by the pituitary gland. The TSH binds to a TSH receptor (TSH-R) on the surface of thyroid cells. The TSH-R is a member of the superfamily of receptors that have 7 transmembrane domains and are coupled to heterotrimeric guanine nucleotide binding proteins (ie, G proteins). In most mammals, binding of TSH to a TSH-R stimulates growth and differentiation of thyroid cells and subsequent secretion of thyroid hormone via a G protein-mediated cAMP-dependent signal transduction pathway.

Heterotrimeric G proteins consist of α, β, and γ subunits that, in the inactive form, exist in a membrane-associated heterotrimer. After ligand-receptor interaction, GTP replaces GDP on G_s and G_i activates signal-transduction effectors such as adenyl cyclase to form cAMP. There are 4 main families of G proteins, classified on the basis of differences in their α subunits. Classically, it is believed that the G_i subfamily inhibits adenyl cyclase and decreases cellular cAMP concentrations, whereas the G_s subfamily stimulates adenyl cyclase and, thus, increases cellular cAMP concentrations. It is postulated that the relative expression of G_s and G_i in a cell determines the final cAMP concentration. Therefore, in animals such as dogs, humans, and rats in which cAMP regulates thyroid gland mitogenesis and hormone production, it can be postulated that relative expression of G_s and G_i controls the amount of growth of the thyroid gland as well as thyroid hormone secretion.

Bacterial toxins have been used to examine function of G proteins because of their specific reactions with G_s and G_i. Cholera toxin (CTX) catalyzes the covalent transfer of an ADP-ribose group from NAD to G_s, whereas pertussis toxin (PTX) catalyzes the same reaction with G_i.

Hyperthyroidism in cats is clinically and histologically similar to toxic multinodular goiter, a form of hyperthyroidism in humans. Efforts to elucidate the cause of thyroid adenomatous hyperplasia in humans have concentrated on identifying molecular abnormalities in the TSH-stimulated signal transduction cascade that result in unregulated growth and function of thyroid cells. In some humans, altered expression of G_s and G_i and constitutive increased expression of G_i have been identified and implicated in tumor pathogenesis and clinical signs of hyperthyroidism. In addition, various gain-of-function genetic mutations have been found in thyroid gland adenomas that result in unregulated activity. These include mutations of the genes encoding for G_i and TSH-R. Those mutations result in constitutive activation of G_i and, subsequently, adenyl cyclase with unregulated increases in...
cAMP. It is estimated that approximately 60% of patients with toxic nodular goiter have such gain-of-function mutations, and, therefore, toxic multinodular goiter in humans is believed to be a multifactorial disease.21

Similar experimental approaches directed toward understanding the molecular basis of hyperthyroidism in cats have been lacking. In a series of experiments, investigators examined the effects of TSH stimulation on cAMP formation in cultured thyroid gland adenoma cells. Analysis of results of those experiments pointed to an abnormality in the TSH-R or coupled G proteins. Analysis of results of those experiments pointed to an abnormality in the TSH-R or coupled G proteins. Thus, the authors concluded that G proteins could be implicated in the pathogenesis of this disease.

Materials and Methods

Tissue preparation—Thyroid gland tissue was surgically removed from 8 hyperthyroid cats and 4 age-matched euthyroid cats. All hyperthyroid cats had evidence of the disease in both lobes of the thyroid gland. Tissue was snap frozen in liquid nitrogen and stored at –80 C. The diagnosis of hyperthyroidism in these cats was made on the basis of clinical signs and an increase in serum thyroxine (T₄) concentrations. The diagnosis was confirmed by histopathologic analysis of the thyroid gland tissue. Five of the hyperthyroid cats were treated with methimazole prior to surgery. Euthyroid control cats did not have clinical signs of hyperthyroidism, had serum T₄ concentrations within the reference range, and had normal thyroid gland tissue on histologic analysis.

Thyroid gland tissue was collected at necropsy immediately after cats were euthanatized. Three of the euthyroid cats were clinically normal and were euthanatized immediately after an episode of trauma, whereas the other euthyroid cat was euthanatized because of acute renal failure of approximately 1-day’s duration after ingestion of ethylene glycol.

Mined thyroid gland tissue was placed in ice-cold buffer containing 30 mM tris, 1 mM EDTA, and 250 mM sucrose (pH 7.4) and was homogenized by use of a hand-held homogenizer (20 strokes). Samples were centrifuged at 2,500 X g for 10 minutes at 4 C, and the resultant supernatant was collected and centrifuged at 30,000 X g for 30 minutes at 4 C. The membrane-enriched pellet was resuspended in tris-EDTA buffer, allocated into aliquots, and stored at –80 C. Protein concentration of the membranes was determined, using a BCA protein assay with bovine serum albumin as the standard.

Western immunoblot analysis—Equivalent amounts of protein (30 to 40 µg) of the enriched thyroid membranes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% gel under reducing conditions, in accordance with the procedure described by Laemmli.21 Proteins were then transferred to a polyvinyl difluoride membrane at 30 V (constant) for 18 hours at 4 C.26 The resulting western blots were blocked by incubation for 1 hour with agitation in a buffer solution containing 250 mM tris, 1.5M NaCl, and 0.1% polyoxyethyl-
ene-sorbitan monolaurate, pH 7.4, (TTBS) into which 5% bovine serum albumin was added. Immunoblots were then incubated with nonimmune rabbit serum at 0.2 g/ml or with specific anti-peptide antibodies directed against unique sequences of Gsα and Giα at a dilution of 1:500 for 1 hour. The Gsα antibody was not subtype-specific and was able to recognize all 3 subtypes of Gsα. In some experiments designed to identify potential nonspecific antibody binding, the Gsα antibody was incubated (4 C for 18 hours with constant agitation) with a 10-fold excess of the peptide against which it was generated prior to incubation with the blotted proteins. Immunoblots were rinsed for 10 minutes in TTBS and incubated with horseradish peroxidase-linked anti-rabbit IgG diluted 1:7,500 in TTBS for 35 minutes. Immunoblots then were rinsed for 2 hours in TTBS with at least 5 buffer changes, after which they were developed, using a chemiluminescence kit in accordance with manufacturer’s directions. Quantification of the chemiluminescence signal was performed, using densitometry.

ADP-ribosylation catalyzed by cholera toxin and perussis toxin—Thyroid gland membranes were subjected to ADP-ribosylation catalyzed by CTX and PTX, using the methods of Kopf et al22 and Gill et al23 Thyroid gland membranes were resuspended to a concentration of 1 to 2 mg/ml in ice-cold buffer containing 0.75% Lubrol-PX, 50 mM tris, and 1 mM EDTA, pH 7.4. Forty micrograms of protein were incubated in a solution containing 6 mM ATP, 0.6 mM GTP, 30 mM MgCl₂, 6 mM EDTA, 6 mM dithio-
reitol, 60 mM thymidine, 30 µM NAD, 1 ml of protease-
inhibitor cocktail, 25 µCi of [³²P] NAD, and 50 mg of CTX/ml or 20 mg of PTX/ml at 30 C for 1 hour. Prior to incubation, toxins were activated in a solution of 30 mM HEPES, pH 7.5, containing 24 mM dithiohethiol, 1.2 mg of bovine serum albumin/ml, 0.15% SDS, and 0.3 mg of CTX/ml or 0.12 mg of PTX/ml at 30 C for 30 minutes. The assay was stopped by adding 5 µl of 5% reducing buffer. Samples were subjected to SDS-PAGE, using 10% gels. Gels were fixed in a solution of 7% acetic acid, 5% methanol, and 1% glycerol. They then were dried and exposed to autoradiography film for 3 to 5 days at –80 C. Radiolabeling was quantified, using densitometry.

Statistical analysis—Values were expressed as median (25th percentile, 75th percentile). Each value was compared with that for its own age-matched (defined as samples obtained from euthyroid cats that differed by ≤2 years in age) control samples on the same immunoblot, which was defined as an arbitrary value of 1. The Wilcoxon signed-rank test was used for statistical comparison. A value of P < 0.05 was considered significant.

Results

Western immunoblot analysis of Gsα—Western immunoblots of enriched thyroid gland membranes probed with Gsα antibody revealed an immunoreactive band of 52 kd that was identified as Gsα (Fig 1). A recombinant human Gsα protein standard was analyzed under identical conditions for molecular weight comparison and yielded identical results (data not shown). Under these conditions, expression of Gsα protein appeared visually similar between normal and hyperthyroid membranes. Immunoblots probed with nonimmune rabbit serum did not have evidence of immunoreactivity.

Densitometry was performed to quantify antibody binding to western immunoblots. Densitometric quan-
tification of the chemiluminescent bands revealed that membranes from hyperthyroid cats had 8.1% (29.4%, 0.6%) less Gs\(^{\alpha}\) than normal tissue from euthyroid cats (Fig 2). However, these values did not differ significantly (n = 6, \(P = 0.17\)).

ADP-ribosylation of Gs\(^{\alpha}\) catalyzed by cholera toxin—Cholera toxin was used with \(^{32}\)P NAD to covalently radiolabel the Gs\(^{\alpha}\) subunit. After ADP-ribosylation with CTX, Gs\(^{\alpha}\) was visible in normal and adenomatous thyroid gland tissue in apparently equal amounts (Fig 3). We did not detect radiolabeling in the absence of CTX.

Comparison of radiolabeling by densitometry did not reveal significant differences between membranes from normal and adenomatous thyroid gland tissue. Adenomatous thyroid gland membranes had a decrease of 12.3% (13.5%, 10.5%) in Gs\(^{\alpha}\), compared with values for normal thyroid gland tissue (Fig 4). However, these values did not differ significantly (n = 5, \(P = 0.50\)).

Western immunoblot analysis of Gi\(^{\alpha}\)—Western immunoblots of membrane-enriched thyroid gland preparations from age-matched euthyroid and hyperthyroid cats were probed with Gi\(^{\alpha}\) antibodies and revealed the expected band at 41 kd that corresponds to the molecular weight of Gi\(^{\alpha}\) (Fig 5). Immunoblots of enriched membranes from mouse brain tissue used as positive-control samples revealed identical immunoreactivity (data not shown). Visual inspection revealed that there was an apparent decrease in Gi\(^{\alpha}\) expression in the hyperthyroid membranes, compared with expression for normal thyroid gland membranes. Prior absorption of the antibody with the peptide against which it was generated completely inhibited binding of Gi\(^{\alpha}\) antibody to the 41-kd band.

Densitometry of the bands revealed that hyperthyroid membranes had significantly less expression of Gi\(^{\alpha}\) protein than normal thyroid gland tissue. Membranes from hyperthyroid cats had a significant
AJVR, Vol 61, No. 8, August 2000

(\(n = 8, P = 0.011\)) decrease of 61.6% (80.7%, 49.3%) in \(G_i^\alpha\) content, compared with values for normal tissue (Fig 6).

ADP-ribosylation of \(G_i^\alpha\) catalyzed by pertussis toxin—Using \(^{32}\)P NAD, PTX radiolabeling was seen at 41 kd, corresponding to \(G_i^\alpha\). This radiolabeling confirmed the result obtained from immunoblot analysis in that there visually appeared to be less \(G_i^\alpha\) in hyperthyroid membranes than in euthyroid membranes (Fig 7). We did not detect radiolabeling of thyroid gland membranes in the absence of PTX.

Densitometry of the bands revealed that adenomatous thyroid gland membranes had significantly (\(n = 5, P = 0.027\)) less \(G_i^\alpha\) than membranes from normal thyroid glands. Membranes from hyperthyroid cats had a median of 66.6% (74.6%, 59.6%) less \(G_i^\alpha\) than normal membranes from euthyroid cats (Fig 8).

Discussion

In the study reported here, we used 2 separate methods to detect expression of \(G_i^\alpha\) subunits at the protein level. We found that membranes isolated from adenomatous thyroid gland tissue from hyperthyroid cats had significantly decreased amounts of the \(G_i^\alpha\) subunit, compared with that for membranes from age-matched euthyroid cats. The findings were repeatable in all 8 hyperthyroid cats, indicating that this \(G\) protein abnormality is consistently identifiable in hyperthyroid cats. These results are similar to findings in a study25 of another endocrine tumor, pituitary adenomas in humans, in which all 3 subtypes of \(G_i^\alpha\) were detected in significantly lower amounts than in normal pituitary tissue. However, in contrast to our findings, investigators in 2 other studies26,n found an increase in \(G_i^\alpha\) expression in functional thyroid adenomas in humans.

Analysis of our results documented that there was not a significant change in the protein expression of...
Gαs, between thyroid gland tissue from hyperthyroid and euthyroid cats. These findings are similar to results from 2 studies15,16 of functional or nonfunctional thyroid gland adenomas in humans; in those studies, investigators did not detect changes in Gαs expression. However, our data are in contrast to a report17 documenting overexpression of Gαs in 4 functional thyroid gland adenomas in humans. It is tempting to speculate that Gαs is of little importance in the autonomous growth and function of thyroid cells in hyperthyroid cats. However, our study examined only expression of Gαs, which does not necessarily correlate with Gαs activity.18,19 Point mutations in the Gαs gene have been described in many patients with toxic nodular goiter.20,21 These mutations decrease the intrinsic GTPase activity of Gαs, thus prolonging its activation.22 Until analysis of the Gαs gene and Gαs activity in cats with hyperthyroidism is complete, a role for Gαs in the cause of this disease cannot be ruled out.

It is classically believed that stimulation of Gαs leads to inhibition of adenylyl cyclase and decreased cAMP concentrations in cells.23 Our finding of decreased amounts of Gαs in thyroid gland adenomas suggests the possibility that Gα could play a role in the inhibition of growth and differentiation in the thyroid gland of cats. Decreased amounts of Gαs in adenomaous hyperplasia or adenomas of hyperthyroid cats could possibly reduce the overall inhibitory effect on adenylyl cyclase leading to an increase in total cellular adenylyl cyclase activity and abnormally high concentrations of cAMP. The result would be that cells could grow and differentiate despite little or no circulating TSH. This, in turn, would result in adenomatous hyperplasia of the tissue producing a palpable goiter. Because the adenylyl cyclase system also appears to control hormone secretion, abnormal cells with overexpression of Gαs would have unregulated hyperscretion of thyroid hormones. A similar mechanism has been proposed for pituitary adenomas in humans that have decreased expression of Gα1, Gα2, and Gα3.24

Concentrations of thyroid hormones alter expression of G proteins in other tissues, so it is important to consider the possibility that our observed differences could have been a result of excess thyroid hormone. Experimentally, the effect of excess thyroid hormone on expression of G proteins varies with the tissues and G protein family. Rats with chemically induced hyperthyroidism did not have alterations in Gα1, Gα2, or Gα3 in their ventricular tissue.25 However, in another study,26 expression of Gα1 and Gα2 were decreased or unchanged in tissues of the heart and vas deferens and unchanged in the cerebral cortex or liver, but expression of Gα3 was increased in cardiac tissues. Rats treated with triiodothyronine to induce hyperthyroidism had reduced expression of Gα1 in adipose cells.27 Specific effects of thyroid hormone itself on concentrations of G proteins in the thyroid gland have not been examined, and it is possible that the reduced expression of Gαs seen in the hyperthyroid cats reported here may be an effect of increased concentrations of thyroid hormone. However, it is less likely that these results are attributable to nonspecific effects of changes in cell size or density, because Gαs expression was unchanged.

Five cats in our study were treated with methimazole. That drug affects the production of active hormone by preventing incorporation of active iodine into tyrosyl residues of thyroglobulin. It would not be anticipated that treatment with methimazole would affect cellular dysregulation resulting in autonomously functioning thyroid gland adenomas. We observed similar significant decreases of Gαs expression in cats regardless of whether they were treated with methimazole, and, therefore, we believe that methimazole treatment did not have an effect on our results. However, the number of cats examined is too small to make a direct comparison between methimazole-treated and untreated cats; therefore, we cannot discount the possibility that methimazole treatment affected the degree of decreased Gαs in these cats.

Analysis of these data suggests a role for Gαs proteins in the pathogenesis of hyperthyroidism in cats. Because of the frequency of this disease in cats, the affected cats reported here offered a unique opportunity to study the molecular basis of this endocrinopathic disease. It is interesting that decreased expression of Gαs was consistent in all hyperthyroid cats in the study. If this is representative of the entire population of hyperthyroid cats, it will provide an intriguing contrast to the diverse G protein abnormalities found in various groups of humans with toxic thyroid adenomas.28 It is important to mention that functionality of the G proteins was not examined, but this should be evaluated in future experiments.

1. Pierce Chemical Co, Rockford, Ill.
3. SC-263, SC-262, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
4. SC-823, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
5. SC-263P, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
8. Sigma-Aldrich, St Louis, Mo.
9. P8340, Sigma-Aldrich, St Louis, Mo.
10. BLU 023 (800 Ci/mmol), NEN Life Science Products, Boston, Mass.
11. Sigma-Aldrich, St Louis, Mo.
12. P 0317, Sigma-Aldrich, St Louis, Mo.

References
and function of cultured thyroid follicles from cats with spontaneous hyperthyroidism. Thyroid 1991;1:331–338.