Evidence for episodic but not circadian activity in plasma concentrations of adrenocorticotrophin, cortisol and thyroxine in dogs

R. J. Kemppainen and J. L. Sartin
Department of Physiology and Pharmacology, School of Veterinary Medicine, Auburn University, Auburn, Alabama 36849, U.S.A.

RECEIVED 27 March 1984

ABSTRACT

Concentrations of immunoreactive (i) ACTH, cortisol and thyroxine were determined in plasma samples obtained at 20-min intervals for 25 h in nine normal and two adrenalectomized dogs. The dogs were exposed to a 12 h light:12 h darkness photoperiod for 30 days before the sampling period. Episodic secretion of iACTH and cortisol was evident in each normal dog, with an average of 9-0 iACTH peaks and 10-1 cortisol peaks in a 24-h period. Levels of iACTH and cortisol were significantly correlated in each normal dog, but periods of dissociation between levels of the two hormones were apparent. A sex difference in 24-h mean iACTH and cortisol levels, numbers of cortisol peaks, and amplitude of iACTH peaks was observed, with females showing higher mean levels and greater peak frequency and amplitude in each instance. Adrenalectomy resulted in a 50- to 150-fold increase in mean iACTH concentrations with an apparent increase in iACTH peak amplitude. Cortisol levels were unchanging in the adrenalectomized dogs.

Thyroxine concentrations showed episodic variation in each of the normal dogs, but the mean number of peaks (3-3/24-h period) was considerably less than for iACTH or cortisol. Female dogs had significantly higher 24-h mean levels of thyroxine than did males. No circadian rhythmicity was obvious for the plasma levels of any of the three hormones measured.


INTRODUCTION

While considerable information exists concerning the daily profiles of circulating pituitary, adrenal and thyroid hormones for a number of species, comparatively few data are available for the dog. Plasma levels of adrenocorticotrophin (ACTH), corticosteroids or both have been shown to vary episodically and with circadian periodicity in several species including man (Krieger, Allen, Rizzo & Krieger, 1971; Gallagher, Yoshida, Roffwarg et al. 1973), rhesus monkeys (Quabbe, Gregor, Bumke-Vogt & Härdel, 1982), sheep (Fulkerson & Tang, 1979) and cattle (Thun, Eggenberger, Zerobin et al. 1981). In each of these studies, hormonal profiles were characterized in blood samples collected at a frequency of every 5 to 30 min throughout the day. In contrast, previous studies in the dog which supported (Rijnberk, der Kinderen & Thijssen, 1968) or refuted (Johnston & Mather, 1978) the existence of a circadian rhythm in plasma corticosteroid levels based their conclusions on analyses of only eight blood samples per day. An exception to these studies is the work of Takahashi, Ebihara, Nakamura & Takahashi (1981) who bled dogs every 30 min for 28 h and could not demonstrate a circadian cortisol rhythm. Plasma ACTH and corticosteroid relationships have been examined in dogs subjected to haemorrhage (Dempsher & Gann, 1983), hypoxia (Raff, Tzankoff & Fitzgerald, 1981), insulin-induced hypoglycaemia (Keller-Wood, Shinsako, Keil & Dallman, 1981) and ACTH infusion (Wood, Shinsako & Dallman, 1982), but not in resting, normal dogs throughout the day.

Peripheral levels of thyroxine (T₄) vary during the day in man (O'Connor, Wu, Gallagher & Helfman, 1974; Azukizawa, Pekary, Hershman & Parker, 1976), rhesus monkeys (Giannella-Neto, Quabbe & Witt, 1981) and rats (Rookh, Azukizawa, DiStefano et al. 1979), but there is a paucity of information regarding the daily T₄ patterns in the dog. Characterization of the daily peripheral profiles would provide important information regarding regulation of normal secretion
and removal of hormones and would serve as a base for future studies during altered conditions. The present study was initiated to examine changes in plasma levels of ACTH, cortisol and T₄ during a 24-h period in normal and adrenalectomized dogs.

MATERIALS AND METHODS

Eleven healthy adult cross-bred dogs (six intact males and five anestrous females) weighing between 14·9 and 32·6 kg were used. The dogs, ranging in age from 1·5 to 2·5 years, were raised within our laboratory animal facility and were permitted regular daily exercise. Each dog was shown to have a normal response to injection of bovine thyrotrophin (Dermathycin; Jensen-Salsbery, Kansas City, Missouri, U.S.A.), ACTH(1-24) (Cortrosyn; Organon, West Orange, New Jersey, U.S.A.) and dexamethasone (Azium; Schering, Kenilworth, New Jersey, U.S.A.), before being included in the study. Normal responses were defined as a plasma T₄ concentration greater than 51 nmol/litre 5 h after an i.v. injection of thyrotrophin, a plasma cortisol concentration less than 41 nmol/litre 4 h after an i.v. dose of 0·01 mg dexamethasone/kg and a plasma cortisol level between 220 and 413 nmol/litre 1 h after ACTH administration. The dogs were housed for 1 month in individual cages in a room with constant temperature (24 °C) and were exposed to 12 h light:12 h darkness (lights on at 07·00 h). Over this period, the dogs were acclimatized to the continual presence of people in their living quarters. The diet consisted of a standard pelleted ration fed once daily in the morning with water continually available.

Two dogs were anaesthetized with sodium thiopental, intubated and maintained on a mixture of halothane and oxygen for bilateral adrenalectomy through a midline abdominal incision. These operations were performed 2 weeks before the start of each bleeding period. After surgery the dogs developed a reduced serum sodium:potassium ratio and were then given 5 mg cortisol acetate i.m. (Rugby Laboratories, Rockville Centre, New York, U.S.A.) and 0·1 mg fludrocortisone acetate orally (Florinef; E. R. Squibb, Princeton, New Jersey, U.S.A.) each day. These treatments were discontinued 2 days before the sampling periods. Following the sampling periods, postmortem examination of both dogs revealed no visible adrenal gland tissue.

The dogs were divided into two groups for the collection of blood samples. The first group consisted of five normal and one adrenalectomized dog, the second of the remaining four normal and one adrenalectomized dog. Sampling of dogs commenced at 09·00 h in the first group after a 12-h fast while the other group was bled starting at 18·00 h 6 days later. Blood samples were obtained at 20-min intervals for 25 h through cannulae (Tygon S-54-HL; inner diameter 1·27 mm, outer diameter 2·29 mm; Norton, Akron, Ohio, U.S.A.) surgically inserted (using sodium thiostyl anaesthesia 2 days before sampling) into the jugular vein and passed to the level of the right atrium. The cannulae were filled with heparinized saline and the exteriorized portion was wrapped with gauze and securely positioned beneath nylon jackets (Alice King Chatham, Los Angeles, California, U.S.A.) which were placed on the dogs 1 week previously. One hour before the first sample collection commenced, the cannulae were connected to extension tubing of the same material which exited the top of the cage through a flexible steel cable which permitted the dog unrestrained movement. The free ends of the cannulae extended to a position behind the cages, out of sight of the dogs. During the period of darkness, sample collection was facilitated by the use of a dim light, whose field of illumination was restricted to an area immediately around the sampling port of the cannulae. Blood samples (2 ml) were taken after first removing 3 ml fluid (including 0·5 ml blood). To replace the lost fluid, 2 ml 0·9% (w/v) NaCl solution were administered followed by 3 ml 2% sodium citrate in a 0·9% NaCl solution. The blood samples were immediately placed in chilled polystyrene tubes containing 3 mg EDTA and 1000 Kallikrein Inhibitor Units of the proteinase inhibitor, aprotonin (Trasylo; Mobay Chemical, New York, U.S.A.). Plasma was harvested within 15 min and stored at -20 °C. Haematocrits declined by an average of 4·1% over the 25-h period. No attempt was made to record sleep periods although periodic observation suggested polyphasic sleep patterns in each animal. The dogs appeared to be relaxed throughout the sampling period and were not disturbed by the sampling procedure.

Hormone analyses

Plasma cortisol (Kemppainen, Thompson & Lorenz, 1983) and T₄ levels were determined using radioimmunoassay kits (Diagnostic Products, Los Angeles, California, U.S.A.). Serial dilution and assay of a dog plasma pool gave inhibition curves with slopes similar (P > 0·05) to the T₄ standard. When varying amounts of T₄ were added to charcoal-treated dog plasma and assayed, the resulting data described a line with the equation \( y = 1·01x + 0·07 \) (x = amount added; y = amount measured) with a correlation coefficient of 0·99. Cross-reactions with the T₄ antiserum were determined for 3·3·5-tri-iodothyronine (2·3%), 3·3·5'-tri-iodothyronine (1·9%), diiodothyronine, monoiiodotyrosine and diiodotyrosine (all <0·01%). The sensitivity of the T₄ assay was 1·9 nmol/l; for the cortisol...
sol assay, 4.4 nmol/l. Intra- and interassay coefficients of variation were approximately 5 and 10%, respectively, for both assays.

Plasma immunoreactive (i) ACTH was measured in non-extracted plasma following the methods of Nicholson, Davis, Sherrell & Orth (1984). The antisera used (immunoglobulin G (IgG)-ACTH-1; IgG Corporation, Nashville, Tennessee, U.S.A.) was produced in rabbits immunized with ACTH(1–24) conjugated to bovine serum albumin and is specific for the 5–18 sequence of ACTH (Nicholson et al. 1984). Porcine ACTH was used for the standard (79.3 i.u./mg; IgG Corporation) and for $^{125}$I-labelled ACTH (Damon Diagnostics, Needham Heights, Massachusetts, U.S.A.). The $^{125}$I-labelled ACTH was purified immediately before use with Quso G32 glass (Philadelphia Quartz, Philadelphia, Pennsylvania, U.S.A.) as described by Berson & Yalow (1968). Parallel inhibition curves were obtained between serial dilutions of normal and adrenalectomized dog plasma, and standards. When known amounts of porcine ACTH were added to dog plasma containing low concentrations of iACTH and assayed, linear regression analysis of the results gave an equation $y = 1.15x - 2.9$ with a correlation coefficient of 0.99. The sensitivity of the assay was 1.1 pmol/l, intra- and interassay coefficients of variation were approximately 10.3 and 15.5% respectively. Plasma levels of iACTH were not detectable in dogs 6 h after an i.v. injection of 0.1 mg dexamethasone/kg; conversely, iACTH increased six-fold 30 min after an i.v. injection of 0.5 i.u. regular insulin/kg.

Data analyses

Hormonal profiles were analysed for episodic peaks using the Fortran program, PULSAR, designed and described by Merriam & Wachter (1982). Confidence limits were determined by multiple assay of plasma pools containing three different concentrations of each hormone (Baxter, 1980). The coefficients describing the relationship between dose of hormone and intra-assay standard deviation were included in the PULSAR program to adjust for assay precision at varying concentrations. Peak cut-off criteria were selected using the empirical method of visual inspection of random hormone profiles as described by Merriam & Wachter (1982).

The presence of a circadian rhythm in hormone concentrations was evaluated by calculating the hourly moving means for each hormone, which minimizes short-term fluctuations while maintaining overall trends (Fulkerson & Tang, 1979). Also, the frequencies of hormone peaks were compared during different periods of the day. Correlations between levels of iACTH and cortisol were determined using the Pearson Product Movement correlation procedure provided by the Statistical Analysis System (SAS Institute, Cary, North Carolina, U.S.A.). Where appropriate, Student's $t$-test for unpaired data and a $\chi^2$ contingency analysis were also used.

RESULTS

Hormonal profiles of dogs bled sequentially starting at 09.00 h were similar to those bled starting at 18.00 h. Hence, the data from both groups were combined in the analyses.

All nine normal dogs showed episodic fluctuations in iACTH and cortisol levels during the 24-h period (Table 1 and Fig. 1). Considerable variation was apparent between individuals in the number of peaks per day (range: cortisol 1–17, iACTH 6–12) and in the peak amplitude (cortisol 24.8–85.5 nmol/l, iACTH 1.3–5.1 pmol/l) of each hormone. Some of the variability was explained on the basis of a sex difference (Table 2), with females showing significantly greater 24-h mean cortisol and iACTH levels, numbers of cortisol peaks, and amplitude of iACTH peaks than males. Individual profiles of iACTH and cortisol showed a generally good correlation of the two hormones (Fig. 1), which was confirmed by the finding of a significant ($P < 0.01$) correlation in the levels of the two hormones in all normal dogs compared with no time lag between determinations. However, time points where a dissociation between concentrations of the

| TABLE 1. Analyses of hormonal profiles obtained from plasma samples from nine normal dogs bled at 20-min intervals for 25 h. Values are means ± s.d. |
|--------------------------|--------------------------|--------------------------|
| **Hormone**              | **iACTH**                | **Cortisol**             | **Thyroxine**            |
| **Determination**        | **(nmol/l)**             | **(nmol/l)**             | **(nmol/l)**             |
| 24-h mean                | 3.6 ± 1.6                | 58.5 ± 16.0              | 17.8 ± 4.4               |
| Number of peaks/24 h     | 9.0 ± 2.0                | 10.1 ± 6.3               | 3.3 ± 2.9                |
| Peak amplitude (nmol/l)  | 2.9 ± 1.2                | 41.4 ± 20.1              | 9.5 ± 2.9                |
| Peak duration (min)      | 51.0 ± 10.8              | 46.2 ± 19.8              | 76.2 ± 55.2              |

* Immunoreactive (i) ACTH concentrations in pmol/l.
two hormones existed were apparent (for example, see Fig. 1b).

A few individual profiles of iACTH and cortisol concentrations showed a tendency for a moving mean baseline during the 24-h period (Fig. 1b). However, these variations were of small magnitude, not consistent as to time of day, and non-existent in most dogs.

**TABLE 2.** Sex differences in hormonal data obtained from plasma samples from nine normal dogs bled at 20-min intervals for 25 h. Values are means ± S.D.

<table>
<thead>
<tr>
<th>Determination</th>
<th>Female (n = 4)</th>
<th>Male (n = 5)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-h mean cortisol (nmol/l)</td>
<td>69.5 ± 16.0</td>
<td>47.7 ± 1.7</td>
<td>0.025</td>
</tr>
<tr>
<td>Number of cortisol peaks/24 h</td>
<td>15.2 ± 1.7</td>
<td>5.0 ± 1.2</td>
<td>0.005</td>
</tr>
<tr>
<td>24-h mean iACTH (pmol/l)</td>
<td>4.9 ± 0.5</td>
<td>2.1 ± 0.9</td>
<td>0.01</td>
</tr>
<tr>
<td>Amplitude of iACTH peaks (pmol/l)</td>
<td>3.8 ± 0.9</td>
<td>1.9 ± 0.4</td>
<td>0.02</td>
</tr>
<tr>
<td>24-h mean thyroxine (nmol/l)</td>
<td>20.7 ± 3.6</td>
<td>15.4 ± 3.6</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* P value determined using Student’s t-test; i, immunoreactive.
When an hourly moving mean profile was calculated for cortisol using data from the nine normal dogs (Fig. 2a), no obvious circadian rhythm in concentrations was apparent. An hourly moving mean profile for iACTH concentrations similarly showed no obvious daily variation. There were no differences in the frequencies of hormonal peaks throughout the 24-h period when the numbers of peaks were compared between four periods (6-h intervals) for either iACTH or cortisol.

Levels of iACTH were increased approximately 50- to 150-fold in both adrenalectomized dogs (mean levels, 390 pmol/l; Fig. 2b) over normal dogs. Large amplitude episodic variations in iACTH concentrations were evident while the PULSAR analysis detected no cortisol peaks. One dog had ten iACTH peaks, the other had 12 peaks; the mean amplitude of the peaks were 205 and 200 pmol/l in the first and second adrenalectomized dog respectively. Cortisol was measurable in both dogs which was probably a result of the previous cortisol acetate treatment. Circadian periodicity in iACTH levels was not evident in either dog.

Thyroxine concentrations fluctuated less dramatically than did either iACTH or cortisol (Fig. 3 and Table 1). A sex difference in the 24-h mean concentration of T₄ was found (Table 2). As with the other hormones, no circadian rhythm in T₄ levels was detected.

**DISCUSSION**

The results of this study clearly show that plasma levels of iACTH and cortisol vary episodically in dogs, which presumably reflects episodic pituitary and adrenocortical secretion. Also, concentrations of iACTH and cortisol showed a high correlation although periods of dissociation were apparent in individual profiles. Such a dissociation along with a significant overall correlation has also been reported in man (Krieger & Allen, 1975) and may relate to the 20-min sampling interval and the differing half-lives of ACTH (1.8–2.1 min, Wood et al. 1982) and cortisol (50 min, McCormick, Herman, Lien & Egdahl, 1974) in the canine species as well as to yet undefined factors (Krieger & Allen, 1975). The average number of cortisol secretory episodes (10.1) in the 24-h period was similar to the number shown to occur in humans (5–10, Krieger et al. 1971; or 7–13, Weitzman, Fukushima, Nogeire et al. 1971), from whom blood samples were collected at about the same frequency as from the dogs in the present study. The frequency of cortisol peaks were equally distributed throughout the 24-h period in the dogs, in contrast to humans, who showed an increase in the number of episodes of secretion in the early morning hours, coincident with the zenith in circadian periodicity (Krieger et al. 1971; Weitzman et al. 1971).

Considerable individual variability in pituitary-adrenocortical activity was apparent in the dogs, some
of which could be explained on the basis of a sex difference, in that mean levels and episodic activity of iACTH and cortisol were generally greater in females. A similar sex difference in corticosteroid levels has been reported in rats (Retiene, Zimmerman, Schindler et al. 1968) while in man, males had higher mean cortisol levels (Zumoff, Fukushima, Weitzman et al. 1974). The reasons for or effects of such sex differences are unknown.

Surgical placement and the continued presence of cannulae constitute a stress which may alter pituitary-adrenocortical activity. Further, repeated blood sampling and fluid replacement may also perturb this endocrine system. To minimize these potential adverse effects, the dogs in this study were acclimatized to the conditions necessary for repeated blood removal. The cannulation procedure was performed rapidly using an ultra-short-acting barbiturate anaesthetic. To reduce the potential for infection and the influences of chronic cannulae bearing, the bleed periods were started 48 h after cannulae placement. To examine whether the sampling procedure itself might influence the hormonal profiles, the dogs were divided into two groups for the sampling periods, with the initiation of sampling in the morning (09.00 h) in one group and in the early evening (18.00 h) in the other. No influences relating to the start of blood sampling were discernable. Further, the effects of stress did not appear to be profound since plasma cortisol concentrations measured in each dog at the beginning of the sampling periods were within normal baseline ranges reported by others (Johnston & Mather, 1978; Takahashi et al. 1981) and levels did not continue to increase consistently throughout the sampling period. Nevertheless, the procedures necessary to conduct experiments of this type may subtly influence normal endocrine events.

The maintenance of episodic iACTH activity in the adrenalectomized dogs suggests that the genesis of such activity does not require adrenal steroid feedback. Cortisol was detected in the plasma of both dogs which was probably due to the relatively prolonged release of the hormone from the i.m. injections of cortisol acetate (last dose given 48 h before blood sampling). However, this level of cortisol did not maintain iACTH concentrations in the normal range nor did it prevent the episodic release of iACTH. Similar findings were reported in patients with Addison's disease who, in addition, maintained a circadian rhythmicity in plasma ACTH concentrations (Krieger & Gewirtz, 1974). Plasma ACTH concentrations in the patients in this latter study were apparently more resistant to glucocorticoid negative feedback influences than concentrations in healthy subjects. Data from the present study are not sufficient to determine whether a similar condition exists in the glucocorticoid-deficient dog. The present results do suggest that normal episodic variations in plasma cortisol concentrations influence factors which regulate the amplitude of pituitary ACTH release, since the magnitude of the iACTH peaks was considerably raised in the adrenalectomized dogs over the normal dogs. Further studies employing a greater number of adrenal-deficient dogs are needed to confirm and expand upon these observations.

Concentrations of T4 showed episodic variation during the day in all dogs, but the number of peaks were considerably less than for either iACTH or cortisol. Fluctuations in peripheral T4 concentrations have been reported in man (O'Connor et al. 1974; Azukizawa et al. 1976), rhesus monkeys (Giannella-Neto et al. 1981) and rats (Rookh et al. 1979). Since T4 has a relatively long biological half-life in the dog (16-6 h; Ganjam, Wyckoff, Comerci & Ravis, 1980), it is unlikely that these short-lived fluctuations result from pulsatile secretion. Instead, they may in part result from haemodynamic and plasma protein fluxes (Azukizawa et al. 1976) or to factors not yet known (Giannella-Neto et al. 1981). A sex difference in T4 concentrations has not been reported for the dog, and the finding of higher 24-h mean T4 levels in females over males contrasts with data obtained in the rat (Fukuda, Greer, Roberts et al. 1975).

The absence of any evidence of a circadian periodicity in concentrations of cortisol or iACTH further supports the findings of Johnston & Mather (1978) and Takahashi et al. (1981) that the dog does not have a circadian rhythm in pituitary-adrenocortical activity. The usefulness of calculation of an hourly moving mean of a hormone concentration versus clock time to evaluate circadian rhythmicity in a group of animals relies on a relatively consistent pattern of overall peaks and troughs amongst individuals. Such a consistency would be anticipated when the subjects are maintained under similar environmental conditions (Aschoff, 1979). The plasma profiles of iACTH and cortisol concentrations for some dogs in the present study did show minor variations in mean baseline levels during the 24 h which were not consistent as to time of day. Additional studies are necessary whereby dogs are sampled for longer periods (48–72 h) to see if such variations persist across days. Similar minor daily differences in mean baseline concentrations of plasma cortisol were reported by Takahashi et al. (1981) who found that one dog had a raised cortisol level during the light period while another dog tended to have a lowered cortisol level during this period. Sleep–wake patterns may also influence endocrine circadian periodicity (Aschoff, 1979) and were not recorded in the present study. However, sleep–wake periods were recorded in the dogs studied by Takahashi et al. (1981) and mean baseline plasma cortisol concentrations
appeared to correlate only to a minor degree with these periods. As documented in the study by Takahashi et al. (1981) and observed in the present study, the sleep–wake patterns of dogs are polyphasic. Hawking, Lobban, Gammage & Worms (1971) found that circadian variations in physical activity, body temperature and urinary water and electrolyte excretion were non-existent or only weakly apparent in dogs, which contrasts with findings in many other species, including man. The findings of these previous studies, taken together with our results, support the view of Moore-Ede, Sulzman & Fuller (1982) that dogs possess a fundamentally different circadian organization from most other mammalian species.

ACKNOWLEDGEMENTS

This work was supported by an Auburn University Grant-in-Aid, number 2-13635 and is published as number 1678, School of Veterinary Medicine, Auburn University, Alabama, U.S.A. We wish to thank Dr B. Reed for assistance with the statistical evaluation and Drs C. Branch and L. Swango for use of facilities and technical advice. We should also like to thank the Biomedical Computing Technology Information Center, Nashville, Tennessee, U.S.A., for providing a copy of the PULSAR program.

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