

Measurement of plasma chromogranin A concentrations for assessment of stress responses in dogs with insulin-induced hypoglycemia

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Objective—To determine whether cross-reactivity exists between canine chromogranin A (CgA) and anti-human CgA antibody and investigate the usefulness of plasma CgA concentration measurements as an index of acute stress responses in dogs.

Animals—12 healthy Beagles.

Procedure—Canine CgA was extracted and purified from canine adrenal glands of cadaver dogs for studying cross-reactivity with anti-human CgA antibody. Western blotting with anti-human CgA antibody was performed. Blood samples were collected from dogs at 0, 10, 20, 30, 40, 60, 120, and 180 minutes after IV administration of saline (0.9% NaCl) solution or insulin. Canine plasma CgA concentrations were determined by use of a CgA ELISA kit with rabbit antiserum against the carboxy-terminal fragment of human CgA. Plasma cortisol and catecholamine (ie, norepinephrine and epinephrine) concentrations were measured by use of an ELISA and a high-performance liquid chromatography method, respectively.

Results—Purified canine CgA was specifically detected by use of western blot analysis and an ELISA with anti-human CgA antibody. An increase in plasma CgA concentrations was observed in insulin-induced hypoglycemic dogs. Changes in plasma CgA concentration were correlated with changes in plasma cortisol or catecholamine concentrations of hypoglycemic dogs.

Conclusions and Clinical Relevance—Use of the CgA ELISA kit for determination of human plasma CgA concentrations is applicable to the measurement of canine plasma CgA concentrations. Canine plasma CgA concentrations, along with measurements of plasma cortisol and catecholamine concentrations, correctly reflect insulin-induced hypoglycemic stressed conditions in dogs. Measurement of canine plasma CgA concentrations may provide a useful index for evaluation of an acute stress response. (*Am J Vet Res* 2005;66:1830–1835)

or norepinephrine from chromaffin granules of the adrenal medulla.^{1,2} Chromogranin A is stored in the secretory granules in a wide variety of neuroendocrine tissues and neuroendocrine tumors.^{3,5} Chromogranin A of various animal species shares considerable homology, and mammalian CgA immunologically cross-reacts with CgA of reptiles, amphibians, fish, and *Drosophila* spp.⁶ Although the functions of CgA are still poorly understood, CgA is associated with hormone packaging, stabilization of the granule against osmotic pressure, and excretion of intracellular calcium. Extracellular peptidases cleave CgA into biologically active peptides, which act as regulators of hormone secretion.⁷ Most of the CgA-derived peptides decrease hormone secretion.

The assessment of cortisol and catecholamine concentrations in the circulation is well recognized as a valuable way to investigate stress because these hormones reflect the activity of 2 important stress-responsive axes: the hypothalamic-pituitary-adrenal (HPA) axis and sympathoadrenal-medullary (SAM) axis. Human CgA is used not only as a marker of pheochromocytoma but also as an index of stressed conditions.⁸ Because CgA is stable in the circulatory system, compared with catecholamines, plasma CgA concentration may be a more accurate index of activated SAM system at the initial phase of the stress response. The presence of canine CgA has been immunologically demonstrated in endocrine and endocrine tumor tissues, including pheochromocytoma, by use of human and bovine antibodies against CgA.⁹⁻¹¹ Plasma CgA concentrations in dogs with pancreatic islet cell tumor are increased, as measured by use of a radioimmunoassay with antibody against human CgA.¹² However, it is not yet known whether canine CgA is a useful marker at the initial phase of the stress response. The purpose of the study reported here was to investigate the usefulness of measuring plasma CgA concentrations as an index of an acute stress response in dogs. Because it is not easy to obtain a useful antibody against canine CgA at the present time, attempts were made to study whether the ELISA kit for measurements of human CgA is applicable for measurement of canine plasma CgA concentrations, as some immunologic cross-reaction between human and canine CgA has been found.⁹⁻¹² To study whether CgA is a candidate as an acute-phase marker in the stress response, we also measured plasma CgA concentrations in hypoglycemic dogs that had received an injection of insulin.^{13,14}

Chromogranin A (CgA) belongs to a family of highly acidic proteins and is released with epinephrine

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Materials and Methods

Preparation of canine CgA and human CgA—Canine adrenal glands were collected from dogs undergoing necropsies. The crude 68-kd protein (CgA) was extracted from canine adrenal glands by use of a previously reported method.¹⁵ The crude 68-kd protein (CgA) was then purified by use of previously reported methods.^{15,16} The human CgA used in this study was the standard CgA from the CgA ELISA kit.³ Concentrations ($\mu\text{g/mL}$) of canine CgA were determined by use of the Lowry method¹⁷ with bovine serum albumin as the standard. The concentration (U/L) of human CgA was calculated and expressed according to the specification of the manufacturer.³

Amino acid sequence—For amino acid sequencing, the purified canine 68-kd protein that was separated by use of SDS-PAGE was electrophoretically transferred onto a polyvinylidene difluoride membrane.^b The polyvinylidene difluoride membrane was stained with Coomassie brilliant blue R250, and the transferred 68-kd band was excised and subjected to NH_2 -terminal amino acid sequence analysis.^c

SDS-PAGE and western blotting—The SDS-PAGE and western blotting were performed by use of previously described methods.^{18,19} For western blotting, the purified canine CgA and human CgA that were separated by SDS-PAGE were transferred to a nitrocellulose membrane.^d After blotting, the nitrocellulose membrane was treated with horseradish peroxidase-conjugated anti-human CgA antibody (diluted to 1:100).³ After washing the nitrocellulose membrane 5 times in 0.01M phosphate buffer (pH, 7.2) containing 0.15M NaCl and 0.05% Tween 20, the specific antigen was detected by use of 3,3'-diaminobenzidine.^e

Induction of hypoglycemia in dogs and blood sample collection—Experiments were performed with 12 healthy Beagles (mean \pm SEM body weight, 11.59 ± 1.68 kg). Dogs were individually housed. Food was provided once daily at 6:00 PM, and water was available ad libitum.

Dogs were randomly assigned to receive either insulin ($n = 6$) or saline (0.9% NaCl) solution (6) treatments. Insulin (0.2 U/kg)^f or an equal volume of saline solution was administered IV at 10:00 AM. Blood samples (2 mL/sample) were obtained by venipuncture at 0, 10, 20, 30, 40, 60, 120, and 180 minutes after IV injection. Blood samples were collected in tubes containing EDTA (for measurement of cortisol, catecholamines, and CgA concentrations) and in a heparinized tube (for measurement of glucose concentration). Plasma samples were obtained by centrifugation at 4°C and stored at -80°C until use.

Experiments complied with institutional guidelines for animal care of Osaka Prefecture University. Dogs were observed throughout the study, and the color of oral mucous membranes and capillary refill times were monitored. Heart and respiratory rates were measured at the time of blood sample collection. If any abnormalities (eg, hypoglycemic seizure, weakness, or collapse) were observed in a dog, the experiment was stopped immediately and suitable medical treatment was given in the Veterinary Medical Center of Osaka Prefecture University.

CgA concentration—To prepare curves of absorbance at 450 nm versus purified canine CgA and human CgA concentrations, the CgA ELISA kit was used according to the protocol of the manufacturer.³ Canine plasma CgA concentrations were similarly determined by use of the ELISA. Canine plasma CgA concentrations were expressed as units per liter and calculated from absorbance at 450 nm. Inter- and intra-assay coefficients of variation were 3.9% and 5.9%, respectively. The minimum level of detection of canine CgA by use of the ELISA was 0.44 U/L. Dose-response data were obtained with

canine plasma CgA concentrations of 0.44 to 53.65 U/L. To determine whether circadian variation of canine plasma CgA concentrations exists, we performed a pilot study to measure plasma CgA concentrations from 10 healthy Beagles (5 females and 5 males) every 6 hours for 24 hours. The circadian variation was 5.5% (range, 0.4% to 9.6%), and a significant difference in plasma concentrations between times of day was not observed. All determinations were examined in duplicate.

Plasma glucose, cortisol, and catecholamine concentrations—Plasma glucose concentrations were determined by use of a blood chemical autoanalyzer.⁸ Plasma cortisol concentrations were determined by use of a canine cortisol ELISA kit according to the protocol of the manufacturer.^h Intra-assay and interassay coefficients of variation were 1.3% and 5.6%, respectively. The sensitivity of this cortisol assay was 2.0 ng/mL. The measurable range was 2.0 to 600 ng/mL.

Plasma catecholamine (ie, norepinephrine and epinephrine) concentrations were measured by use of a slightly modified isocratic procedure, as previously described,^{20,22} with high-performance liquid chromatography. In brief, plasma norepinephrine and epinephrine concentrations were analyzed by a reversed-phase high-performance liquid chromatography system with an electrochemical detectorⁱ; the carbon electrode was held at 0.75 V relative to the reference electrode. The mobile phase consisted of 0.1M tartaric acid-sodium acetate buffer containing 0.5mM disodium EDTA, sodium 1-octanesulfonate (200 mg/L), and 5% methanol (pH, 4.0). The minimum detectable amount of catecholamines was 20 pg. All determinations were examined in duplicate.

Statistical analyses—Values are expressed as mean \pm SD. Data were analyzed for significant differences in parameters between dogs that received insulin and dogs that received saline solution by use of an unpaired Student *t* test.^j Pearson correlation coefficients were calculated to investigate linear relationships between the various response parameters.^k Values of $P < 0.05$ were considered significant.

Results

Characterization of canine CgA—Purified canine protein had a protein band at 68 kd on SDS-PAGE (Figure 1). To confirm whether the 68-kd protein is canine CgA, the NH_2 -terminal amino acids of the protein were sequenced. The first 12 amino acids sequence at the NH_2 -terminal end had 83.3% (ie, 10/12 sequences) homology with that of human CgA, as reported previously,²³ indicating that the purified protein is canine CgA.

In western blots with antibody against human CgA, the canine CgA had a well-defined 68-kd band, whereas human CgA had a 66-kd band (Figure 1). These results were confirmed by use of the CgA ELISA kit. Curves of absorbance at 450 nm versus canine CgA (range, 200 to 0.5 $\mu\text{g/mL}$) and human CgA (range, 120 to 0.6 U/L) concentrations were obtained (Figure 2).

Insulin-induced hypoglycemia in dogs—Plasma glucose concentrations in dogs that received insulin significantly decreased from 10 to 60 minutes after injection, compared with those of dogs that received saline solution (Figure 3). In dogs that received insulin, plasma glucose concentrations had a nadir of 38.5 ± 9.6 mg/dL at 20 minutes after injection and increased thereafter to return to greater than baseline values by 120 minutes. These results indicate that

hypoglycemic stress had been obtained in insulin-treated dogs.

In response to hypoglycemia, plasma cortisol concentrations were significantly higher from 20 to 60 minutes after injection in insulin-treated dogs, compared with saline solution-treated dogs (Figure 4). The highest plasma cortisol concentrations (21.3 ± 3.4 ng/mL) were observed at 60 minutes after insulin injection.

Following insulin administration, plasma norepinephrine and epinephrine concentrations rapidly increased to 432.95 ± 77.78 pg/mL and 523.66 ± 215.63 pg/mL at 40 minutes after insulin injection, respectively (Figure 5). Plasma norepinephrine and

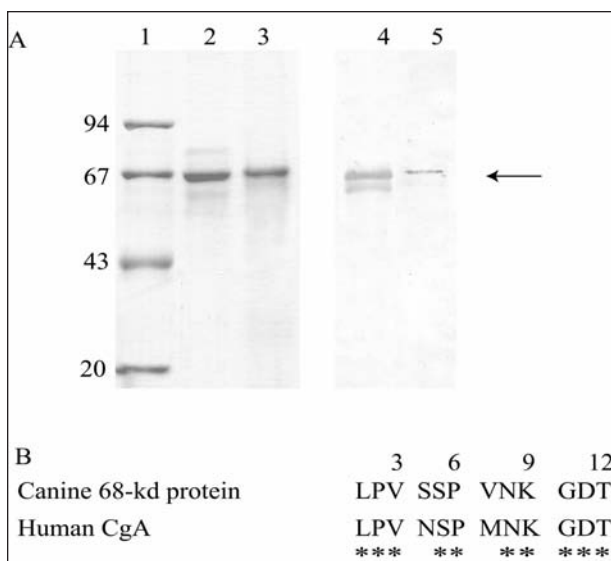


Figure 1—A—SDS-PAGE and western blot analysis of human chromogranin A (CgA) and purified canine CgA. Lanes 1 through 3 represent the results of SDS-PAGE, where lane 1 contains molecular weight markers, lane 2 contains human CgA, and lane 3 contains the purified canine 68-kd protein. Lanes 4 and 5 represent the results of western blot analysis, where lane 4 contains human CgA and lane 5 contains the purified canine 68-kd protein. Notice the expected molecular weight of CgA (arrow). B—The amino acid sequence of the NH₂ terminal of the canine 68-kd protein. The first 12 amino acids from NH₂ terminal were sequenced, and the sequence had 83.3% (10/12 amino acids) homology with the published human CgA sequence.²³ Notice the amino acids that are conserved between the canine 68-kd protein and human CgA (asterisks).

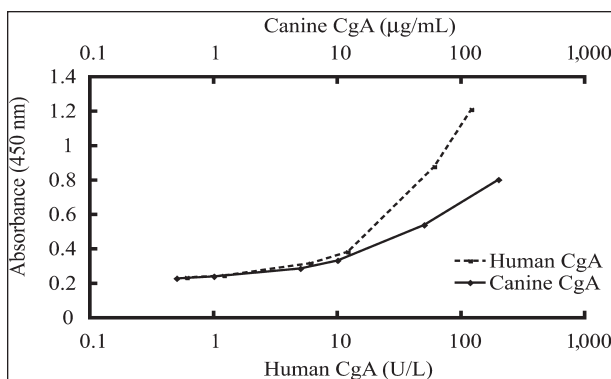


Figure 2—Curves of absorbance at 450 nm versus human CgA and purified canine CgA concentrations as determined by use of the CgA ELISA kit.⁹

epinephrine concentrations were significantly increased from 30 to 40 minutes and from 20 to 60 minutes, respectively, in insulin-treated dogs, compared with saline solution-treated dogs.

Plasma CgA concentrations significantly increased from 30 to 120 minutes after injection in insulin-treated dogs, compared with saline solution-treated dogs (Figure 6). The significant increase in plasma CgA concentration persisted until 120 minutes after insulin injection, which was a longer period of increase than observed for plasma cortisol and catecholamine concentrations. The highest plasma CgA concentration (6.98 ± 1.51 U/L) was observed at 60 minutes after insulin injection. Plasma CgA concentration measured by use of the ELISA before administration of saline solution or insulin was 2.67 ± 0.20 U/L. Saline solution administration did not significantly alter plasma glucose (Figure 3), cortisol (Figure 4), catecholamine (Figure 5), and CgA concentrations.

The plasma CgA concentration was correlated with plasma cortisol ($r = 0.67$; $P < 0.001$) and epinephrine ($r = 0.75$; $P < 0.001$) concentrations until 60 minutes after insulin administration ($n = 6$; Figure 7). The plasma CgA concentration continued to correlate with plasma cortisol ($r = 0.59$; $P < 0.001$) and epinephrine ($r = 0.67$; $P < 0.001$) concentrations until

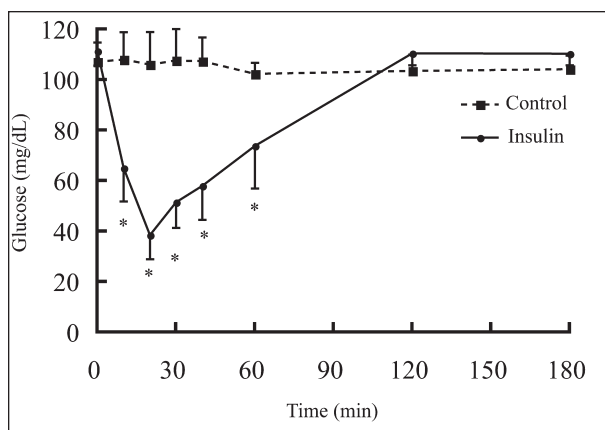


Figure 3—Mean \pm SD plasma glucose concentrations versus time after insulin ($n = 6$) and saline solution (control [6]) administration at time 0 in dogs. *Significant ($P < 0.05$) difference between saline solution and insulin administration.

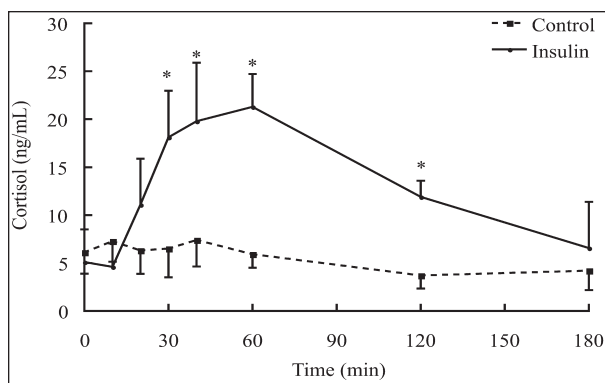


Figure 4—Mean \pm SD plasma cortisol concentrations versus time after insulin ($n = 6$) and saline solution (control [6]) administration at time 0 in dogs. See Figure 3 for key.

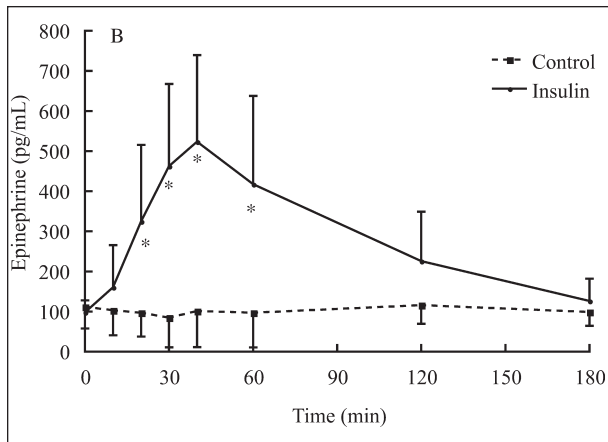
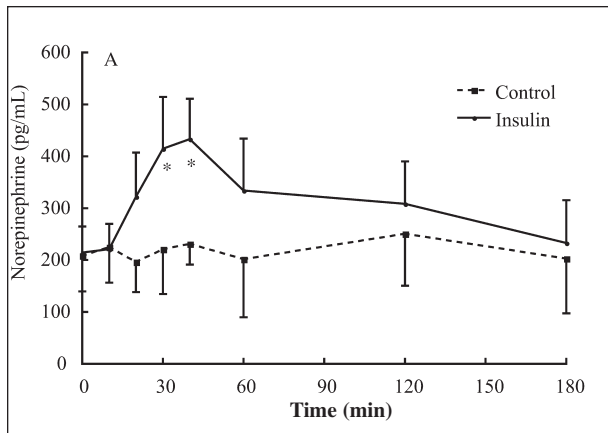


Figure 5—Mean \pm SD plasma catecholamine (A, norepinephrine; B, epinephrine) concentrations versus time after insulin ($n = 6$) and saline solution (control [6]) administration at time 0. See Figure 3 for key.

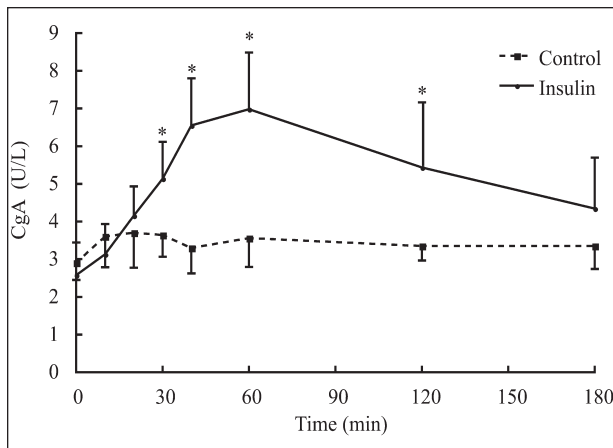


Figure 6—Mean \pm SD plasma CgA concentrations versus time after insulin ($n = 6$) and saline solution (control [6]) administration at time 0 in dogs. See figure 3 for key.

180 minutes after insulin injection (data not shown). Plasma CgA concentration was also correlated with plasma norepinephrine ($r = 0.50$; $P = 0.024$) concentration until 60 minutes after insulin administration. The plasma CgA concentration continued to be correlated with the plasma norepinephrine concentration ($r = 0.44$; $P < 0.001$) until 180 minutes after insulin injection (data not shown).

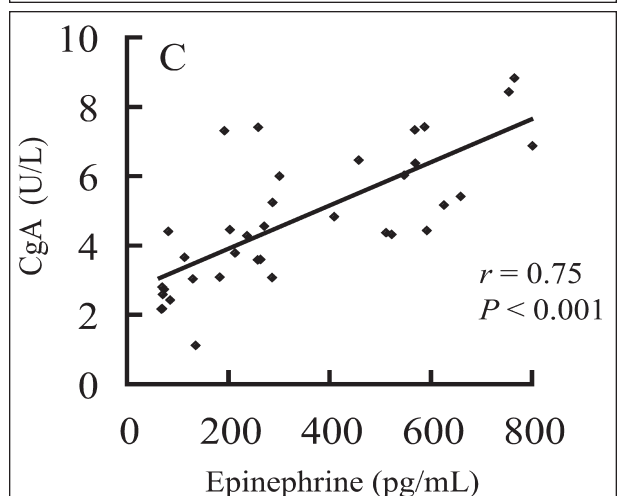
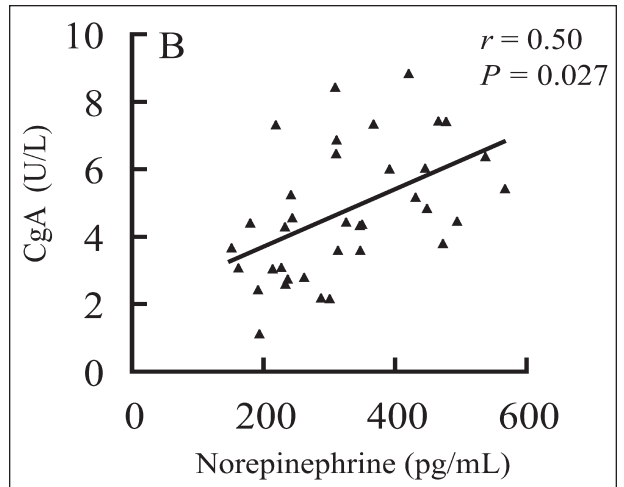
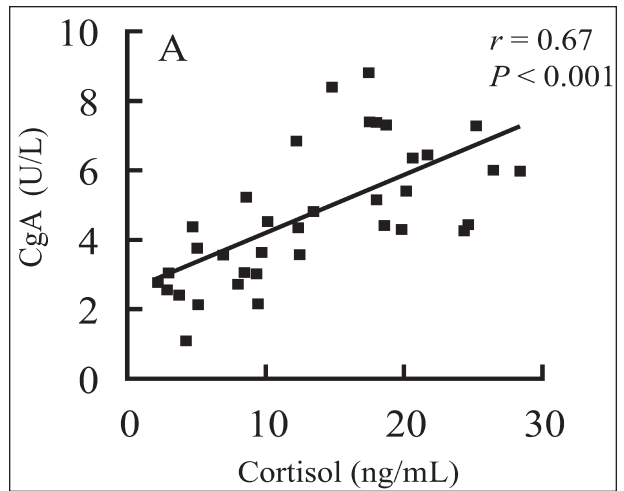


Figure 7—Relationship between plasma CgA concentration and plasma cortisol (A), plasma norepinephrine (B), and plasma epinephrine (C) concentrations from 0 to 90 minutes after insulin administration in dogs.

Discussion

Stress responses consist of 3 stages of time-limited physical and behavioral changes (ie, alarm reaction, resistance, and exhaustion). The SAM system allows for the flight or fight response during the alarm reaction stage. Recently, the magnitude of activation in this

system has been evaluated by monitoring epinephrine and norepinephrine or by studying psychophysiological measures (heart rate, blood pressure, and skin-conductance responses).²⁴ During activation of the SAM system, not only catecholamine but also an abundance of jointly stored proteins, the chromogranins, are released by exocytosis into the extracellular environment and subsequently into the circulation.^{1,2,6,25,26} Increases in plasma cortisol and catecholamine concentrations support the hypothesis that cortisol and catecholamine interact synergistically in mounting a glycemic recovery response.²⁷ Results of our study on insulin-induced hypoglycemia in dogs revealed that insulin administration leads to a significant decrease in plasma glucose concentration and subsequently to a significant increase in plasma cortisol, catecholamine, and CgA concentrations.

Other stages of stress response, stages of resistance and exhaustion, have been regarded as the central component of the stress response and are regulated by the HPA axis. Quantitative indices of these stages have mainly been provided by biochemical monitoring of the functions of the hypothalamus, pituitary, and adrenal cortex (eg, release of corticotropin-releasing hormone, ACTH, and cortisol). However, it is difficult to evaluate the stress response by measurement of plasma cortisol concentration because circadian variation of circulatory cortisol concentration exists in healthy dogs, as previously reported.²⁸ On the other hand, plasma CgA concentration has recently been reported to increase simultaneously with plasma catecholamine concentrations during brief high-intensity exercise on a bicycle ergometer.²⁹ Thus, the plasma CgA concentration appears to be a better index of sympathetic activity and will be a useful marker in evaluating stressed conditions in dogs.

The amino acid sequences at the NH₂- and COOH-terminal regions of CgA are highly conserved among animal species,^{23,30-32} indicating that some immunologic cross-reactivity exists among various CgA. This is confirmed by findings in previous studies⁹⁻¹² and the results of our study in which an antibody against human CgA cross-reacted with the canine CgA that had a molecular weight of 68 kd. Use of the ELISA kit with anti-human CgA revealed that canine CgA can be detected in a concentration-dependent manner. Moreover, the NH₂-terminal amino acid sequence of canine CgA had 83.3% homology with human CgA, as reported previously.²³

Linear relationships were clearly observed between plasma CgA concentrations and plasma cortisol and epinephrine concentrations. The plasma CgA concentration was slightly correlated with the norepinephrine concentrations. These results suggest that plasma CgA concentration may reflect sympathoadrenal activity in dogs. They also suggest that the released catecholamines were rapidly removed or catabolized and then decreased to baseline concentrations within 60 minutes. In contrast, the increase in plasma CgA concentration remained at 120 minutes after insulin injection, suggesting that CgA may remain intact far longer than catecholamines in circulation. By comparison, plasma CgA concentrations are kept low in

healthy dogs and circadian variations in plasma CgA concentrations have not been observed in humans.³³

Results of our study indicate that plasma CgA concentrations before administration of saline solution or insulin were low in comparison to established reference range values for humans (range, 9.3 to 15.3 U/L; mean, 12.3 U/L) that were obtained by use of the same method³⁴; similar findings have been reported previously.¹² Although these low values of plasma CgA concentrations in our study cannot be accounted for at the present time, they may be the result of the use of a heterologous antigen-antibody reaction.

In conclusion, results of our study indicate that use of the ELISA kit for determination of human plasma CgA concentrations is applicable to the measurement of canine plasma CgA concentrations. Canine plasma CgA concentrations, along with measurements of plasma cortisol and catecholamine concentrations, correctly reflect hypoglycemic stressed conditions that are induced by insulin administration in dogs. Further research is needed to measure canine plasma CgA concentrations by use of an ELISA with an antibody against canine CgA to evaluate activation of the SAM system and regulation by the HPA axis of acute stress responses in dogs.

- a. Chromogranin A ELISA kit, DAKO Cytomation, Glostrup, Denmark.
- b. Polyvinylidene difluoride membrane, Atto Co Ltd, Tokyo, Japan.
- c. HP G1005A protein sequencing system, Hewlett-Packard, Palo Alto, Calif.
- d. ADVANTEC MEMBRANE, Toyo Roshi Kaisha Ltd, Tokyo, Japan.
- e. 3,3'-diaminobenzidine tetrahydrochloride tablets, Sigma Chemical Co, St Louis, Mo.
- f. Novolin R 100, Novo Nordisk A/S, Bagsvaerd, Denmark.
- g. COBAS READY, Roche Diagnostics Japan, Tokyo, Japan.
- h. K9 cortisol ELISA test kit, Endocrine Technologies Inc, Newark, Calif.
- i. LC-4B, Bioanalytical Systems Inc, West Lafayette, Ind.
- j. Microsoft Excel, Microsoft Corp, Redmond, Wash.

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