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

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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-ribosylation.

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.[1-3] Adenomatous thyroid gland tissue functions autonomously, and the result is increased secretion of thyroid hormones causing clinical onset of disease.[1] Most hyperthyroid cats have multiple hyperplastic nodules that are surrounded by inactive paranodular tissue in both lobes of the thyroid gland.[2-4]

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.[5-7]

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_α, and G_α activates signal-transduction effectors such as adenylyl cyclase to form cAMP. There are 4 main families of G proteins, classified on the basis of differences in their α subunits.[8] Classically, it is believed that the G_i subfamily inhibits adenylyl cyclase and decreases cellular cAMP concentrations, whereas the G_s subfamily stimulates adenylyl 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.[9] 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 $G_{s\alpha}$,[10] whereas **pertussis toxin (PTX)** catalyzes the same reaction with $G_{i\alpha}$.[11]

Hyperthyroidism in cats is clinically and histologically similar to toxic multinodular goiter, a form of hyperthyroidism in humans.[12] 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[13,14] and constitutive increased expression of G_{i1}[15] 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_s[16,17] and TSH-R.[18-20] Those mutations result in constitutive activation of G_s and, subsequently, adenylyl cyclase with unregulated increases in

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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,[a] 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. In another study,[22] investigators examined a small portion of feline TSH-R in an attempt to identify mutations similar to those found in humans; however, such mutations were not identified in the 11 hyperthyroid cats examined.

In an effort to identify signaling abnormalities that may be involved in the pathogenesis of hyperthyroidism in cats, we examined expression of G proteins in thyroid gland adenomas obtained from hyperthyroid cats to determine whether alterations in expression of 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 (T_4)** 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 T_4 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 euthanized. Three of the euthyroid cats were clinically normal and were euthanized immediately after an episode of trauma, whereas the other euthyroid cat was euthanized because of acute renal failure of approximately 1-day's duration after ingestion of ethylene glycol.

Minced thyroid gland tissue was placed in ice-cold buffer containing 50 m*M* tris, 1 m*M* EDTA, and 250 m*M* sucrose (pH 7.4) and was homogenized by use of a hand-held homogenizer (20 strokes). 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 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[b] 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.[23] Proteins were then transferred to a polyvinyl difluoride membrane[c] at 30 V (constant) for 18 hours at 4 C.[24] The resulting western blots were blocked by incubation for 1 hour with agitation in a **buffer solution containing 250 m*M* tris, 1.5*M* NaCl, and 0.1% polyoxyethylene-sorbitan monolaurate, pH 7.4, (TTBS)** into which 5% teleost gelatin 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 $G_{s\alpha}$[d] and $G_{i\alpha}$[e] at a dilution of 1:500 for 1 hour. The $G_{i\alpha}$ antibody was not subtype-specific and was able to recognize all 3 subtypes of $G_{i\alpha}$. In some experiments designed to identify potential nonspecific antibody binding, the $G_{i\alpha}$ antibody was incubated (4 C for 18 hours with constant agitation) with a 10-fold excess of the peptide against which it was generated[f] prior to incubation with the blotted proteins. Immunoblots were rinsed for 10 minutes in TTBS and incubated with horseradish peroxidase-linked anti-rabbit IgG[g] 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[h] in accordance with manufacturer's directions. Quantification of the chemiluminescence signal was performed, using densitometry.

ADP-ribosylation catalyzed by cholera toxin and pertussis toxin—Thyroid gland membranes were subjected to ADP-ribosylation catalyzed by CTX and PTX, using the methods of Kopf et al[10] and Gill et al.[11] Thyroid gland membranes were resuspended to a concentration of 1 to 2 mg/ml in ice-cold buffer containing 0.75% Lubrol-PX,[i] 50 m*M* tris, and 1 m*M* EDTA, pH 7.4. Forty micrograms of protein were incubated in a solution containing 6 m*M* ATP, 0.6 m*M* GTP, 30 m*M* $MgCl_2$, 6 m*M* EDTA, 6 m*M* dithiothreitol, 60 m*M* thymidine, 30 µ*M* NAD, 1 ml of protease-inhibitor cocktail,[j] 25 µCi of ^{32}P NAD,[k] and 50 mg of CTX[l]/ml or 20 mg of PTX[m]/ml at 30 C for 1 hour. Prior to incubation, toxins were activated in a solution of 30 m*M* HEPES, pH 7.5, containing 24 m*M* dithiothreitol, 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.[24] 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 $G_{s\alpha}$—Western immunoblots of enriched thyroid gland membranes probed with $G_{s\alpha}$ antibody revealed an immunoreactive band of 52 kd that was identified as $G_{s\alpha}$ (**Fig 1**). A recombinant human $G_{s\alpha}$ protein standard was analyzed under identical conditions for molecular weight comparison and yielded identical results (data not shown). Under these conditions, expression of $G_{s\alpha}$ 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-

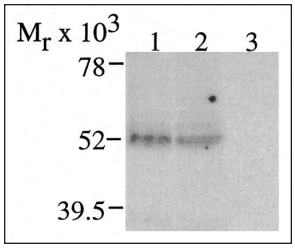

Figure 1—Representative western immunoblot revealing immunologic detection of $G_{s\alpha}$ in normal and adenomatous thyroid gland tissues from cats. Membrane-enriched thyroid gland preparations of normal (30 μg) and adenomatous (30 μg) tissues were evaluated by use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% gel. Samples were probed with $G_{s\alpha}$ antibodies (lanes 1 and 2) or nonimmune rabbit serum (lane 3) and developed by use of enhanced chemiluminescence autoradiography.

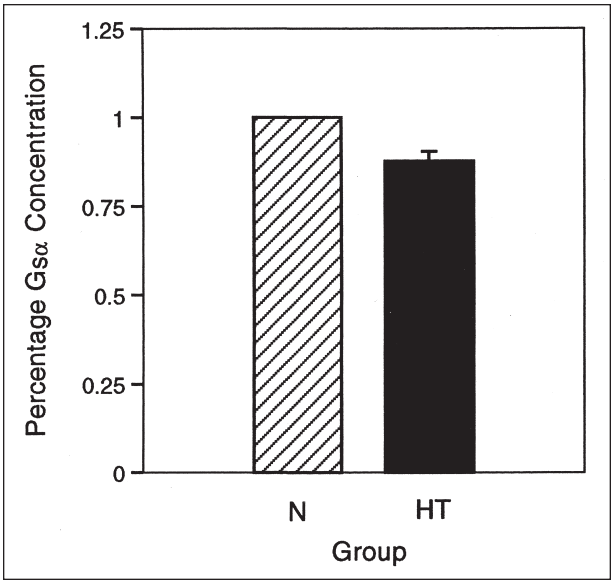

Figure 2—Quantification of $G_{s\alpha}$ concentrations in normal tissues from euthyroid cats (N) and adenomatous thyroid gland tissues from hyperthyroid cats (HT), using densitometry. Each value was adjusted for its age-matched control sample, which was defined as an arbitrary value of 1. Adenomatous tissue had a nonsignificant (n = 6, P = 0.17) decrease of 8.1% (25th and 75th percentile values = 29.4 and 0.6%, respectively) in $G_{s\alpha}$ content, compared with that of normal tissues. Median values are reported, and the error bar represents the 75th percentile value.

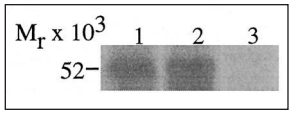

Figure 3—Representative western immunoblot revealing ADP-ribosylation of $G_{s\alpha}$ catalyzed by cholera toxin in normal and adenomatous thyroid gland tissues from cats. Membrane-enriched thyroid gland preparations from normal (40 μg) and adenomatous (40 μg) tissues were incubated with cholera toxin, subjected to SDS-PAGE on a 10% gel, and developed with autoradiography (lanes 1 and 2). A control sample received the identical treatment, except it was not incubated with cholera toxin (lane 3).

tification of the chemiluminescent bands revealed that membranes from hyperthyroid cats had 8.1% (29.4%, 0.6%) less $G_{s\alpha}$ than normal tissue from euthyroid cats (**Fig 2**). However, these values did not differ significantly (n = 6, P = 0.17).

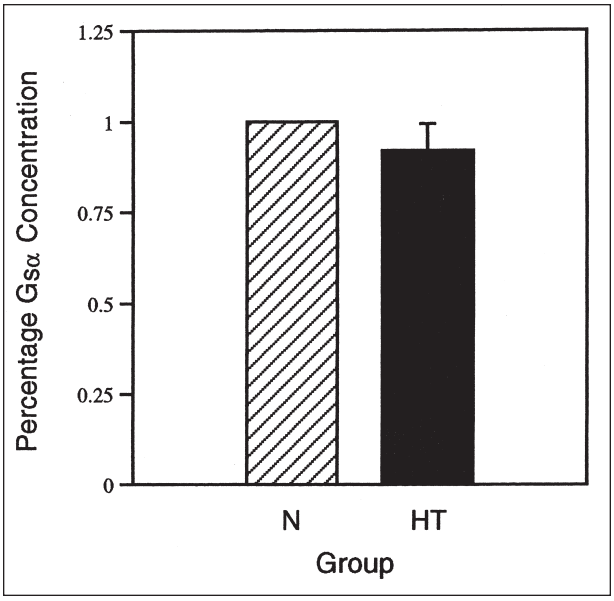

Figure 4—Quantification of ADP-ribosylation of $G_{s\alpha}$ catalyzed by cholera toxin in normal tissues from euthyroid cats (N) and adenomatous thyroid gland tissues from hyperthyroid cats (HT), using densitometry. Each value was adjusted for its own age-matched control sample, which was defined as an arbitrary value of 1. Adenomatous tissue had a nonsignificant (n = 5, P = 0.50) decrease of 12.3% (25th and 75th percentiles, 13.5 and 10.5%) in $G_{s\alpha}$ content, compared with that of normal tissues. Median values are reported, and the error bar represents the 75th percentile value.

ADP-ribosylation of $G_{s\alpha}$ catalyzed by cholera toxin—Cholera toxin was used with ^{32}P NAD to covalently radiolabel the $G_{s\alpha}$ subunit. After ADP-ribosylation with CTX, $G_{s\alpha}$ 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 $G_{s\alpha}$, 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 $G_{i\alpha}$—Western immunoblots of membrane-enriched thyroid gland preparations from age-matched euthyroid and hyperthyroid cats were probed with $G_{i\alpha}$ antibodies and revealed the expected band at 41 kd that corresponds to the molecular weight of $G_{i\alpha}$ (**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 $G_{i\alpha}$ 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 $G_{i\alpha}$ antibody to the 41-kd band.

Densitometry of the bands revealed that hyperthyroid membranes had significantly less expression of $G_{i\alpha}$ protein than normal thyroid gland tissue. Membranes from hyperthyroid cats had a significant

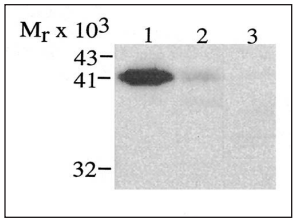

Figure 5—Representative western immunoblot revealing immunologic detection of $G_{i\alpha}$ in normal and adenomatous thyroid gland tissues from cats. Membrane-enriched thyroid gland preparations of normal (30 μg) and adenomatous (30 μg) tissues were evaluated by use of SDS-PAGE on a 12.5% gel. Samples were probed with $G_{i\alpha}$ antibodies (lanes 1 and 2) or antibody previously absorbed with the peptide to which it was generated (lane 3) and developed with enhanced chemiluminescence autoradiography.

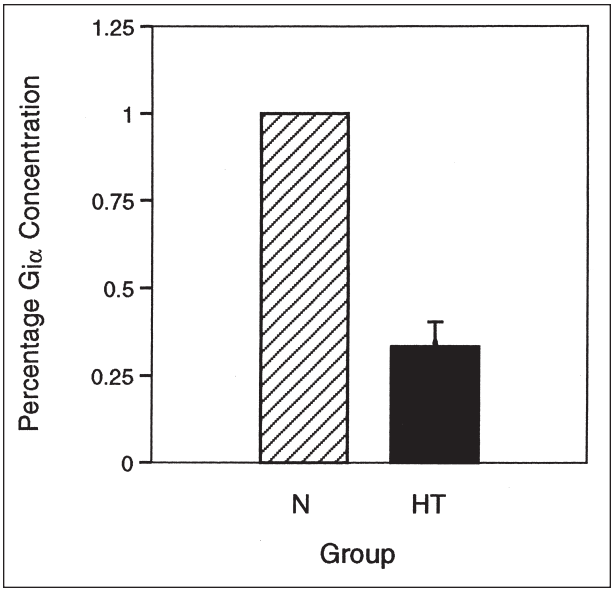

Figure 6—Quantification of $G_{i\alpha}$ concentrations in normal tissues from euthyroid cats (N) and adenomatous thyroid gland tissues from hyperthyroid cats (HT), using densitometry. Each value was adjusted for its own age-matched control sample, which was defined as an arbitrary value of 1. Adenomatous tissue had a significant (n = 8, $P = 0.011$) decrease of 61.6% (25th and 75th percentiles, 80.7 and 49.3%) in $G_{i\alpha}$ content, compared with that of normal tissues. Median values are reported, and the error bar represents the 75th percentile value.

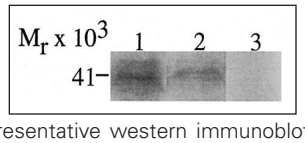

Figure 7—Representative western immunoblot revealing ADP-ribosylation of $G_{i\alpha}$ catalyzed by pertussis toxin in normal and adenomatous thyroid gland tissues from cats. Membrane-enriched thyroid gland preparations from normal (40 μg) and adenomatous (40 μg) tissues were incubated with pertussis toxin, subjected to SDS-PAGE on a 10% gel, and developed with autoradiography (lanes 1 and 2). A control sample received the identical treatment, except it was not incubated with pertussis toxin (lane 3).

(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

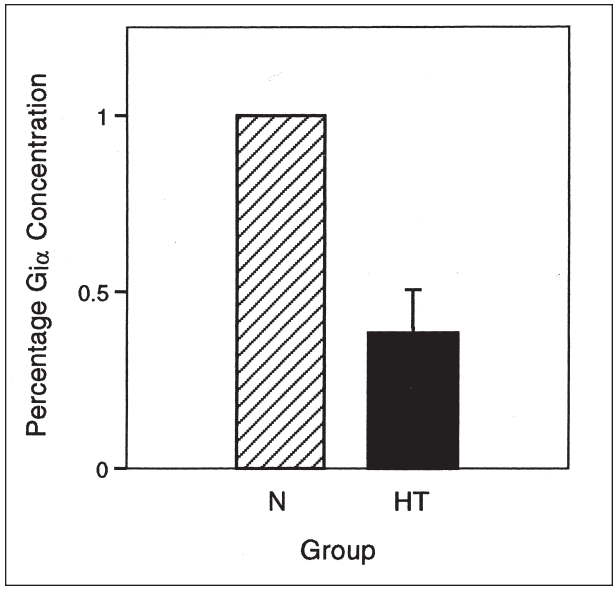

Figure 8—Quantification of ADP-ribosylation of $G_{i\alpha}$ catalyzed by pertussis toxin in normal tissues from euthyroid cats (N) and adenomatous thyroid gland tissues from hyperthyroid cats (HT), using densitometry. Each value was adjusted for its own age-matched control sample, which was defined as an arbitrary value of 1. Adenomatous tissue had a significant (n = 5, $P = 0.027$) decrease of 66.6% (25th and 75th percentiles, 74.6 and 59.6%) in $G_{i\alpha}$ content, compared with that of normal tissues. Median values are reported, and the error bar represents the 75th percentile value.

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_α 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 study[25] of another endocrine tumor, pituitary adenomas in humans, in which all 3 subtypes of $G_{i\alpha}$ ($G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$) were detected in significantly lower amounts than in normal pituitary tissue. However, in contrast to our findings, investigators in 2 other studies[26,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

$G_{s\alpha}$ between thyroid gland tissue from hyperthyroid and euthyroid cats. These findings are similar to results from 2 studies[27,28] of functional or nonfunctional thyroid gland adenomas in humans; in those studies, investigators did not detect changes in $G_{s\alpha}$ expression. However, our data are in contrast to a report[29] documenting overexpression of $G_{s\alpha}$ in 4 functional thyroid gland adenomas in humans. It is tempting to speculate that $G_{s\alpha}$ is of little importance in the autonomous growth and function of thyroid cells in hyperthyroid cats. However, our study examined only expression of $G_{s\alpha}$, which does not necessarily correlate with $G_{s\alpha}$ activity.[30] Point mutations in the $G_{s\alpha}$ gene have been described in many patients with toxic nodular goiter.[16,17] These mutations decrease the intrinsic GTPase activity of $G_{s\alpha}$, thus prolonging its activation.[31] Until analysis of the $G_{s\alpha}$ gene and $G_{s\alpha}$ activity in cats with hyperthyroidism is complete, a role for $G_{s\alpha}$ in the cause of this disease cannot be ruled out.

It is classically believed that stimulation of $G_{i\alpha}$ leads to inhibition of adenylyl cyclase and decreased cAMP concentrations in cells.[9] Our finding of decreased amounts of $G_{i\alpha}$ in thyroid gland adenomas suggests the possibility that G_i could play a role in the inhibition of growth and differentiation in the thyroid gland of cats. Decreased amounts of $G_{i\alpha}$ in adenomatous 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 $G_{i\alpha}$ would have unregulated hypersecretion of thyroid hormones. A similar mechanism has been proposed for pituitary adenomas in humans that have decreased expression of $G_{i\alpha 1}$, $G_{i\alpha 2}$, and $G_{i\alpha 3}$.[25]

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 G_s, G_{i2}, or G_{i3} in their ventricular tissue.[32] However, in another study,[33] expression of G_{i1} and G_{i2} were decreased or unchanged in tissues of the heart and vas deferens and unchanged in the cerebral cortex or liver, but expression of G_s was increased in cardiac tissues. Rats treated with triiodothyronine to induce hyperthyroidism had reduced expression of G_i in adipose cells.[34] 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 $G_{i\alpha}$ 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 $G_{s\alpha}$ 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 $G_{i\alpha}$ 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 G_i in these cats.

Analysis of these data suggests a role for $G_{i\alpha}$ 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 $G_{i\alpha}$ 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.[30] It is important to mention that functionality of the G proteins was not examined, but this should be evaluated in future experiments.

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[b]Pierce Chemical Co, Rockford, Ill.
[c]Immobilon-P, Millipore, Bedford, Mass.
[d]SC-823, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
[e]SC-262, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
[f]SC-262P, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
[g]Na 934, Amersham Life Science, Arlington Heights, Ill.
[h]Renaissance Chemiluminescence Kit, NEN Life Science Products, Boston, Mass.
[i]D5628, Sigma-Aldrich, St Louis, Mo.
[j]P8340, Sigma-Aldrich, St Louis, Mo.
[k]BLU 023 (800 Ci/mmol), NEN Life Science Products, Boston, Mass.
[l]C 8052, Sigma-Aldrich, St Louis, Mo.
[m]P 0317, Sigma-Aldrich, St Louis, Mo.
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