Expression of inhibitory G proteins in adenomatous thyroid glands obtained from hyperthyroid cats

Cynthia R. Ward, VMD, PhD; Sara E. Achenbach, BA; Mark E. Peterson, DVM; Kenneth J. Drobatz, DVM; David Holt, BVSc

Objective—To identify within guanosine triphosphate–binding proteins (G proteins) the subset of inhibitory G proteins (G_i) that have decreased expression in adenomatous thyroid glands obtained from hyperthyroid cats.

Sample Population—Adenomatous thyroid glands obtained from 5 hyperthyroid cats and normal thyroid glands obtained from 3 age-matched euthyroid cats.

Procedure—Expression of G_{i1}, G_{i2}, and G_{i3} in enriched membrane preparations from thyroid glands was quantified by use of immunoblotting with G_i subtype-specific antibodies.

Results—Expression of G_{i2} was significantly decreased in tissues of hyperthyroid glands, compared with expression in normal thyroid tissue. Expression of G_{i1} and G_{i3} was not significantly different between normal thyroid tissues and tissues from hyperthyroid glands.

Conclusions and Clinical Relevance—A decrease in G_{i2} expression decreases inhibition of adenylyl cyclase and allows a relative increase in stimulatory G protein expression. This results in increased amounts of cAMP and subsequent unregulated mitogenesis and hormone production in hyperthyroid cells. Decreased G_{i2} expression may explain excessive growth and function of the thyroid gland in cats with hyperthyroidism. (*Am J Vet Res* 2005;66:1478–1482)

Hyperthyroidism is a common endocrinopathy among cats that is evident most often in middle-aged to older cats. Although clinical signs of and diagnostic tests for the disease have been described, the cause is unknown. This is particularly intriguing because the disease appears to be seen with increasing frequency since its initial description in 1979.[1-4] In most cases, hyperthyroidism in cats results from benign adenomatous hyperplasia of the thyroid gland with single adenomas accounting for a smaller portion of the disease.[5] Studies[6-9] indicate that the disease is at the level of the thyroid gland with hyperthyroid cells of cats functioning autonomously to cause unregulated cell growth and hormone secretion. Although nutritional and environmental factors have been implicated in the cause of the disease, no distinct factor has been identified that causes thyroid cells to become autofunctional.[4,10-12]

Synthesis and secretion of thyroid gland hormones are directly regulated by **thyroid-stimulating hormone (TSH)**, which 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 **guanosine triphosphate–binding proteins (G proteins)** that control cAMP concentrations in the thyroid cells. Activation of this signal transduction system and subsequent increases in intracellular cAMP concentrations result in growth and differentiation of thyroid cells and subsequent secretion of thyroid gland hormones.[13-15] Thus, abnormalities of any part of the receptor–G protein–cAMP signal transduction system could potentially result in unregulated growth of thyroid cells and excessive hormone production seen in cats with hyperthyroidism.

Receptor-coupled heterotrimeric G proteins consist of α, β, and γ subunits that form separate signaling entities when activated by their coupled receptors.[16,17] The G proteins are categorized into 4 major families on the basis of differences in the α subunit.[18] The **stimulatory G protein (G_s)** and **inhibitory G protein (G_i)** families control intracellular cAMP concentrations, with G_{sa} causing activation of adenylyl cyclase and increases of cAMP concentrations and G_{ia} resulting in inhibition of adenylyl cyclase and decreases in intracellular cAMP concentrations. Therefore, the relative expression or activation of G_s and G_i in each thyroid cell ultimately determines the intracellular cAMP concentration that directly stimulates cell growth and proliferation as well as hormone production.[19] For example, when the ratio for expression of $G_{s\alpha}$ to expression of $G_{i\alpha}$ is higher in a disease state than the ratio typically found in normal tissue, the cAMP concentrations may be increased in an unregulated manner. The G_i family of G proteins contains 3 G_i subtypes (G_{i1}, G_{i2}, and G_{i3}).[18] Although closely related in structure, these subtypes can couple selectively to different receptors and activate specific intracellular signal transduction proteins.[20] In humans, the thyrotropin receptor couples to G_s as well as to all 3 G_i subtypes.[21]

Hyperthyroidism in cats is clinically and histopathologically similar to a hyperthyroid disease in humans (ie, toxic nodular goiter). Abnormalities of the G protein and cAMP-signaling pathway have been implicated in the pathogenesis of human toxic nodular goiter. Gain-of-function mutations of $G_{s\alpha}$ and the TSH

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From the Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104-6010 (Ward, Achenbach, Drobatz, Holt); and the Caspary Research Institute, Animal Medical Center, 510 E 62nd St, New York, NY 10021 (Peterson). Dr. Ward's present address is the Department of Small Animal Medicine and Surgery, College of Veterinary Medicine, University of Georgia, Athens, GA 30602.
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Address correspondence to Dr. Ward.

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receptor have been described[22-29] and result in unregulated growth and activation of affected thyrocytes. Altered expression of $G_{s\alpha}$ and $G_{i\alpha}$ has also been detected in human patients with toxic nodular goiter.[30,31] Similar mechanisms appear to be involved in the pathogenesis of hyperthyroidism in cats. In another study[32] conducted by our laboratory group, we documented a specific decrease of G_i expression in membranes isolated from hyperthyroid cells. To further define signaling abnormalities in hyperthyroid cells, the study reported here was conducted in an attempt to identify the G_i subtype or subtypes that have decreased expression in thyroid gland tissues of hyperthyroid cats.

Materials and Methods

Sample population—Thyroid gland tissue was surgically removed from 5 hyperthyroid cats and obtained immediately after euthanasia from 3 aged-matched euthyroid cats that were euthanatized for reasons unrelated to thyroid gland disorders. Medical examinations of hyperthyroid cats were performed by a board-certified specialist in veterinary internal medicine (CRW), and a diagnosis of hyperthyroidism was determined on the basis of appropriate clinical signs and high serum **thyroxine (T_4)** concentration. Histologic analysis of thyroid gland tissue was used to confirm the diagnosis of hyperthyroidism. All hyperthyroid cats in the study had disease in both lobes of the thyroid gland. Euthyroid cats did not have clinical signs of hyperthyroidism, had serum T_4 concentrations within the reference range, had normal thyroid gland tissue on histologic analysis, and did not have evidence of systemic disease. All cats used in the study were client-owned animals; written consent was obtained from all owners to permit use of their cats in the study. Conduct of the study was approved by an institutional animal care and use committee.

Tissue preparation—Immediately after they were collected, thyroid gland tissues were snap-frozen in liquid nitrogen and stored at –80°C until use. Minced thyroid gland tissue was placed in ice-cold buffer containing 50mM Tris, 1mM EDTA, and 250mM sucrose (pH, 7.4) and then homogenized. Samples were centrifuged (2,500 $\times$ *g* for 10 minutes at 4°C), and the resultant supernatant was collected and centrifuged (30,000 $\times$ *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 by use of a protein assay[a] in which bovine serum albumin was used as the standard.

Western immunoblot analysis—Equivalent amounts of protein (30 to 40 µg) from the enriched thyroid membranes were subjected to SDS-PAGE on a 12.5% gel by use of reducing conditions.[33] Proteins were then transferred to a polyvinyl difluoride membrane[b] by use of 30 V (constant voltage) for 18 hours at 4°C.[34] The resulting western blots were blocked by incubation for 1 hour (with agitation) in a buffer containing **250mM Tris, 1.5M NaCl, and 0.1% polyoxyethyene-sorbitan monolaurate** (**[TTBS]**; pH, 7.4) into which 5% teleost gelatin was added. Immunoblots were then incubated for 1 hour with nonimmune rabbit serum (0.2 g/mL) as negative control samples or with specific rabbit antipeptide antibodies[c] directed against unique sequences of all $G_{i\alpha}$ ($G_{i\alpha 1-3}$; antiserum 8730)[35] or against each specific subtype ($G_{i\alpha 1}$ [antiserum 3646],[36] $G_{i\alpha 2}$ [antiserum 1521],[35] and $G_{i\alpha 3}$ [antiserum 1518][36]) at a dilution of 1:500. These antibodies exclusively recognize the $G_{i\alpha}$ subunit against which they were raised.[35,36] Immunoblots were rinsed for 10 minutes in TTBS, followed by incubation for 35 minutes with horseradish peroxidase–linked anti-rabbit IgG diluted 1:7,500 in TTBS. Immunoblots then were rinsed for 2 hours in TTBS with at least 5 buffer changes, after which they were developed by use of a chemiluminescence kit[d] used in accordance with the manufacturer's directions.

Quantification of the chemiluminescence signal was performed by use of densitometry. Blots were scanned in a multiple-image light cabinet[e] and analyzed by use of densitometry software.[f] Each blot was scanned once. Identically sized areas of immunoreactivity were compared between the euthyroid and hyperthyroid samples. Values were expressed as densitometry of immunoreactivity in the hyperthyroid tissue divided by densitometry of immunoreactivity in the euthyroid tissue.

Statistical analysis—Values were expressed as median, minimum, and maximum. Each value was compared with its own age-matched control sample on the same immunoblot; the control sample was assigned an arbitrary value of 1. The Wilcoxon signed rank test was used for statistical comparisons. Values of $P < 0.05$ were considered significant.

Results

To determine $G_{i\alpha}$ expression at the protein level, western immunoblots of membrane-enriched thyroid gland preparations from age-matched euthyroid and hyperthyroid cats were probed by use of nonimmune rabbit serum, antipeptide antibodies that recognized all $G_{i\alpha}$ subtypes (ie, $G_{i\alpha 1-3}$), and antipeptide antibodies that specifically recognized $G_{i\alpha 1}$, $G_{i\alpha 2}$, or $G_{i\alpha 3}$ **(Figure 1)**. Immunoreactive bands at 40 kd corresponded to the molecular weights of $G_{i\alpha 1}$, $G_{i\alpha 2}$, and $G_{i\alpha 3}$. There was a decrease in binding of the common antibody for all subtypes (ie, $G_{i\alpha 1-3}$) in membranes from hyperthyroid tissues, compared with binding in membranes from euthyroid tissues. Examination of the specific subtypes revealed that $G_{i\alpha 1}$ and $G_{i\alpha 3}$ had similar antibody binding, whereas $G_{i\alpha 2}$ had a decrease in antibody binding in the hyperthyroid membranes. We did not detect immunoreactive bands in the 40-kd region in the lanes probed by use of nonimmune serum (data not shown).

Densitometry was performed to quantify antibody binding and, hence, protein expression on the western immunoblots **(Figure 2)**. Membranes from hyperthyroid tissue had a significant ($P = 0.043$) median decrease of 48% (minimum, 41%; maximum, 65%) in expression for all $G_{i\alpha}$ subtypes (ie, $G_{i\alpha 1-3}$). However,

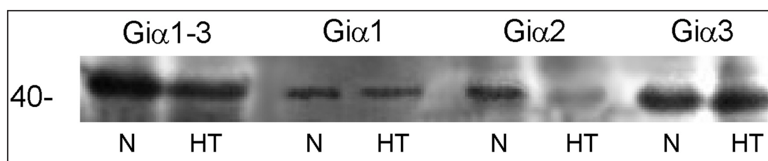

Figure 1—Western immunoblot revealing immunologic detection of the guanosine triphosphate-binding proteins (G proteins) for all subtypes of inhibitory G protein (Gi) α subtypes ($G_{i\alpha 1-3}$) and each specific subtype ($G_{i\alpha 1}$, $G_{i\alpha 2}$, and $G_{i\alpha 3}$) in normal thyroid gland tissues obtained from euthyroid cats (N) and adenomatous thyroid gland tissues obtained from hyperthyroid cats (HT). Membrane-enriched thyroid gland preparations (25 mg) were evaluated by use of SDS-PAGE. Samples were probed with antibodies that recognized all subtypes (ie, $G_{i\alpha 1-3}$) or were specific for each subtype (ie, $G_{i\alpha 1}$, $G_{i\alpha 2}$, and $G_{i\alpha 3}$), and gels were developed by use of enhanced chemiluminescence autoradiography. The number on the left side of the figure represents a molecular weight marker (in kilodaltons)

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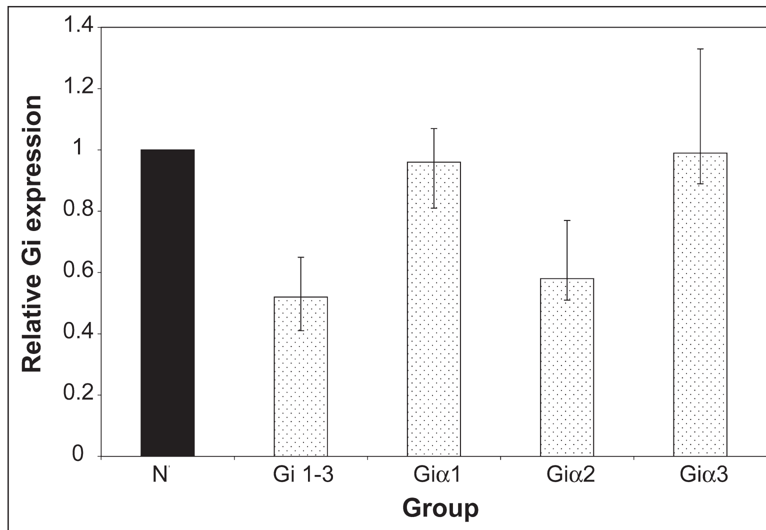

Figure 2—Quantification of expression of $G_{i\alpha}$ in normal thyroid gland tissues obtained from euthyroid cats (N; black bar) and adenomatous thyroid gland tissues obtained from hyperthyroid cats (HT; dotted bars). Densitometry was performed on immunoblots that compared expression of $G_{i\alpha}$ for the HT group with expression of $G_{i\alpha}$ to its own age-matched N group on the same blot. Expression for the N group was assigned an arbitrary value of 1. Results represent the median, minimum, and maximum for 5 separate experiments.

relative protein expression differed among the specific $G_{i\alpha}$ subtypes. Protein expression of $G_{i\alpha1}$ in hyperthyroid membranes had a median decrease of 3.9% (minimum, 0.81%; maximum, 1.106%) from that for euthyroid membranes; however, this decrease was not significantly (*P* = 0.50) different. Similarly, protein expression of G_{ia3} did not differ significantly (*P* = 0.68) between the euthyroid and hyperthyroid membranes (median decrease for membranes from both groups, 1.3%; minimum, 0.92%; maximum, 1.26%). However, protein expression of $G_{i\alpha2}$ in hyperthyroid membranes had a significant (*P* = 0.043) median decrease of 55% (minimum, 48%; maximum, 73%), compared with results for euthyroid membranes.

Discussion

Results for the study reported here are in agreement with results of another study[32] conducted by our laboratory group in that $G_{i\alpha}$ expression was decreased in hyperthyroid cells, compared with expression in normal thyroid cells. In the study reported here, we were able to document that the decrease in $G_{i\alpha}$ expression was attributable exclusively to a decrease in the expression of the $G_{i\alpha2}$ subtype.

We postulate that the decrease of $G_{i\alpha2}$ expression changes the ratio for the expression of $G_{i\alpha}$ to expression of $G_{s\alpha}$ in thyroid cells, which results in a relative overactivity of $G_{s\alpha}$. Subtypes $G_{i\alpha}$ and $G_{s\alpha}$ regulate intracellular cAMP concentrations by mediating the activation and inhibition, respectively, of separate isoforms of adenylyl cyclase.[19,37] Thus, an imbalance of the ratio for expression of $G_{s\alpha}$ to expression of $G_{i\alpha}$ in favor of $G_{s\alpha}$ expression may result in relative overactivity of adenylyl cyclase and excess production of cAMP, which result in unregulated growth of the thyroid gland and excessive hormone production characteristic of hyperthyroidism in cats. We did not examine adenylyl cyclase concentrations or $G_{s\alpha}$ activity in this study; therefore, this proposed mechanism is only for contemplation, and additional studies need to be conducted to test its validity.

On activation by a specific ligand, most G protein–coupled receptors can activate only a limited set of G protein classes that control downstream signaling activity. The TSH receptor is a relatively promiscuous receptor and couples to many classes of G proteins.[21] We chose to examine the G_s and G_i families of G proteins because they directly control cAMP production that leads to hormone production and mitogenesis. The TSH receptor activates all subtypes of $G_{i\alpha}$ after binding to the TSH ligand. The fact that only the $G_{i\alpha2}$ subtype had decreased expression in hyperthyroid cells indicated that there may have been factors causing this specific effect on the thyroid gland cells obtained from these cats, such that only $G_{i\alpha2}$ was affected or involved. Factors that may have caused this effect are unknown but could be contaminants of food or the environment.

Tissues used in this study to compare expression of G proteins in euthyroid and hyperthyroid cats were matched on the basis of age such that the age of a control cat was within 1 year of the age of the hyperthyroid cat to which it was matched. To our knowledge, no studies have directly examined expression of $G_{i\alpha}$ isoforms in the thyroid gland, but age-related changes in the expression of specific G_{α} subunits have been reported in the brain of rats,[38] neural cell lines,[39] the brain of humans,[40,41] and the aorta of rats.[42] Moreover, marked age-related changes in signal transduction for β-adrenergic receptors have also been described[43] in peripheral cells obtained from humans. Therefore, to remove potential variability attributable to age and not to the disease state, we only compared tissues obtained from cats of similar age.

Most cases of toxic nodular goiter in humans in which a cause can be determined are the result of mutations in the TSH receptor, $G_{s\alpha}$, or both.[22,23,26,29] These mutations cause constitutive activation of the affected protein such that there is hormone production and mitogenesis without TSH regulation. In 21 cats with hyperthyroidism, investigators did not detect mutations in the TSH receptor.[44,45] In a study[45] conducted in Europe, polymorphisms in the feline $G_{s\alpha}$ gene detected in 4 of 10 hyperthyroid cats may have been associated with hyperthyroidism. These mutations were in areas similar to those that have been documented to result in constitutive activation of $G_{s\alpha}$ in humans; however, no activation studies have been performed in cells from hyperthyroid cats. Because of the high incidence of mutations in the TSH receptor and $G_{s\alpha}$ in humans with toxic nodular goiter, clonal expansion of mutated cells resulting in adenomas is a common pathologic change of this disease.[46] Most cases of hyperthyroidism in cats do not result from single adenomas in the thyroid gland; instead, they are the result

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of adenomatous hyperplasia in both lobes of the thyroid gland. However, clonality of these hyperplastic cells has not been determined. Therefore, it is unexpected that mutations would play a large part in the pathogenesis of the disease in cats. However, there may be regional differences in the pathologic characteristics of the disease such that adenomas may be more common in cats in certain areas of the world.

Analysis of data for the study reported here suggested that a decrease in $G_{i\alpha 2}$ expression may have played a part in the pathogenesis of hyperthyroidism in cats. This decreased expression allowed for unregulated growth and function of the affected thyroid cells, and we postulate that the affected cells will take over the normal thyroid gland, resulting in the enlarged hyperplastic glands found during histologic examination. Additional studies may reveal the specific factor or factors responsible for the decrease in this signal transduction protein. Such information will help us better understand the pathogenesis of hyperthyroidism in cats.

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a. Micro BCA Assay, Pierce Chemical Co, Rockford, Ill.
b. Immobilon-P, Millipore, Bedford, Mass.
c. Provided by Dr. David Manning, Department of Pharmacology, School of Medicine, University of Pennsylvania, Philadelphia, Pa.
d. Renaissance, NEN Life Science Products, Boston, Mass.
e. Multi-Cabinet, Alpha Innotech Corp, San Leandro, Calif.
f. AlphaImager 2000, version 4.0, Alpha Innotech Corp, San Leandro, Calif.

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