Assessment of calcitonin response to experimentally induced hypercalcemia in cats

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Objective—To characterize the dynamics of calcitonin secretion in response to experimentally induced hypercalcemia in cats.

Animals—13 healthy adult European Shorthair cats.

Procedures—For each cat, the calcitonin response to hypercalcemia (defined as an increase in ionized calcium concentration > 0.3mM) was investigated by infusing calcium chloride solution and measuring circulating calcitonin concentrations before infusion (baseline) and at various ionized calcium concentrations. Calcitonin expression in the thyroid glands of 10 of the cats was investigated by immunohistochemical analysis.

Results—Preinfusion baseline plasma calcitonin concentrations were very low in many cats, sometimes less than the limit of detection of the assay. Cats had a heterogeneous calcitonin response to hypercalcemia. Calcitonin concentrations only increased in response to hypercalcemia in 6 of 13 cats; in those cats, the increase in calcitonin concentration was quite variable. In cats that responded to hypercalcemia, calcitonin concentration increased from 1.3 ± 0.3 pg/mL at baseline ionized calcium concentration to a maximum of 21.2 ± 8.4 pg/mL at an ionized calcium concentration of 1.60mM. Cats that did not respond to hypercalcemia had a flat calcitonin-to-ionized calcium concentration curve that was not modified by changes in ionized calcium concentration. A significant strong correlation (r = 0.813) was found between the number of calcitonin-positive cells in the thyroid gland and plasma calcitonin concentrations during hypercalcemia.

Conclusions and Clinical Relevance—Healthy cats had very low baseline plasma calcitonin concentrations. A heterogeneous increase in plasma calcitonin concentration in response to hypercalcemia, which correlated with the expression of calcitonin-producing cells in the thyroid, was identified in cats. (Am J Vet Res 2013;74:1514–1521)

Calcitonin participates in the control of extracellular calcium concentrations. In plasma, calcium is found in 3 fractions: protein-bound calcium, complexed calcium, and ionized calcium. The stimulation of the calcium-sensing receptor located in thyroid gland C cells by ionized calcium promotes calcitonin secretion. Calcitonin inhibits osteoclastic bone resorption, has some positive influence on renal calcium excretion, and in the long term, may impair intestinal calcium absorption. Although the hypocalcemic role of calcitonin is very consistent in the species in which it has been studied, the relative importance of this hormone in calcium metabolism seems to be species specific. Thus, although calcitonin is very important in the regulation of mineral metabolism in some species (eg, rats), calcitonin seems to have a minor role in others (eg, humans).

Information about calcitonin in domestic animals is fragmentary. A specific assay for quantification of canine calcitonin has been described, and measurement of plasma calcitonin concentrations in horses has been recently reported, but no similar data are available for cats, to our knowledge.

The relationship between circulating calcitonin and ionized calcium concentrations can be studied through creation of a calcitonin-to-ionized calcium concentration curve, which describes the response of calcitonin concentration to changes in extracellular ionized calcium concentration. Calcitonin-to-ionized calcium concentration curves have been studied in clinically normal and uremic rats and in humans with chronic renal failure. For both species, a sigmoidal calcitonin-to-ionized calcium concentration curve has been reported. Among domestic animals, increases in circulating calcitonin concentration secondary to acute increases in ionized calcium concentration in dogs and horses have been reported. Nevertheless, no information is available regarding the dynamics of calcitonin secretion in response to changes in extracellular ionized calcium concentration in cats. The purpose of the
study reported here was to characterize the dynamics of calcitonin secretion in response to experimentally induced hypercalcemia and the related changes in extracellular ionized calcium concentration in cats.

Materials and Methods

Animals—Thirteen European Shorthair cats of both sexes (6 males and 7 females), 16 to 18 months of age, that weighed 3.8 ± 0.3 kg were included in the study. Cats were randomly chosen from 5 litters born in a research colony. Cats were kept in a cattery belonging to the Animal House Faculty of the University of Cordoba and were fed a diet containing calcium (1.1%), phosphorus (1%), and vitamin D (1,500 U/kg). Cats were considered healthy on the basis of physical examination findings and results of hematologic assessment and plasma biochemical profile. In addition, blood samples were obtained from all cats to evaluate mineral metabolism and thyroid gland function by measuring circulating concentrations of calcium (total and ionized), phosphorus, parathyroid hormone, 25-hydroxyvitamin D (calcidiol), 1,25-dihydroxyvitamin D (calcitriol), and free thyroxine. To study the response to hypercalcemia (defined as an increase [from the preinfusion baseline value] in whole blood ionized calcium concentration > 0.3mM), cats were anesthetized with a combination of ketamine hydrochloride (15 mg/kg, IM) and midazolam (0.4 mg/kg, IM). Once the experiments were finished, some cats were used for another study (an acute surgical procedure) that required euthanasia. Samples of thyroid gland tissue were obtained after death from 10 of the cats in which the dynamics of calcitonin secretion had been studied. All experimental procedures were approved by the Ethics Committee of the University of Cordoba.

Assessment of calcitonin response to ionized hypercalcemia—For each cat, the calcitonin-to-ionized calcium concentration curve was obtained following IV infusion of calcium chloride solution. A jugular vein and the contralateral cephalic vein were cannulated with 18- and 20-gauge catheters, respectively. The cephalic venous catheter was used for calcium chloride infusion, and the jugular venous catheter was used for blood sample collection. Hypercalcemia was achieved by IV infusion of calcium chloride solution, started at 0 minutes, at a rate of 0.27 mEq of calcium/kg/h. Infusion of calcium chloride was increased every 5 minutes up to a final rate of 0.55 mEq of calcium/kg/h after 50 minutes. Before initiation of the calcium chloride infusion, 3 blood samples (1 mL/sample) were obtained from each cat to provide baseline data (ie, mean values of whole blood ionized calcium and plasma calcitonin concentrations); thereafter, 10 blood samples (1 mL/sample) were obtained from each cat. Each one of the 10 samples was collected every 5 minutes until the end of the experiments (at 50 minutes). This protocol was extrapolated from a previous study of calcitonin-to-ionized calcium concentration curves in other species and from our investigation of parathyroid hormone-to-ionized calcium concentration curves in cats.

Individual calcitonin-to-ionized calcium concentration curves were constructed by adjusting the horizontal and vertical ionized calcium concentration

Plasma concentrations of 25-hydroxyvitamin D (calcidiol) and 1,25-dihydroxyvitamin D (calcitriol)
were measured with radioimmunoassays that have been validated for cats. Plasma parathyroid hormone concentration was measured with an immunoradiometric assay designed for the quantitative determination of human whole parathyroid hormone and intact parathyroid hormone concentrations validated by our laboratory for measurement of feline parathyroid hormone concentration. Plasma free thyroxine concentration was measured by radioimmunoassay.

Histologic and immunohistochemical evaluation—Following euthanasia by an IV overdose of barbiturate solution, thyroid glands from 10 of the 13 cats were collected and fixed in neutral-buffered 10% formalin. Formalin-fixed samples were embedded in paraffin, sectioned at 5 μm, and stained with H&E stain.

For immunohistochemical evaluation by the avidin-biotin-peroxidase complex method, tissue sections were dewaxed and rehydrated. Endogenous peroxidase activity was exhausted by incubation of the sections with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature (approx 25°C). The antigen retrieval method used was microwave heating in 0.01M citrate buffer (pH 6). Sections were incubated at 4°C overnight (approx 18 hours) in a humid chamber with the primary polyclonal rabbit antihuman calcitonin antibody diluted 1:200. This antibody has been used successfully to detect calcitonin in other animal species. After primary incubation, slides were washed in PBS solution and incubated with a biotinylated secondary antibody, diluted 1:200, for 1 hour at room temperature. After washes in PBS solution, samples were incubated with the avidin-biotin-peroxidase complex for 1 hour at room temperature. All tissue sections were finally rinsed in PBS solution, incubated for 1 minute with chromogen solution, and counterstained with hematoxylin. Cells that reacted were counted by 2 independent investigators (CP and AIR) who were masked to details about the experimental group from which the sample was obtained. Positive cells in 40 nonoverlapping fields of 0.20 mm² chosen randomly in 4 diagonally positioned squares were counted. The mean number of positive cells per field was also calculated. For negative controls, nonimmune serum was used in place of primary antibody.

Statistical analysis—Statistical analysis was performed with statistical software. Variables were normally distributed. For cats with plasma calcitonin concentrations below the limit of assay detection, a value of 0.9 pg/mL was assigned and used for statistical analysis. Plasma calcitonin concentrations at various ionized calcium concentrations were compared with hormonal concentrations at baseline ionized calcium concentration by means of paired t tests. Comparisons between subgroups of cats with different calcitonin responses to hypercalcemia (ie, responders vs nonresponders) were made by means of unpaired t tests. The Pearson test was used to determine correlation. Values of P < 0.05 were considered significant.

Results
Baseline plasma calcitonin concentrations—Measurement of plasma calcitonin concentration in baseline samples ranged from less than the limit of detection (< 0.9 pg/mL) to 3.2 pg/mL. For the 11 cats with calcitonin concentrations below the limit of assay detection, a value of 0.9 pg/mL was assigned and used for statistical analysis. Among the 13 cats, the mean ± SE whole blood ionized calcium concentration was 1.20 ± 0.01 mM.

Figure 1—Mean ± SE whole blood ionized calcium concentration (circles) and plasma calcitonin concentration (squares) in 13 cats that received an IV infusion of calcium chloride (started at 0 minutes) at a rate of 0.27 mEq of calcium/kg/h, which was then increased every 5 minutes up to a final rate of 0.55 mEq of calcium/kg/h after 50 minutes, to induce hypercalcemia (defined as an increase in whole blood ionized calcium concentration to > 0.3mM). Cats were assigned to 1 of 2 subgroups on the basis of whether their plasma calcitonin concentration did (responders n = 6; black symbols) or did not (nonresponders 7; white symbols) increase in response to hypercalcemia.

Figure 2—Calcitonin-to-ionized calcium concentration curve obtained after induction of hypercalcemia in the 13 cats in Figure 1. Plasma calcitonin concentration increased in response to the increasing severity of hypercalcemia in only 6 cats (responders [black circles]) and did not change in the remaining 7 cats (nonresponders [white circles]). *Value is significantly (P < 0.05) different from the plasma calcitonin concentration determined at the baseline whole blood ionized calcium concentration (ie, mean of the value determined from 3 samples obtained before infusion).
Plasma calcitonin concentrations during hypercalcemia—Plasma calcitonin concentrations were plotted against time (Figure 1) and against whole blood ionized calcium concentration (Figure 2). Examination of the calcitonin-to-ionized calcium concentration curve for individual cats revealed that the calcitonin response to hypercalcemia was varied. Plasma calcitonin concentration only increased in response to hypercalcemia in 6 of the 13 cats. In cats that responded to hypercalcemia (responders), basal calcitonin concentration increased from 1.3 ± 0.3 pg/mL (range, 0.9 to 3.2 pg/mL) at baseline ionized calcium concentration to a maximum calcitonin concentration of 21.2 ± 8.4 pg/mL (range, 8.0 to 43.5 pg/mL) at an ionized calcium concentration of 1.60 mM. Nonresponders had a flat calcitonin-to-ionized calcium concentration curve that was not modified by changes in ionized calcium concentration. It is also interesting to note that a heterogeneous calcitonin response was observed in the subgroup of responders, with the maximum calcitonin concentration ranging from 8.0 to 43.5 pg/mL (Figure 3). Variables derived from the calcitonin-to-ionized calcium concentration curve were calculated on the basis of data from responders. The ratio of basal to maximum calcitonin concentration was 3.20 ± 1.45% (range, 0.02% to 9.09%), and the set point of the calcitonin-to-ionized calcium concentration curve was 1.46 ± 0.02 mM (range, 1.40 to 1.52 mM). A good correlation between ionized calcium and calcitonin concentrations (r = 0.576; P < 0.001) was found when the values obtained in normo- and hypercalcemic responders were pooled together.

To confirm that the lack of calcitonin response to hypercalcemia in some cats was not an erroneous finding, repeated calcitonin-to-ionized calcium concentration curves were determined for 6 cats (2 responders and 4 nonresponders). The calcitonin responses to hypercalcemia in the repeated experiments were consistent with the previous findings in each subgroup of cats.
(nonresponders) increase in response to hypercalcemia. Their plasma calcitonin concentration did (responders) or did not increase in whole blood ionized calcium concentration every 5 minutes up to a final rate of 0.55 mEq of calcium/kg/h. The reason for this was unclear. It may have been similar to the results reported by Titlbach et al.12 Interestingly, calcitonin-positive cells were identified in thyroid gland tissue from responders and nonresponders. However, both the total number of calcitonin-positive C cells and mean number of calcitonin-positive C cells per field were significantly (P = 0.03) higher in responders (342.8 ± 62.9 cells and 8.6 ± 1.6 cells/field, respectively) than in nonresponders (126.5 ± 31.1 cells and 3.2 ± 0.8 cells/field, respectively). In addition, a significant correlation (r = 0.813; P = 0.004) was found between maximum calcitonin concentration and both the total number of calcitonin-positive C cells and mean number of calcitonin-positive C cells per field.

**Discussion**

Abnormalities of ionized calcium metabolism (eg, hypercalcemia of malignancy and hypo- or hypercalcemia associated with renal failure and nutritional disorders) are common in cats.13,14 Moreover, cats can develop derangements of ionized calcium metabolism, such as idiopathic hypercalcemia, with unknown etiopathogenesis.15 To have a comprehensive understanding of these disorders, it is important to know the dynamics of secretion of calciotropic hormones. To our knowledge, this report is the first to describe in detail the response of calcitonin to experimentally induced hypercalcemia in cats.

In cats as well as in most domestic animals, endocrine studies are often complicated by the lack of assays with specific antibodies. Even though this dearth may be regarded as an important problem for quantification of calciotropic hormones in animals, it should be noted that calcitonin molecules are quite similar among different species.16 Sequencing of calcitonin has revealed homology ranging from 53% to 90% among the mammals (humans, rats, dogs, and horses) in which it has been studied.3,16,17 Thus, heterologous assays (ie, assays incorporating antibodies against the human calcitonin molecule) can be used to reliably measure calcitonin concentration in some other mammals. Recently, the usefulness of a human calcitonin assay for quantification of equine calcitonin concentration has been demonstrated.8

Very little information is available regarding plasma calcitonin concentrations in domestic animals, and to our knowledge, calcitonin concentrations in cats have not been reported. Our results show very low

<table>
<thead>
<tr>
<th>Variable</th>
<th>Responders (n = 6)</th>
<th>Nonresponders (n = 7)</th>
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<tbody>
<tr>
<td>Initial ionized calcium (mM)</td>
<td>1.20 ± 0.03</td>
<td>1.19 ± 0.01</td>
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<tr>
<td>Final ionized calcium (mM)</td>
<td>1.58 ± 0.04</td>
<td>1.58 ± 0.02</td>
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<tr>
<td>Difference (increase) in ionized calcium* (mM)</td>
<td>0.38 ± 0.03</td>
<td>0.39 ± 0.03</td>
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<tr>
<td>Initial intact parathyroid hormone (pg/mL)</td>
<td>10.4 ± 1.6</td>
<td>11.2 ± 2.5</td>
</tr>
<tr>
<td>Final intact parathyroid hormone (pg/mL)</td>
<td>5.9 ± 0.5</td>
<td>5.8 ± 0.5</td>
</tr>
<tr>
<td>Difference (decrease) in intact parathyroid hormone (pg/mL)</td>
<td>4.5 ± 1.5</td>
<td>5.4 ± 2.5</td>
</tr>
<tr>
<td>Initial whole parathyroid hormone (pg/mL)</td>
<td>15.5 ± 3.7</td>
<td>16.5 ± 5.8</td>
</tr>
<tr>
<td>Final whole parathyroid hormone (pg/mL)</td>
<td>4.9 ± 0.5</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>Difference (decrease) in whole parathyroid hormone (pg/mL)</td>
<td>10.6 ± 3.4</td>
<td>12.2 ± 5.8</td>
</tr>
<tr>
<td>25-hydroxyvitamin D (ng/mL)</td>
<td>61.3 ± 8.0</td>
<td>58.0 ± 13.1</td>
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<tr>
<td>1,25-dihydroxyvitamin D (pg/mL)</td>
<td>117.1 ± 15.9</td>
<td>111.0 ± 12.4</td>
</tr>
<tr>
<td>Free thyroxine (ng/dL)</td>
<td>1.09 ± 0.10</td>
<td>1.12 ± 0.12</td>
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Concentrations of parathyroid hormone (intact and whole), 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, and free thyroxine were measured in plasma samples; ionized calcium concentrations were measured in whole blood samples.

*The differences in ionized calcium concentration were calculated as final concentration minus initial concentration for each subgroup. The differences in parathyroid hormone concentration were calculated as initial concentration minus final concentration for each subgroup.
calcitonin concentrations in clinically normal cats. Of note, it is not unusual to find low calcitonin concentrations in other species. In fact, healthy humans may have basal calcitonin concentrations similar to what we have found in cats (0.9 to 3.2 pg/mL) and nearly undetectable calcitonin concentrations are considered normal in humans.18

In domestic animals, the calcitonin response to changes in ionized calcium concentration has been studied in dogs and horses that received an IV bolus of calcium.3,4 Basal circulating calcitonin and maximum calcitonin concentrations seem to be higher both in dogs3 and horses4 than in cats. Although previous studies3,4 provide evidence of calcitonin response to changes in circulating calcium concentration in domestic animals, those data do not allow a thorough evaluation of the calcitonin-to-ionized calcium concentration curve. Thus, we had to compare results of the present study with data from rats, the only animal species for which a detailed calcitonin-to-ionized calcium concentration curve has been described.5–7 The shape and the setpoint of the calcitonin-to-ionized calcium concentration curves for cats and rats are similar. However, basal calcitonin and maximum calcitonin concentration are much higher in rats than in cats. In addition, the heterogeneity in the calcitonin response to hypercalcemia has not been reported in other mammals, to our knowledge. The importance of calcitonin in the control of mineral metabolism is quite different between species. In rats, calcitonin seems to be very important for avoiding development of hypercalcemia. Results of 1 study7 have indicated a tendency for rats to become hypercalcemic after thyroparathyroidectomy or after selective thyroidecomy. By contrast, in thyroidectomized cats, hypocalcemia is reportedly the main complication; hypocalcemia that develops in some cats after total thyroidectomy is assumed to be related to damage to the parathyroid glands.19 Nonetheless, the fact that the ablation of thyroid gland tissue does not result in hypercalcemia suggests that calcitonin has less influence than parathyroid hormone on ionized calcium metabolism in cats. Taken together, the results of the present study (low baseline calcitonin concentration plus moderate and heterogeneous response to hypercalcemia) would support the contention that, in contrast to the case in rats, calcitonin seems to have a secondary role in calcium homeostasis in cats.
One of the more interesting findings of the present study was the fact that some cats did not have an increase in plasma calcitonin concentration in response to hypercalcemia. This surprising discovery was confirmed through repeated experiments. Given that plasma calcitonin concentrations were not increased at any time in nonresponders, the differences between the responders and nonresponders should be at the level of secretion rather than at the level of clearance. No significant differences were found in baseline calcitonin concentration between responders and nonresponders; however, considering that baseline calcitonin was very low in both subgroups, it would be unlikely to find such differences. When analyzing the ionized calcium concentration changes as a result of the calcium chloride infusion, it was interesting to note that there was no difference between the 2 subgroups of cats. The rate of calcium change, which has been shown to influence calcitonin secretion,21 was almost identical in both responders and nonresponders (Figure 1). The mean ± SE difference in ionized calcium concentration (final concentration minus initial concentration) was 0.38 ± 0.03mM in responders and 0.39 ± 0.03mM in nonresponders. Thus, the absence of calcitonin secretion did not result in a more profound hypercalcemia in nonresponders. To determine whether the lack of calcitonin would be compensated by changes in parathyroid hormone concentration, final parathyroid hormone concentrations at the end of hypercalcemia and the difference in parathyroid hormone concentration (initial concentration minus final concentration) in both subgroups were compared. Again, no significant difference in either variable was found between responders and nonresponders on the basis of data obtained with intact or whole parathyroid hormone assays. Also, no differences in vitamin D status or in thyroid gland function or whole parathyroid hormone assays. Also, no differences in vitamin D status or in thyroid gland function were found between the 2 subgroups of cats. Immuno-histochemical analysis of thyroid gland tissues revealed that the density of cells expressing calcitonin was higher in responders than in nonresponders. Thus, a histologic basis to explain the disparate response to hypercalcemia between the 2 subgroups of cats was found.

In humans, gender is known to affect the calcium response of calcitonin. Men have higher basal calcitonin concentrations and a more pronounced calcitonin response to hypercalcemia.20 It is interesting to note that there was no difference between the 2 subgroups of cats. The rate of calcium change, which has been shown to influence calcitonin secretion,21 was almost identical in both responders and nonresponders (Figure 1). The mean ± SE difference in ionized calcium concentration (final concentration minus initial concentration) was 0.38 ± 0.03mM in responders and 0.39 ± 0.03mM in nonresponders. Thus, the absence of calcitonin secretion did not result in a more profound hypercalcemia in nonresponders. To determine whether the lack of calcitonin would be compensated by changes in parathyroid hormone concentration, final parathyroid hormone concentrations at the end of hypercalcemia and the difference in parathyroid hormone concentration (initial concentration minus final concentration) in both subgroups were compared. Again, no significant difference in either variable was found between responders and nonresponders on the basis of data obtained with intact or whole parathyroid hormone assays. Also, no differences in vitamin D status or in thyroid gland function were found between the 2 subgroups of cats. Immuno-histochemical analysis of thyroid gland tissues revealed that the density of cells expressing calcitonin was higher in responders than in nonresponders. Thus, a histologic basis to explain the disparate response to hypercalcemia between the 2 subgroups of cats was found.

The results of the present study indicated that the calcitonin response to hypercalcemia in cats was heterogeneous. Two subgroups of cats were distinguished: cats in which plasma calcitonin concentration increased during hypercalcemia and cats in which calcitonin concentrations remained very low even at very high ionized calcium concentrations (up to 1.60mM) that are consistent with concentrations in cats with clinical hypercalcemia. Although no deleterious effects in the acute response to hypercalcemia were detected in the nonresponders, cats in which plasma calcitonin concentration did not increase could be predisposed to disorders of ionized calcium metabolism. Therefore, further investigation of this topic is warranted. In any case, additional studies exploring calcitonin concentrations in naturally hypercalcemic cats (idiopathic or otherwise) seem necessary.

### References

9. Pineda C, Aguiler-Tejero E, Raya AI, et al. Feline parathyroid...