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The Use of Saliva Cortisol, Urinary Cortisol, and Catecholamine Measurements for a Noninvasive Assessment of Stress Responses in Dogs

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A problem in assessing animal welfare is that collecting data in itself may be stressful to the animals. Therefore, noninvasive methods for collecting data have to be devised and tested. A first step in investigating saliva cortisol, urinary cortisol, and urinary catecholamine as noninvasive indicators of canine well-being is the validation of these hormonal measures as alternatives for those in plasma. Using a model of insulin (0.2 U/kg)-induced hypoglycemia, we report on stress-induced responses in saliva cortisol, urinary cortisol, and urinary catacholamines relative to cortisol and catecholamine responses in plasma. Hypoglycemia in six dogs induced significant ($P < 0.05$) increases in plasma cortisol and adrenaline but not noradrenaline. Saliva cortisol responses expressed as net area under the response curve correlated significantly with plasma cortisol responses ($r > 0.92$). Saliva cortisol levels measured 7 to 12% of plasma cortisol concentrations. Cortisol/creatinine ratios in urine were significantly higher when voided after insulin administeration, compared to when voided after saline treatment. Insulin-induced increments in cortisol/creatinine ratios were nonsignificant when urine samples were assayed after dichloromethane extraction. Although urinary adrenaline/creatinine (A/C) ratios were significantly correlated with maximum plasma adrenaline values after insulin administration, A/C ratios did not differ significantly between insulin and saline treatment. The present experiment provides strong support for using saliva sampling and urine collection as noninvasive methods to establish stress-induced cortisol responses. For measuring acute plasma adrenaline responses, measuring A/C ratios may not be a valid alternative.

Poor housing conditions, harsh training sessions, and uncontrollable or unpredictable social environments are just some of the situations that may lead to the poor welfare of dogs. The detection of poor welfare can be established through the assessment of stress responses (Beerda, Schilder, de Vries, and van Hooff, accepted for publication) and, as such, requires tools that measure stress. The measurement of stress responses bears the risk of misinterpreting results with regard to well-being. Anticipatory reactions and responses to the collection of samples that are not specific for stress (Murphree, Peters, and Dykman, 1967; Lynch and McCarthy, 1969) may bias real stress responses and lead to misinterpretation. Noninvasive sampling procedures may minimally disturb the subject under study and, thus, partly prevent anticipatory or stress nonspecific responses. Noninvasive sampling procedures are furthermore advantageous in that samples are collected easily. From the above it follows that noninvasive sampling techniques that may be used to detect stress responses in the dog deserve attention.

The assessment of levels of cortisol and catecholamine is an established way to investigate stress since these hormones reflect the activity of two important stress responsive axes: the hypothalamic–pituitary–adrenal (HPA) axis and the sympatho–adrenal–medullary (SAM) axis. In humans, measuring saliva cortisol has been validated as a noninvasive alternative for measuring plasma cortisol responses (for a review see Kirschbaum and Hellhammer, 1989). Studies in man (Schwab, Heubel, and Bartels, 1992) and sheep (Houpt, Kendrick, Parrott, and De La Riva, 1987) rejected salivary catecholamine concentrations as a reliable alternative for identical measures in plasma. Naturally voided urine has been reported to be another useful substrate to study stress hormones in humans in a noninvasive

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way. Elevated urinary cortisol has been implied in major depression (Roger, Anton, Noyes, and Gehris, 1989), whereas elevated urinary catecholamines were found in severely stressed women (Ende, Gertner, and Socha, 1990) and men (Kosten, Mason, Giller, Ostroff, and Harkness, 1987).

Therefore, in this study we aimed to validate saliva cortisol, urinary cortisol, and urinary catecholamine as noninvasive ways to measure stress in dogs. To this end we used a model of insulin-induced hypoglycemia, which stimulates the HPA axis and predominantly the adrenomedullary component of the SAM axis (Goldstein, Garty, Bagdy, Szemeredi, Sternberg, Listwak, Pacak, Deka-Starosta, Hoffman, Chang, Stull, Gold, and Kopin, 1993). This makes it a valid paradigm to correlate plasma cortisol and catecholamine responses with those in saliva and urine.

METHODS

Animals

Experiments were performed on 16 kennel dogs of different ages, sexes, and breeds. Dogs were individually housed and allowed outside between 07.00 and 11.00 AM. Food was provided once a day at 08.00 AM, whereas water was available *ad libitum.*

Experiments

Starting at 08.00 AM dogs were transferred to kennels similar to their own. Here, blood samples were taken every half hour from 11.00 AM to 16.00 PM by vena puncture, collected in heparinized tubes, and kept on ice for a maximum of 1 hr. Plasma was obtained by centrifugation for 10 min (1000*g;* 4°C) and stored at −20°C until further analysis for cortisol. During the day, spontaneously voided urine samples were collected and adjusted to pH 3–4 using formic acid. pH-adjusted samples were frozen at −20°C until further analysis for cortisol, adrenaline and noradrenaline. One milliliter of each urine sample, to be assayed for creatinine, was processed differently in that no acid was added.

On the second experimental day, dogs were randomly assigned to either insulin ($n = 8$) or saline ($n = 8$) treatment. Insulin (0.2 U/kg) or saline was administered in the vena femoralus. Blood samples were taken by vena puncture at −5, 0, 5, 10, 20, 30, 40, 60, 90, 120, 180, and 240 min from injection at 11.00 AM. Samples to be assayed for cortisol were collected in heparinized tubes, whereas blood to be assayed for adrenaline, noradrenaline, and glucose was collected in tubes coated with natrium fluoride. Similar to the blood sampling regime, saliva was collected at −5, 5, 10, 20, 30, 60, 75, 90, 105, 120, 180, and 240 min. Saliva samples were obtained from the cheek pouches by gentle sucking through a collection tube. A filter within the tube prevented the mixture of canine and human saliva. A double-acting pump supplied the over pressure that was used to recollect the canine saliva from the collection tube. Samples were stored at −20°C within the hour. Prior to the taking of a sample the saliva flow of dogs was stimulated through the oral administration of pellets of citric acid. Natural voided urine samples collected on the second experimental day were processed as described previously.

Determinations

Plasma glucose was determined by the glucose oxidase method on a WAKO-20R Biochemical Analyzer (Wako Chemicals Gmbh, Neuss, Germany). Creatinine concentrations were measured using the Jaffé kinetic method using initial rate reaction.

Cortisol concentrations in saliva, plasma, and urine were established in a solid-phase ^{125}I radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA) following standard procedures. The mean interassay coefficient of variation was 7%. Urine samples were assayed before and after extraction with 2 vol of dichloromethane.

After solid-phase extraction, plasma and urinary catecholamine concentrations were determined by HPLC followed by radiochemical detection. For the plasma extraction a commercially available kit was used (Pharmacia LKB, Woerden, The Netherlands). Fifty microliters internal standard (3,4-dihydroxybenzylamine, DHBA; 600 pg) was added to a 1-ml plasma sample. This sample was mixed end over end for 10 min with aluminum oxide dissolved in 0.5 ml extraction buffer. After three wash steps with 1 ml extraction buffer the catecholamines were eluted with 120 μl elution buffer. The extraction efficiency amounted to 70%. Six milliliters 0.1% (w/v) Na_2EDTA, 100 ml internal standard (DHBA; 2500 pg), and 180 μl sodium acetate (0.5 *M;* pH 5.0) were added successively, to 3 ml of the urine samples. This mixture was extracted on cation exchange columns (Pharmacia LKB). After three wash steps with 1 ml bidistilled water the catecholamines were eluted with 6 ml 4% (w/v) boric acid. The HPLC system consisted of a Model 112 solvent delivery system (Beckmann, Mijdrecht, The Netherlands) and a Model 460 electrochemical detector (Waters, Milford, MA). Appro-

priate volumes of the extracts (20–50 μl) were injected on a C_{18} reversed-phase column (LiChroCART 250-4; Merck, Darmstadt, Germany) and eluted with a mixture of 0.5% sodium acetate, 0.5% citric acid, 0.05% sodium-EDTA, 0.1% octanesulfonic acid, 0.05% di-*n*-butylamine, and 0.25% methanol. The electrochemical detector data were analyzed using a Model 3396 integrator (Hewlett Packard, Amsterdam, The Netherlands). The concentration of catecholamine was calculated relative to the internal standard concentration after a multi-level calibration with adrenaline, noradrenaline, and dopamine.

Some urine samples could not be successfully assayed for all hormones, due to limited sample volumes, concentrations below the detection limit, and unreliable duplos.

Data Processing and Statistical Analysis

Urine samples voided before 11.00 AM were labeled pretreatment, whereas samples voided after 12.30 PM were considered to reflect posttreatment conditions. Hormonal concentrations in urine were expressed as ratios to urinary creatinine concentrations.

Results are presented as mean values ± the standard error of the mean. Mean levels of plasma cortisol obtained under undisturbed conditions were checked for extreme values. Animals that showed mean levels higher than three times the interquartile range (the distance between the 25th and the 75th percentile value) on top of the 75th percentile value were considered extreme and omitted from further analysis. Raw data from the remaining dogs were analyzed for significant differences between animals assigned to insulin or saline by an analysis of variance (ANOVA). Differences in time were tested using an analysis of variance for repeated measurements. Pearson correlation coefficients were calculated to investigate linear relationships between the various response parameters. The level of significance was set at $P < 0.05$.

RESULTS

Undisturbed Levels of Plasma Cortisol, Urinary Cortisol, and Urinary Catecholamines

On the first day, mean plasma cortisol concentrations between 11.00 AM and 16.00 PM were 58.8 ± 9.1 nmol/liter. Of the 16 dogs, 2 showed high cortisol levels of 102.8 and 181.6 nmol/liter. These animals were considered extreme and omitted from further analysis. The pooled data from the remainder of the animals showed a mean plasma cortisol concentration of 46.9 ± 1.3 nmol/liter. Over the period we measured, cortisol levels did not show diurnal variation.

Mean urinary concentrations of cortisol, adrenaline, and noradrenaline are presented in Table 1. In more detail, the mean nonextracted urinary cortisol/creatinine (C^{NE}/C) ratio established in the morning was 49% higher than the value established in the afternoon. Since samples of only 4 animals could be collected during the morning, this difference was not statistically tested. Dichloromethane extraction lowered the mean cortisol/creatinine (C^{E}/C) ratio to 57% of its nonextracted counterpart but affected the difference between morning and afternoon means minimally (morning ratios were on average 56% higher than in the afternoon). No correlation of significance was detected between undisturbed levels of plasma cortisol and urinary cortisol.

Compared to afternoon levels, urinary adrenaline/creatinine (A/C) ratios were on average 45% lower in the morning. On the other hand, mean noradrenaline/creatinine (N/C) ratios were 29% higher in the morning. A significant correlation ($r = 0.65$, $n = 10$) was observed between urinary N/C and C^{E}/C ratios. N/C ratios as measured in the course of the experiment are not discussed in further detail, as these showed only minor fluctuations, around $2.4 \pm 0.5 \times 10^{-6}$ (34 urine samples).

None of the parameters measured on the first experimental day deviated significantly between animals assigned to saline or insulin treatment the next day.

Hormonal Levels in Plasma, Saliva, and Urine before and after Saline Administration

Saline administration in eight dogs did not significantly alter plasma glucose (Fig. 1), cortisol (Fig. 2a) or catecholamine concentrations (Fig. 3a). Mean glucose levels of 5.0 ± 0.1 nmol/liter coincided with plasma cortisol levels of 49.0 ± 2.5 nmol/liter and saliva cortisol levels of 4.7 ± 0.4 nmol/liter. Cortisol levels in plasma and saliva before saline or insulin treatment were correlated significantly ($r = 0.58$, $n = 14$). Mean levels of plasma adrenaline and plasma noradrenaline were calculated at 0.34 ± 0.02 and 1.09 ± 0.07 nmol/liter, respectively.

Saline administration was never associated with significant changes in any of the urinary cortisol parameters (Table 1). In a similar way, saline administration did not disturb the difference between mean A/C ratios in the morning and afternoon as found under undisturbed conditions (Table 1). Urinary cortisol and cate-

TABLE 1
Hormonal Levels in Urine Naturally Voided by Dogs Subjected to Different Conditions

	Undisturbed conditions	Saline treatment		Insulin treatment	
		Pretreatment	Posttreatment	Pretreatment	Posttreatment
$Cortisol^{NE}$/creatinine	11.9 ± 1.2^{12}	13.2 ± 2.2^{7}	13.2 ± 2.3^{8}	13.1 ± 1.8^{5}	$28.2 \pm 7.1^{6*}$
$Cortisol^{E}$/creatinine	6.2 ± 1.0^{12}	6.9 ± 1.9^{7}	7.5 ± 1.9^{8}	5.6 ± 1.3^{5}	$17.1 \pm 6.3^{6**}$
Adrenaline/creatinine	3.9 ± 1.3^{10}	2.8 ± 0.9^{5}	5.0 ± 2.0^{8}	1.2 ± 0.6^{5}	3.2 ± 1.7^{5}
Noradrenaline/creatinine	1.5 ± 0.3^{10}	4.0 ± 2.1^{5}	3.6 ± 2.2^{8}	1.8 ± 0.8^{5}	2.4 ± 1.1^{3}

Note. Catecholamine/creatinine ratios ($\times 10^{-6}$) and cortisol/creatinine ratios ($\times 10^{-6}$) in natural voided urine samples; mean values ± SEM of *n* dogs (presented as a superscript) that were subjected to no (undisturbed), saline, or insulin treatment; cortisol was determined in dichloromethane extracted $^{(E)}$ and nonextracted $^{(NE)}$ urine samples; * and ** indicate the relevant differences between saline- and insulin-treated animals and represent *P* values of 0.03 and 0.12, respectively.

cholamine parameters, established before saline or insulin treatment, correlated significantly with their counterparts established in undisturbed animals: $r > 0.80$, $n = 12$ and $r > 0.71$, $n = 9$, respectively.

Hormonal Levels in Plasma and Saliva before and after Insulin Administration

Insulin administration in six dogs was associated with significantly lower levels of glucose from 5 up to 40 min postinjection (Fig. 1). Plasma glucose concentrations decreased to a nadir of 1.9 ± 0.3 mmol/liter after 17 ± 2 min and increased thereafter to return above baseline by 90 min. In response to this hypoglycemia, plasma cortisol was significantly increased from 30 up to 90 min postinjection (Fig. 2b). Peak responses of on average 205.0 ± 27.1 nmol/liter were demonstrated after 45 ± 5 min. Coincident with plasma cortisol, highest mean saliva cortisol concentrations of 22.1 ± 3.4 nmol/liter were measured after 45 ± 10 min (Fig. 2b). Saliva cortisol values were significantly elevated from 20 up to 105 min postinsulin administration. Peak values in plasma and saliva correlated significantly ($r = 0.93$). The correlation remained significant when responses were expressed as net area under the response curves (NAURC): $r = 0.94$. Cortisol concentrations in saliva ranged between 7.2% ($t = +5$ min) and 11.9% ($t = +30$ min) of comparative plasma levels.

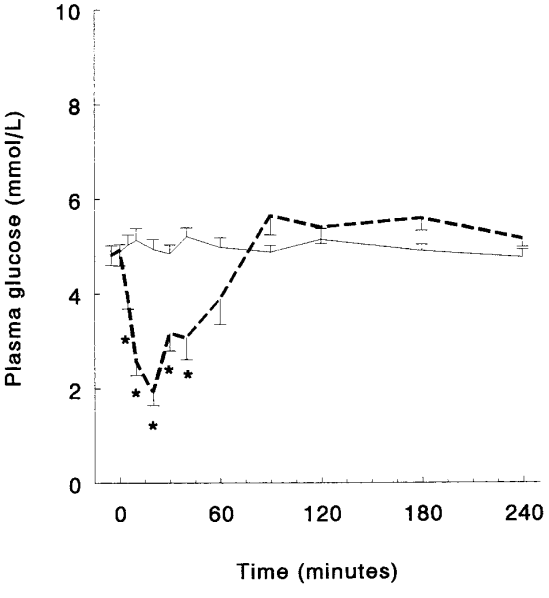

FIG. 1. Mean (±SEM) levels of plasma glucose after insulin (dotted line, $n = 6$) and saline (solid line, $n = 8$) administration at Time = 0 min. (*) Significant ($P < 0.05$) differences between saline and insulin treatment.

Following insulin administration, mean plasma adrenaline concentrations rapidly increased to a peak value of 3.25 ± 1.13 nmol/liter (Fig. 3b). Levels were significantly elevated from 20 up to 120 min. Plasma adrenaline responses expressed as NAURC correlated significantly with plasma cortisol peak values ($r = 0.83$). In response to insulin treatment mean levels of noradrenaline increased nonsignificantly to a maximum of 1.94 ± 0.10 nmol/liter (Fig. 3b).

Hormonal Levels in Urine Samples Voided before and after Insulin Administration

Insulin treatment was associated with rises in urinary C^{NE}/C ratios and C^{E}/C ratios (Table 1). However, only C^{NE}/C ratios differed significantly from mean levels in saline-treated animals. Although the mean A/C ratio increased 164% after insulin administration, it remained below the mean level observed after saline administration (Table 1). A/C ratios correlated signifi-

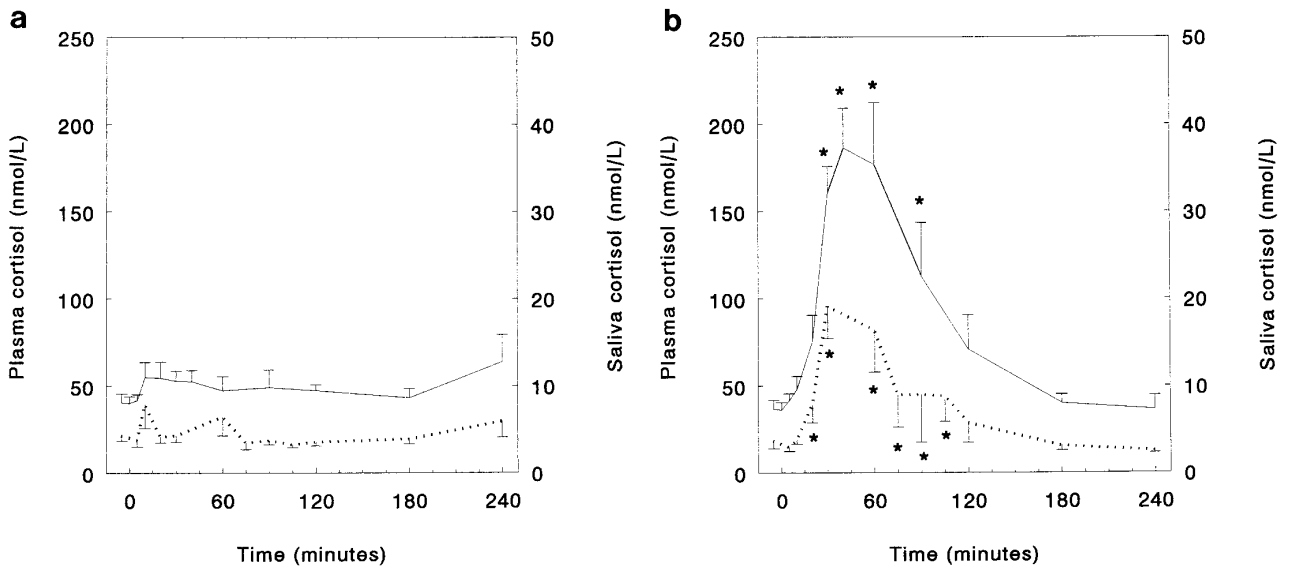

FIG. 2. Mean (±SEM) levels of plasma cortisol (solid line, $n = 6$) and saliva cortisol (dotted line, $n = 6$) after the administration of saline (a) or insulin (b) at Time = 0 min. (*) Significantly ($P < 0.05$) higher than the levels that were established after saline administration.

cantly with highest adrenaline concentrations in plasma ($r = 0.90$, $n = 5$).

DISCUSSION

Hormonal Levels in Undisturbed Experimental Dogs

Plasma cortisol concentrations obtained in undisturbed dogs showed a mean level (46.9 nmol/liter) that agrees with levels reported by other authors (Kemppainen and Sartin, 1984; Palazollo and Quadri, 1987). The observed mean urinary C^{NE}/C ratio of 6.2×10^{-6}, on the other hand, is lower than the previously reported value of 9.4×10^{-6} (Jones, Refsal, Lippert, Nachreiner, and Schwacha, 1990). Differences in the time and method of urine collection are likely to contribute to this discrepancy. Under undisturbed conditions, the levels of urinary cortisol were not significantly correlated to levels of plasma cortisol. This correlation was significant when the bladder was experimentally emptied before and at the termination of a period of blood sampling (Jones *et al.*, 1990). Differences in the periods of cortisol secretion that reflect in plasma or urine were thus prevented in the latter but not the present study. Clear relationships between plasma and urinary measurements may also have been clouded by an episodic release of cortisol (Kemppainen and Sartin, 1984). Fluctuating levels of plasma cortisol will hamper the establishment of reliable base levels when only a limited number of samples are taken, as in the present study.

Although levels of plasma cortisol did not change from morning to afternoon, urinary concentrations tended to be higher in the morning. Possible acute cortisol responses during the transfer of the animals into the experimental room would have been cleared from the blood before the commencement of the blood sampling. However, such cortisol increases may have accumulated in the urine that was eventually voided during the late morning. This interpretation is supported by the tendency of higher C^{NE}/C and C^{E}/C ratios during the morning of allocation compared to similar measures achieved after 24 hr.

Base levels of cortisol in nonextracted urine correlated significantly with levels of urinary noradrenaline. Individual variability in the levels of activity may have contributed to this relationship since physical strain in humans and rats is known to increase both glucocorticoid and noradrenaline releases (Weicker and Werle, 1991; Boer de, Koopmans, Slangen, and Gugten van der, 1990).

Hormonal Levels in Dogs Subjected to Saline or Insulin Treatment

Insulin administration was associated with significant decreases in plasma glucose and subsequent increases in plasma cortisol and adrenaline. The latter

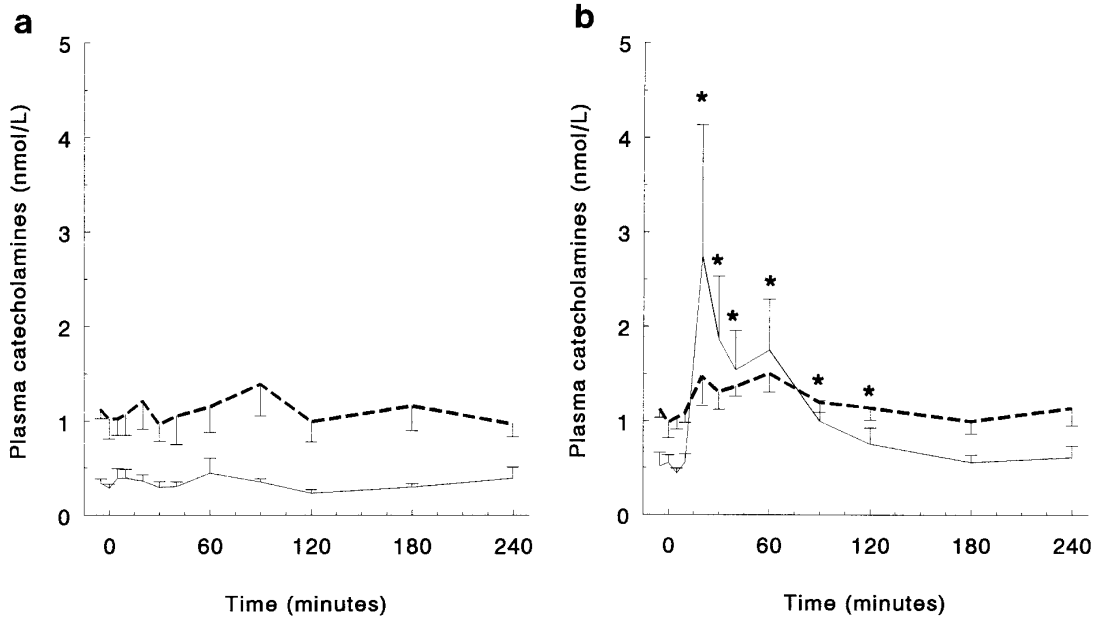

FIG. 3. Mean (±SEM) levels of plasma adrenaline (solid line, $n = 6$) and plasma noradrenaline (dotted line, $n = 6$) after the administration of saline (a) or insulin (b) at Time = 0 min. (*) Significantly ($P < 0.05$) higher than the levels that were established after saline administration.

increases are in line with expectations, as cortisol and adrenaline are hypothesized to interact synergistically in mounting a glycemic recovery response (Eigler, Sacca, and Sherwin, 1979). A minor nonsignificant increase in plasma noradrenaline may have originated primarily from the adrenal medulla and not so much from the sympathetic nerve terminals, since insulin-induced rises in noradrenaline were not detected in anaesthetized dogs with functional adrenalectomies (Yamaguchi, Briand, and Brassard, 1989).

Hypoglycemic-induced plasma cortisol responses were significantly correlated to responses in saliva cortisol. Comparative moderate, but still significant, correlations were found when plasma and saliva cortisol levels were achieved before insulin or saline treatment. Since a clear linear relationship was detected between plasma and saliva cortisol values, a pronounced influence of the saliva flow rate on saliva cortisol concentrations appears unlikely. This is supported by the finding in humans that cortisol levels are unaffected by the saliva flow rate (for a review see Kirschbaum and Hellhammer, 1989).

Saliva cortisol concentrations in our dogs were between 7.2 and 11.9% of comparable plasma values. These findings are more or less in line with the 4 to 10% range as previously reported by Vincent and Michell (1992a). Saliva cortisol levels are relatively low, since only up to 15% of the total cortisol fraction in plasma, namely the unbound fraction, transfers into saliva. Conversion of cortisol into corticosterone by an 11β-hydroxysteroid dehydrogenase may further reduce saliva cortisol levels down to 50% of the unbound levels in plasma (Kirschbaum and Hellhammer, 1989). In the present study we were not able to detect any delay in saliva cortisol responses relative to plasma responses. A major delay is not to be expected since unbound cortisol is a small and highly lipid soluble molecule which will easily pass through cell membranes into saliva. Although a small transfer time (1–2 min) of cortisol from blood into saliva has been reported in humans (Kirschbaum and Hellhammer, 1989), the present sampling regime possibly was too crude to detect delays in this order of magnitude.

Our study suggests that saliva cortisol is a valid and less invasive alternative for the assessment of plasma cortisol in the dog. Measuring saliva cortisol may be especially useful in detecting responses of the HPA axis following acute stress.

Hypoglycemia-induced cortisol secretion resulted in elevated urinary C/C ratios. Only when urine samples were not extracted did this increase prove to be significant. Increments in C^E/C ratios were proportionally of an even higher magnitude but may have failed to achieve significance due to a stronger individual variation. Whereas we observed a mean C^{NE}/C ratio of 28.2 $\times 10^{-6}$ in urine voided by dogs exposed to hypoglyce-

mic stress, Jones *et al.* (1990) reported C^{NE}/C ratios above 130×10^{-6} following ACTH administration. In addition to the use of a more intense stressor, a more precise separation of urine excreted before and after treatment may have caused the stronger responses in the study by Jones *et al.* (1990). The later authors achieved such a strict separation by emptying the bladder before treatment. In our study the mixture of pre- and posttreatment urine was somewhat controlled for by introducing a 90-min lag immediately after treatment, but it was not eliminated.

The results of the present study provide strong support for the urinary C^{NE}/C ratio as a valid noninvasive indicator of acute stress in dogs. To the authors' knowledge little is reported on the delay between stressor administration and urinary cortisol responses. Direct urine collection from the kidneys in two dogs showed an increased 11β-hydroxycorticosteroid secretion within 30 min after shock administration (Houser and Paré, 1974). It remains open to further investigation to determine more precisely the delay between the onset of a stressor and the subsequent urinary cortisol response.

Insulin administration did not significantly affect noradrenaline concentrations in plasma or urine. Since no significant noradrenaline responses were elicited in plasma, the validity of N/C ratios for measuring acute stress in dogs remains obscure. Significant adrenaline responses in plasma remained undetected in the urine samples. Although A/C ratios assessed after insulin treatment were on average 164% of the measures obtained before treatment, levels were below those measured after saline administration. Our failure to show acute plasma adrenaline responses reflected in urinary catecholamine excretion is in agreement with findings by Unger, Buu, and Kuchel (1978). Uptake of adrenaline by renal tissue, noradrenaline release by nerve terminals of the kidney, metabolism of conjugated dopamine into free noradrenaline, and a conjugation mechanism for both noradrenaline and adrenaline in the kidney have been hypothesized to cause some of the discrepancy between plasma and urinary levels of catecholamine (Unger *et al.*, 1978). In a study that dealt with 36 anesthetized mongrel dogs, Cuche and Safar (1992) found a significant correlation between urinary excretion and arterial blood levels of noradrenaline. The same did not account for levels of adrenaline. The absence of a significant correlation between plasma and urinary adrenaline was suggested to be due to an intrarenal synthesis of adrenaline.

Supporting earlier studies (Unger *et al.*, 1978; Cuche and Safar, 1992), the present experiment provides no strong support for the urinary A/C ratio as a valid indicator of stress-induced increases in canine SAM activity. A significant correlation between insulin-induced plasma adrenaline responses (expressed as peak values) and posttreatment A/C ratios does, however, suggest some relation between a dog's level of plasma adrenaline and its urinary A/C ratio. It has to be noted that since this correlation was based upon data from only five dogs, its validity and importance remain open to further investigation.

In conclusion, hypoglycemia-induced plasma cortisol responses are well reflected in saliva and precede responses in urinary cortisol/creatinine ratios. Correlations between plasma and urinary cortisol are less pronounced when urine is assayed after dichloromethane extraction. Saliva sampling and urine collection are concluded valid and noninvasive methods to establish stress-induced cortisol responses in the dog. The present study failed to validate urinary catecholamine responses as a noninvasive alternative for acute catecholamine responses in plasma.

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