Plasma Adrenocorticotropic Hormone and Cortisol in Pigs: Effects of Time of Day on Basal and Stressor-Altered Concentrations[1]

(42828)

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Abstract. **An initial study was conducted to establish the presence in plasma of diurnal rhythms of immunoreactive porcine adrenocorticotropic hormone (pACTH) and cortisol in castrated male pigs (barrows). Fourteen barrows with jugular catheters were bled at 6-hr intervals for 24 hr. Significant changes in plasma pACTH were evident with peak levels (61 ± 6 pg/ml) at 0100–0700 hr and a trough (38 ± 4 pg/ml) at 1900 hr. Changes ($P < 0.05$) in plasma cortisol were also present in barrows with a peak (44 ± 6 ng/ml) at 0700 hr and a trough (21 ± 5 ng/ml) at 1900 hr. Plasma norepinephrine and epinephrine were measured at the same time intervals and did not differ among hours. In these unstressed pigs the ratio cortisol/$\log_{10}$ pACTH at 0700 hr (25.3 ± 3.0) was greater than the ratio at 1900 hr (12.9 ± 2.7). Sequential blood samples were subsequently taken on four of the barrows 12 and 26 days later. Plasma pACTH was variable among pigs and did not differ among hours. Plasma cortisol on both dates was greater ($P < 0.05$) in the morning (0100 or 0700 hr) than at 1900 hr. The ratio cortisol/$\log_{10}$ pACTH at 0700 hr was repeatedly greater than at 1900 hr. A second study was conducted to determine whether plasma pACTH and cortisol responses to mild (32°C for 2 hr) or strong (20-min restraint) stressors were dependent on the time of day of stressor application (0800 hr, AM; 1600 hr, PM). Response-associated parameters (maximum concentration, maximum incremental concentration, and integrated response) for pACTH and cortisol did not differ between AM and PM. However, a qualitative difference existed between the AM and PM plasma pACTH responses to restraint +32°C wherein the AM response consisted of a single prolonged surge, and the PM response of an initial major peak followed by a second significant minor peak. A suggested explanation is that the initial 20-min restraint stressor potentiated the hypothalamic-hypophyseal response to 32°C. These studies are the first direct measurements which suggest the presence of diurnal changes in plasma ACTH and cortisol in barrows. The studies also indicate for barrows an absence of diurnal changes in plasma epinephrine and norepinephrine. The responsiveness of the pituitary-adrenocortical axis to stressors did not exhibit quantitative diurnal changes at the time periods measured. However, it is hypothesized that the repeatable AM-PM difference in the ratio cortisol/$\log_{10}$ ACTH reflects a diurnal change in adrenal responsiveness to ACTH in unstressed pigs.** [P.S.E.B.M. 1989, Vol. 190]

A circadian rhythm in adrenocortical function has been described in a variety of species (1–4), including pigs (5–7). Measured circadian changes in corticosteroids are associated with similar changes in plasma adrenocorticotropic hormone (ACTH) in those species in which ACTH has been measured (8–10). Alterations in ACTH presumably reflect the actions of circadian changes in release of hypothalamic corticotropin releasing factor (CRF) (11, 12). Circadian rhythms in ACTH and corticosteroids occur synchronously, and it is generally assumed that the former is in part responsible for the latter (13). Adrenal changes in sensitivity to ACTH (12–14), or efferent neural connections to the adrenal (15), may also mediate circadian release of corticosteroids.

In addition to circadian fluctuations in plasma

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ACTH and corticosteroids, there is also evidence for diurnal changes in stressor or CRF-associated ACTH responses (4, 12, 16, 17). Even more extensive evidence exists in a variety of species for circadian differences in adrenocortical responses to a variety of stimuli (1, 4, 15, 17–24).

In pigs there have been no reported studies concerning the presence or absence of diurnal changes in plasma ACTH, nor has it been determined whether diurnal changes in plasma cortisol, previously reported to occur in boars (5), persist after castration. Orchidectomy of a nonhuman primate was associated with the absence of the normal circadian rhythm in plasma cortisol (25). Additionally, no investigations have been previously conducted to ascertain diurnal differences in ACTH or cortisol responses to stressors. Hence, studies reported herein were performed to evaluate these pituitary-adrenocortical-related phenomena. Previous investigators have demonstrated that diurnal differences in response depend on the intensity and/or duration of the stressor (16, 19), hence both a strong stressor (restraint for 20 min) and a mild stressor (exposure to 32°C for 2 hr) (26) were employed.

Materials and Methods

Animals, Surgery, and Blood-Sampling Techniques. Thirty-nine crossbred barrows (one-fourth Yorkshire: one-fourth Landrace: one-fourth Large White: one-fourth Chester White) weighing 70–102 kg were used in the acute stressor study, while 14 crossbred barrows weighing 34–50 kg were used in the experiment involving a determination of circadian changes in plasma ACTH. Barrows were housed in environmental chambers measuring 4.9 × 5.2 m, capable of maintaining a constant temperature over the range of −20 to 40°C. In the first study involving diurnal changes in plasma hormonal levels, one group of four barrows was housed in a larger room (4 × 17 m). Within these chambers or room, each barrow was housed in an individual pen with dimensions of 1.2 × 0.6 m. Each animal had visual, olfactory, auditory, and tactile contact with another animal in an adjacent pen. Animals were provided a corn and soybean meal-based ration and water *ad libitum*. Throughout both studies animals were maintained in a photoperiod consisting of 12-hr light (lights on 0600–1800 hr).

A microrenathane catheter (2.03 mm o.d. × 1.02 mm i.d.; Braintree Scientific Inc., Braintree, MA) coated on its internal and external surfaces with 7% TDMAC-heparin (Polysciences Inc., Warrington, PA) was surgically implanted in the jugular vein of halothane-anesthetized pigs using previously described techniques (27). Surgery was conducted either 6-7 days (acute stressor study) or 3 weeks (diurnal changes in plasma hormones study) prior to treatment initiation. Patency of these catheters was maintained by periodic flushing with 10 ml of sterile 0.15 M NaCl solution which contained 50 IU/ml of heparin and 1% benzyl alcohol, with pH adjusted to 7.4. Prior to blood sampling a 1.5- to 1.8 m-catheter extension (Silastic Medical Grade, 3.18 mm o.d. × 1.57 mm i.d.) was attached to each animal the night before blood samples were to be taken to allow blood to be obtained from outside the pen and without disturbing the pig. In both studies blood samples were obtained using 10-ml syringes containing 10 mg of EDTA in 200 μl 0.15 M NaCl solution. Blood samples were immediately placed on ice and subsequently centrifuged at 1500g_{av} for 20 min at 2°C. The supernatant from this centrifugation was subsequently recentrifuged at 3584g_{av} for 20 min at 2°C (28). Plasma for cortisol determinations was then stored frozen at −20°C, while an additional aliquot for ACTH determination was rapidly frozen in liquid nitrogen and stored at −100°C.

pACTH Radioimmunoassay. Immunoreactive porcine ACTH (pACTH) in plasma was measured using a homologous double antibody procedure in unextracted plasma (29). This assay uses anti-ACTH serum (produced in a rabbit immunized with ACTH 1-24) and porcine ACTH, with immunoreactivity and biological activity identical to that of synthetic ACTH 1-24, for iodination and as a standard (IgG Corp., Nashville, TN). Details of the iodination and assay procedure have been previously reported in detail (29). Variations from reported procedures include use of a 14- × 0.8-cm column of Sephadex G-25 for initial separation of iodinated ACTH from free iodide subsequent to iodination; a 3-day preincubation period of first antibody and plasma or first antibody and standards prior to addition of ^{125}I-pACTH; and, finally, a 3-day incubation period after addition of ^{125}I-pACTH and prior to addition of the second antibody. This assay was extensively validated for use with porcine plasma. Serial dilutions of four porcine plasma pools (6–100 μl) produced competition curves with an average slope of $b = -1.11$ and whose individual slopes did not differ significantly ($P > 0.05$) from that of the standard curve ($b = -1.09$). A serial dilution of plasma (12.5–100 μl) from which endogenous ACTH was removed via treatment with silicic acid (SIL-A-200; Sigma, St. Louis, MO) demonstrated no competition with labeled pACTH for the first antibody. Accuracy for the pACTH radioimmunoassay was assessed by addition of known amounts of pACTH (0.6–8 pg) to porcine plasma previously treated with silicic acid to remove endogenous ACTH. The average accuracy of estimates was 102%. A plot of expected vs measured picograms of pACTH had a slope ($b = 1.08$) which did not differ from 1 ($P > 0.05$) and a y-intercept (−0.15) which did not differ from 0 ($P > 0.05$). Sensitivity of the assay as determined by calculating the lower 95% confidence limit for the y-intercept (100% tubes) of the seven standard curves reported in this article was 0.4 pg/tube, or as indicated by the lowest standard in the linear range of the standard curve it was 1.5 pg/tube. The interassay coefficient of variability (CV) was 9.61%, and the intraassay CV measured over the 805 plasma samples assayed in duplicate was 3.27%.

Cortisol Radioimmunoassay. To correct for procedural losses, cortisol ([1,2,6,7-^{3}H]hydrocortisone; New England Nuclear, Boston, MA) was added to a 200-μl plasma aliquot, subsequently mixed thoroughly, and equilibrated for 16–20 hr at 4°C. Plasma and internal trace were than extracted with 2 ml of anhydrous 100% ethanol (USI Chemical Co, Tuscola, IL). After thorough mixing for 3 min, the samples were centrifuged at 1900g_{av} for 10 min at 4°C. Two-hundred microliters of ethanol extract were then removed, transferred to a scintillation vial, evaporated at room temperature for 16–20 hr in air, resuspended in liquid scintillation fluid (Budget Solve; Research Products International, Mount Prospect, IL), and counted in a Packard liquid scintillation counter. For the radioimmunoassay proper, additional 200-μl aliquots of ethanol extract were transferred to 12- × 75-mm polypropylene tubes, and the ethanol was evaporated under nitrogen for 10 min at 41–44°C. Contents of the tubes were then resolubilized in 0.1 *M* sodium phosphate buffer containing 0.15 *M* NaCl, 0.002% thimersol, and 1% bovine serum albumin, pH 5.0 (cortisol buffer A). ^{125}I-cortisol (20,000 cpm; Cambridge Medical Diagnostics, Billerica MA) in 50 μl of buffer A and first antibody (Cambridge Medical Diagnostics, Billerica MA) in 50 μl of buffer A, which contained 1% normal rabbit serum in lieu of bovine serum albumin, were then added. The final incubation volume was 500 μl, and the concentration of first antibody in the radioimmunoassay tubes was 1:100,000. The second antibody (100 μl at a 1:20 dilution in buffer A of sheep anti-rabbit serum prepared at the USDA-Meat Animal Research Center, Clay Center, NE) was added, and the incubation continued for 16–24 hr at 4°C. The incubation was terminated by addition of 2 ml of buffer A containing 3% bovine serum albumin and subsequent centrifugation at 3584g_{av} for 30 min at 4°C. After decanting supernatants, radioactivity within pellets was counted on a gamma counter. Serial dilutions (20–200 μl) of three porcine plasma pool extracts had an average slope of $b = -0.93$, and individual slopes which did not differ significantly from that of the standard curve ($b = -0.87$). A serial dilution (25–300 μl) of ethanol extract of plasma that had been previously treated with dextran-coated charcoal to remove endogenous steroids produced no competition whatsoever with labeled cortisol for the first antibody. Accuracy of the assay was determined by addition of known quantities of cortisol (6.25–50 ng) to 200-μl aliquots of two different porcine plasma pools and thereafter proceeding with the extraction process and radioimmunoassay. After correcting for extraction losses the average accuracy in the two pools was 111.9%. Plots of expected vs measured produced slopes ($b = 1.03$ and 1.33) which did not differ significantly from 1 and *y*-intercepts which did not differ from 0 ($P > 0.05$). The precision of the assay as measured by the average coefficient of variability of the 818 samples assayed in duplicate for the studies presented was 3.9%. The interassay CV for the seven assays reported was 11.5%. Sensitivity of the assay as determined by calculating the lower 95% confidence limits for the *y*-intercepts (100% tubes) of the standard curves was 0.74 pg/tube and as indicated by the lowest standard in the linear range of the standard curve, it was 10 pg/tube.

Plasma Catecholamine Assay. Norepinephrine (NE) and epinephrine (E) were measured using high-performance liquid chromatography with electrochemical detection. Equipment was supplied by Bioanalytical Systems, Inc. (BAS, West Lafayette, IN) and included their LC-300 series with a BAS Biophase ODS 5 μ*M* C-18 4.6- × 250-mm column to separate catecholamines. The mobile phase consisted of 0.15 *M* monochloroacetate buffer, pH 3.0, containing 2 m*M* sodium EDTA, and 100 mg of sodium octyl sulfate/liter. With each set of experimental samples, standard curves for E (BAS, Inc.; 66–846 pg) and NE (BAS, Inc.; 47–606 pg) were included.

Plasma samples for measurement of catecholamines were prepared as described above. Prior to freezing, 1 mg/ml of reduced glutathione was added. Samples were stored at −80 to −100°C. Upon thawing, 2 ml of plasma were added to a 5-ml conical polypropylene tube. The following ingredients were then added: 4.5 ng of 3,4-dihydroxybenzylamine (BAS, Inc.) to measure procedural losses, 1 ml of 1.5 *M* Tris-HCl buffer (pH 8.6) at room temperature, and 50 mg of acid-washed alumina (BAS, Inc.). The plasma and associated ingredients were then mixed thoroughly for 15 min at 4°C, centrifuged at 1040g_{av} for 1 min, and the supernatant aspirated. The alumina was then washed with 1 ml of distilled water, recentrifuged at 1040g_{av} for 1 min, and the water wash aspirated. Catecholamines were then extracted from the alumina by addition of 400 μl of 0.1 *M* perchloric acid. This acid extract was then maintained at 4°C until 100 μl were injected into the high-performance liquid chromatography system. Catecholamines were eluted at a flow rate of 1.1 ml/min with a mobile phase temperature of 30°C. The ampometric controller (LC-48; BAS, Inc.) maintained an applied voltage of 0.65 V and employed a IL-5A glassy carbon thin-layer transducer cell with a Model RE-1 Ag/AgCl reference electrode.

The accuracy of this assay was determined by addition of known amounts of E and NE (315–1391 pg) in triplicate to plasma samples. After extraction, measurement, and correction for procedural losses, the average accuracy for NE was 100.9% and for E it was 97.4%. The limit of detection (the amount of catecholamine required to give a response 2-fold greater than noise) for the assay reported was 54.4 pg for NE and 114.6 pg for E. All samples were measured on a single assay which had an intraassay CV of 4.5% for NE and 4.7% for E.

Experimental Design and Procedures

Determination of Diurnal Changes in Plasma pACTH and Cortisol. In this study barrows in environmental chambers were maintained at a constant 20°C and at a relative humidity that ranged from 22–38% (dewpoint temperature of −2 to 5°C). The four barrows kept in the larger room were maintained at a temperature of 22–24°C and at a relative humidity that ranged from 40–75% during a 4-week period. Since hormonal values in these latter four pigs were similar to those of pigs under more controlled environments, it was thought justified to include them in the study. Each animal was bled via the indwelling jugular catheter at 6-hr intervals for a 24-hr period (0700, 1300, 1900, 0100, and 0700 hr). During the 3-week interval between catheterization and removal of blood samples, all pigs were exposed daily to the presence of caretakers and research personnel.

Acute Stressor Study. In this study pigs were allowed to adjust to environmental chambers and to the daily presence of caretakers or research personnel for 6-7 days prior to use. The experiment involved a randomized complete block design. Each block used eight barrows, was conducted over a 2-week period, and included the following eight treatments to which pigs were randomly assigned: (i) control animals sampled during the morning and afternoon hours (0700–1600 hr, n = 5); (ii) control animals sampled during the afternoon and nighttime hours (1500–2400 hr, n = 5); (iii) pigs subjected to a 20-min restraint (0800–0820 hr, n = 5); (iv) pigs subjected to a 20-min restraint (1600–1620 hr, n = 5); (v) pigs subjected to 32°C for approximately a 2-hr period (0800–1000 hr, n = 5); (vi) pigs subjected to 32°C for approximately a 2-hr period (1600–1800 hr, n = 5); (vii) pigs subjected to a 20-min restraint (0800–0820 hr, n = 5) and to 32°C for 2 hr (0800–1000 hr); and (viii) pigs restrained for 20 min (1600–1620 hr, n = 4) and subjected to 32°C for 2 hr (1600–1800 hr). For morning treatments (i, iii, v, vii) blood samples were obtained at 20-min intervals initially (0700–1100 hr) and at hourly intervals thereafter (1200–1600 hr). For afternoon treatments (ii, iv, vi, viii), blood samples were obtained at 20-min intervals initially (1500–1900 hr) and at hourly intervals thereafter (2000–2400 hr).

Pigs subjected to restraint were transferred from their pens to a mobile restraining cage constructed of steel bars padded with foam rubber with cage dimensions of 1.22-m long × 0.25-m wide × 0.7-m high. One side of this cage was adjustable, and hence the pig could be firmly immobilized without causing it physical pain. Transfer time was 2–4 min and blood samples were taken immediately after transfer (0800 or 1600 hr). A subsequent blood sample was obtained just prior to returning the pig to its home pen (0820 or 1620 hr). Because of limitations in the number of chambers available, pigs subjected to 32°C were transferred from their home pen to a similar pen in a second environmental chamber 24 hr prior to treatment. A separate treatment group involving only transfer to a new chamber to ascertain potential effects of a novel environment was not included. Hence, 32°C treatment included not only the 2-hr temperature increase, but also effects of movement (24 hr earlier) to a different chamber. The latter effects, however, are undoubtedly minimal since these barrows had baseline pACTH and cortisol concentrations similar to those of other barrows (see Results). On the day of treatment at either 0800 or 1600 hr, the temperature in the environmental chamber was increased from 20 to 32°C at an average rate of 60°C/hr. There was an average temperature overshoot of 4.2°C which lasted 20 min before a stable 32°C was achieved. Two hours after the initial temperature change, ambient temperature was returned to 20°C at an average rate of 54.4°C/hr. After an average temperature undershoot of 6.4°C lasting 24 min and a rebound to 24°C lasting 26 min, temperatures stabilized at 20°C. The average exposure time to 32°C or higher was 1.8 hr. In this study pigs were maintained at 20°C (dry bulb temperature) and an average dewpoint temperature of 2.8°C (average relative humidity of 33%) except as indicated above—for those animals subjected to a transient 32°C (average dewpoint temperature of 5.3°C and relative humidity of 20%).

Statistical Analysis. Data were analyzed using multiway analysis of variance for repeated measures to test for main effects and interactions and made use of the General Linear Models of the Statistical Analysis System (30). For the initial study concerning determination of diurnal rhythms of plasma ACTH and cortisol, the appropriate error term for hour effects is the residual mean square from the analysis of variance. This error term is also applicable when using the Student-Newman-Keuls test for determining the differences among specific means. In the continuation of that study, involving comparisons of plasma pACTH and cortisol on days 12 and 26, the error term used to test for day effects was pigs × day and for hour effects it was pigs × hour. In the acute stressor study the appropriate error term for treatment effects (control, restraint, 32°C, restraint + 32°C), as well as for general time of day (AM or PM), is the mean square associated with subjects within groups—pigs (treatment × AM-PM). To test for differences among hours (the repeated measure term) the residual mean square is the appropriate error term (31). Subsequent tests to determine significant differences among individual means conformed to procedures detailed by Winer (31) and involved use of the conservative Student-Newman-Keuls a posteriori test for comparison of all means, or use of the more robust a priori linear orthogonal combinations for preplanned comparisons of specific means (32). Comparisons among parameters obtained from the original data (basal levels, maximum levels, maximum changes, and integrated responses) involved multiway analyses of variance, and the above-indicated comparisons of means. Data were tested for normality of distribution via the Shapiro Wilk Statistic (33) and for homogeneity of variance via the $F_{\max}$ test. Data were log or square root transformed where necessary to fulfill assumptions of analysis of variance. A probability level of <0.05 was considered significant. The integrated responses above baseline values were computed using the trapezoidal rule (34), which calculates the area under the response curve. In some instances slopes of response variables were compared by analysis of covariance (32).

Results

Diurnal Changes in Plasma pACTH and Cortisol.

As indicated in Figure 1, average plasma concentrations of immunoreactive pACTH declined steadily ($P < 0.05$) during daylight hours and were increased ($P < 0.05$) during the early morning hours. From minimum to maximum there was a 64% increase in plasma ACTH. Eleven of the pigs (79%) exhibited changes in plasma pACTH similar to the average profile shown in Figure 1. Of these 11 pigs, 91% had peak concentrations of pACTH at 0100–0700 hr and troughs at 1300–1900 hr.

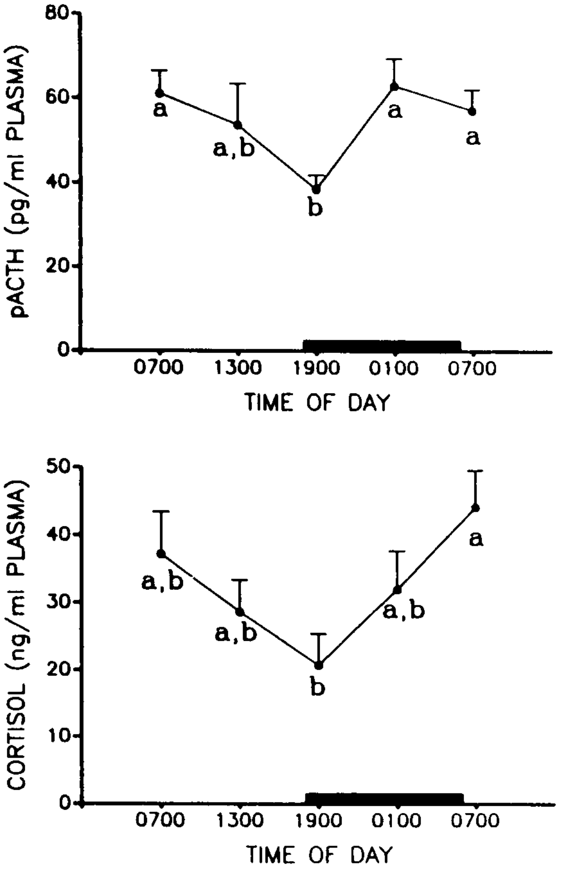

Figure 1. Plasma immunoreactive pACTH and cortisol concentrations in barrows. Blood samples were obtained via indwelling jugular catheters at the time periods indicated. Each datum point represents the mean ± SE of 14 barrows. Means with different subscripts are significantly different ($P < 0.05$).

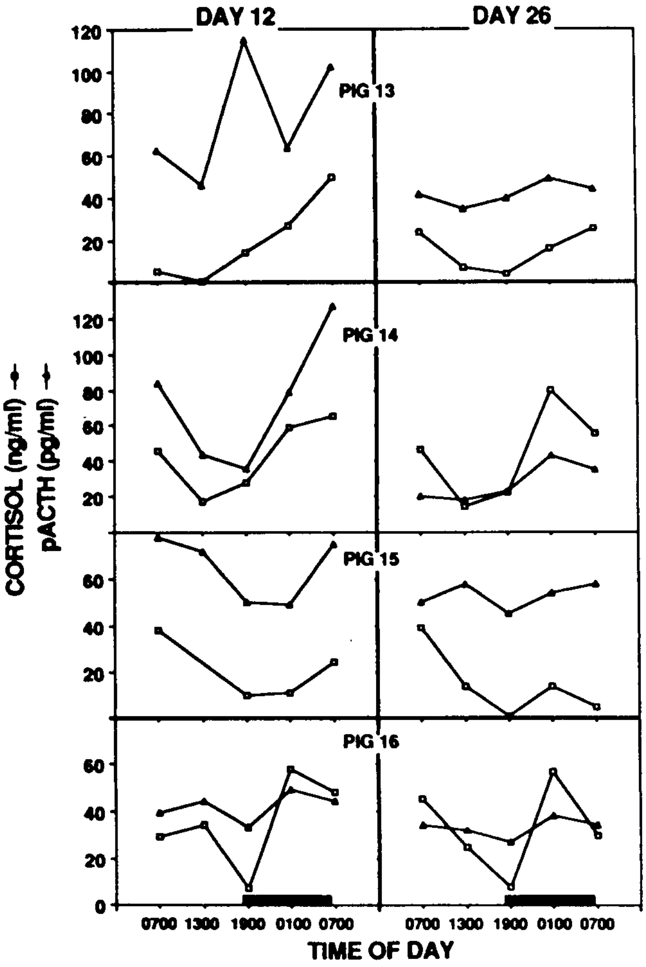

Figure 2. Plasma immunoreactive pACTH and cortisol concentrations in four barrows on 2 days subsequent to the sampling period described in Figure 1. Blood samples were obtained via indwelling jugular catheters at the time periods indicated.

Changes in plasma cortisol concentrations were similar to those of pACTH and from minimum to maximum there was a 114% increase in plasma cortisol. Nine of the 14 barrows (64%) exhibited changes in plasma cortisol similar to the average changes depicted in Figure 1. All of these nine barrows had peaks at 0100–0700 hr and troughs at 1300–1900 hr. The remaining barrows which did not exhibit plasma pACTH or cortisol profiles exactly characteristic of the average trends present in Figure 1 had variations in timing of peak and trough hormonal levels, and in some animals two peaks were present. Such variability was also present on subsequent dates as evidenced by data represented in Figure 2 which is discussed below.

At the time intervals measured, there was a slight ($r = 0.34$) but significant ($P < 0.01$) correlation between plasma pACTH and cortisol. Data in Table I represent

Table I. Ratio of Plasma Cortisol (ng/ml) to Plasma pACTH ($\log_{10}$ pg/ml) in Barrows at 6-hr Intervals on Three Different Days

Day	n	Time of day (hr)				
		0700	1300	1900	0100	0700
0	14	21.1[a] ± 3.5(a,b)[b]	16.8 ± 2.4(a,b)	12.9 ± 2.7(b)	18.5 ± 3.4(a,b)	25.3 ± 3.0(a)
12	4	16.2 ± 4.6(a,b)	10.4 ± 5.9(a,b)	8.8 ± 3.1(b)	21.7 ± 6.6(a,b)	24.4 ± 4.1(a)
26	4	25.8 ± 4.6(a)	10.1 ± 2.6(b)	6.2 ± 3.5(b)	25.7 ± 10.1(a)	18.5 ± 6.8(a,b)

[a] Each value represents the mean ± SE.

[b] Means with different letters in parentheses within a given day are significantly different ($P<0.05$).

Table II. Plasma Catecholamines at 6-hr Intervals in Barrows

	n	Time of day (hr)				
		0700	1300	1900	0100	0700
Norepinephrine (pg/ml)	7	462[a] ± 64(a)[b]	478 ± 56(a)	574 ± 178(a)	443 ± 90(a)	389 ± 42(a)
Epinephrine (pg/ml)	7	339 ± 52(a)	452 ± 71(a)	318 ± 24(a)	403 ± 44(a)	395 ± 28(a)

[a] Each value represents the mean ± SE.

[b] Means with different letters in parentheses for a given hormone are significantly different ($P<0.05$).

the ratio plasma cortisol (ng/ml)/plasma ACTH ($\log_{10}$ pg/ml; since a linear increase in cortisol results from a $\log_{10}$ increase in ACTH (35)) throughout the 24-hr period indicated in Figure 1. There was a 96% increase ($P < 0.05$) in this ratio noted between 1900 hr and the second 0700-hr sampling.

In spite of prior conditioning of pigs to the presence of personnel in the animal rooms, the possibility existed that the presence of persons taking the blood samples might alarm the animals and cause abnormally high levels of pACTH or cortisol. This eventuality might be especially true at night when the pigs were unaccustomed to the presence of people. To ascertain the validity of this supposition, plasma catecholamines, which are rapidly responsive to minor perturbations in an animal's environment (36), were also measured in seven of the animals. The data in Table II indicate that concentrations of both norepinephrine and epinephrine were constant throughout the time periods measured. There were no significant correlations ($P > 0.05$) when plasma pACTH and cortisol were tested with NE and E.

The data above represent a pretreatment period for an experiment designed to ascertain effects of temperature on adrenal function. Subsequent to the described period, four of the barrows remained at 23°C and additional blood samples were taken 12 and 26 days later. As indicated in Figure 2, considerable variability existed among pigs and within pigs on the different days. A recurrent pattern in plasma cortisol is obvious in each barrow on both days, with troughs occurring in the afternoon or evening hours and peaks at 0100 or 0700 hr. Repeated measures analysis of variance indicated a significant hour effect ($P = 0.02$) and no day effect ($P = 0.10$). A multiple range test identified cortisol concentrations at 1900 hr to be below those at 0700 hr on both days ($P < 0.05$).

Changes in plasma pACTH are less easily defined. In some of the barrows on given days (pig 14, days 12 and 26; pig 15, day 12), afternoon or evening troughs and morning peaks were apparent. In other cases, plasma pACTH throughout the day at the time periods measured exhibited but modest changes (pig 13, day 26; pig 15, day 26; pig 16, days 12 and 26). Repeated measures analysis of variance indicated no hour ($P = 0.17$) or day ($P = 0.07$) effects and a lack of day × hour interaction ($P = 0.37$).

As indicated in Table I, the ratio cortisol/$\log_{10}$ pACTH again changed significantly throughout the day with values at 1900 hr being below those of one of the 0700-hr periods and on day 26, also below the ratio at 0100 hr. On day 12 the increase in the ratio after 1900 hr was 1.8-fold and on day 26 it was 3.1-fold.

Acute Stressor Study. From the above-described data and from previous reports which described low cortisol at 1600 hr and significantly greater concentrations in the morning at 0800–1000 hr (5–7, 37), it was concluded that 0800 hr and 1600 hr should represent significantly different periods in the pituitary-adrenocortical diurnal rhythm of function.

As indicated in Figures 3 and 4, neither plasma pACTH nor cortisol concentrations differed significantly in control animals during the morning and early afternoon sampling period (AM). Plasma pACTH was very constant with an AM average of 33.51 ± 1.53 pg/ml, whereas plasma cortisol was somewhat more variable with an AM average of 23.70 ± 1.79 ng/ml. In the late afternoon and nighttime period (PM), plasma pACTH in control animals increased 36% ($P < 0.05$) during the 1600–1640-hr sampling period when com-

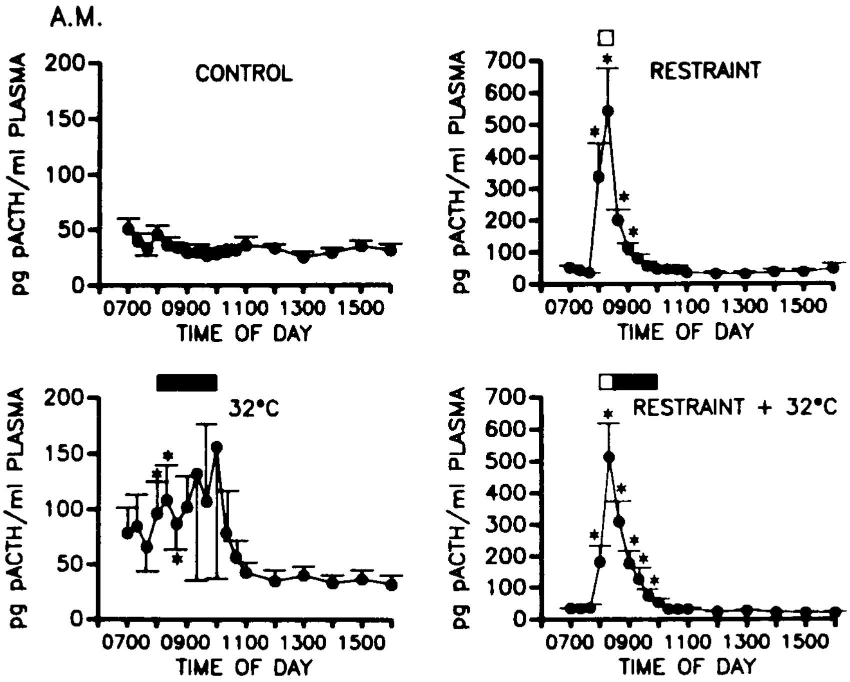

Figure 3. Plasma immunoreactive pACTH concentrations in control or morning (AM) stressor-treated barrows. Control and restraint animals were maintained at 20°C. One group of five barrows was placed in a restraining cage for a 20-min period (0800–0820 hr) as indicated by the open bar. A second group of five barrows was subjected to 32°C for 2 hr (0800–1000 hr) as indicated by the solid bar. A third group of five barrows was concomitantly subjected to restraint (0800–0820 hr) and to 32°C (0800–1000 hr). Blood samples were obtained via jugular catheter and at 20 min (0700–1100 hr) or hourly (1200–1600 hr) intervals. Each datum point represents the mean ± SE of five observations. * Indicates means which are significantly different from pretreatment basal levels as determined by the Student-Newman-Keuls test or by preplanned linear orthogonal combinations ($P < 0.05$). Statistics were computed on log-transformed data. Note that the scales of ordinates for control and 32°C are larger than for restraint and restraint + 32°C.

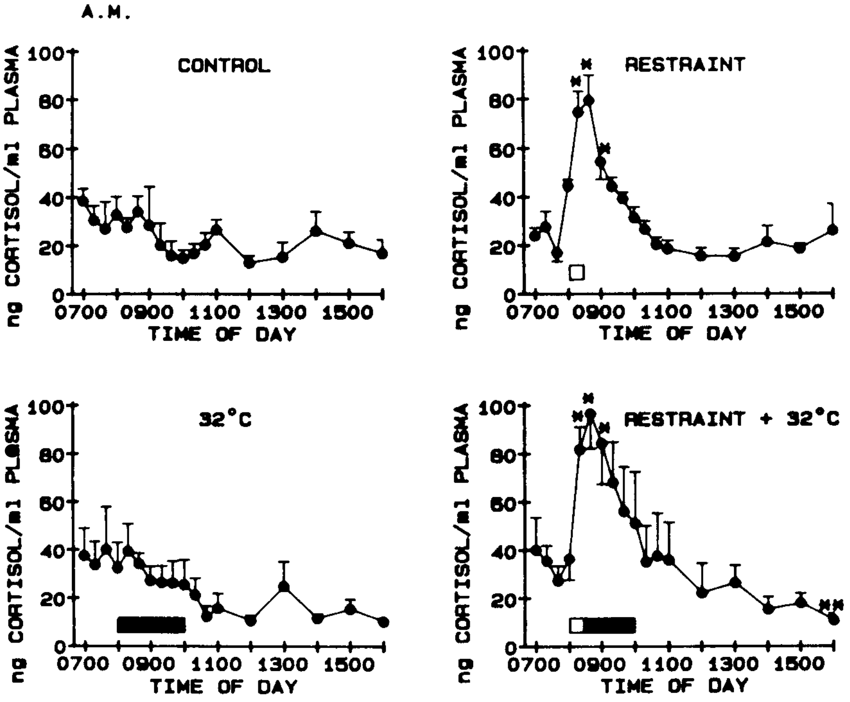

Figure 4. Plasma cortisol concentrations in control or morning (AM) stressor-treated barrows. Experimental conditions, sample sizes, statistical procedures, and significance indicators are identical to those described in the legend to Figure 3.

pared with 1500–1540-hr values (59.1 ± 4.4 vs 43.4 ± 1.5 pg/ml), whereas plasma cortisol underwent a 62% ($P < 0.05$) decline between 1640 and 2000 hr (Figs. 5, 6). In the AM control pigs the ratio cortisol/$\log_{10}$ pACTH at 0700 hr (24.1 ± 3.7) was greater ($P = 0.0004$) than the ratio occurring in the PM controls (4.8 ± 0.9) at 1900 hr.

Restraint of pigs was associated within 5 min with 8-fold increases ($P < 0.05$) in plasma pACTH in both the AM and PM (Figs. 3, 5). Plasma cortisol responded less rapidly and significant increases in plasma cortisol were delayed until 20 min after restraint initiation when 2- to 3-fold increases occurred (Figs. 4, 6). Peak concentrations of plasma pACTH were measured at the end of the restraining period (0820 or 1620 hr), and once pigs were removed from restraint plasma pACTH decreased within 20 min, but remained elevated ($P < 0.05$) for 40–80 min after removal of restraint. However, plasma cortisol continued to increase subsequent to restraint termination, and peak values in both the AM and PM were reached 20 min later (0840 or 1640 hr). Subsequently, elevated values ($P < 0.05$) persisted for another 20 min.

Using a priori statistics which evaluated a preplanned comparison between the three pretreatment levels and only the first three posttreatment levels, it was indicated that exposure of animals to 32°C in the AM was associated with a 28% increase ($P < 0.05$) in plasma pACTH above preexposure values. Subsequently, at 1000 hr a 2-fold increase was measured. In the PM a 74% increase ($P < 0.05$) in plasma pACTH occurred during the first hour after exposure to 32°C (60.6 ± 5.9 vs 34.7 ± 1.0 pg/ml). However, because of interanimal variability plasma ACTH concentrations in 32°C-treated barrows were not significantly different from those of controls regardless of perturbations which were especially apparent in the AM-treated pigs. These increases in plasma ACTH were not accompanied by changes ($P > 0.05$) in plasma cortisol (Figs. 3–6).

A combination of 20-min restraint and 2 hr at 32°C in the AM produced pACTH responses similar to restraint alone. In the PM, however, there was a subsequent second elevation ($P < 0.05$) of pACTH occurring concomitantly with elevated ambient temperature, which did not occur in pigs treated similarly in the AM. Increased pACTH at this time interval after treatment initiation in the PM was greater than ($P < 0.001$) plasma pACTH at the same time interval in the AM. Plasma cortisol concentrations in these treatment groups paralleled changes in pACTH, but a distinct second cortisol peak in the PM group corresponding to the second pACTH peak was not evident.

In order to compare more accurately hormonal responses a number of response-associated parameters

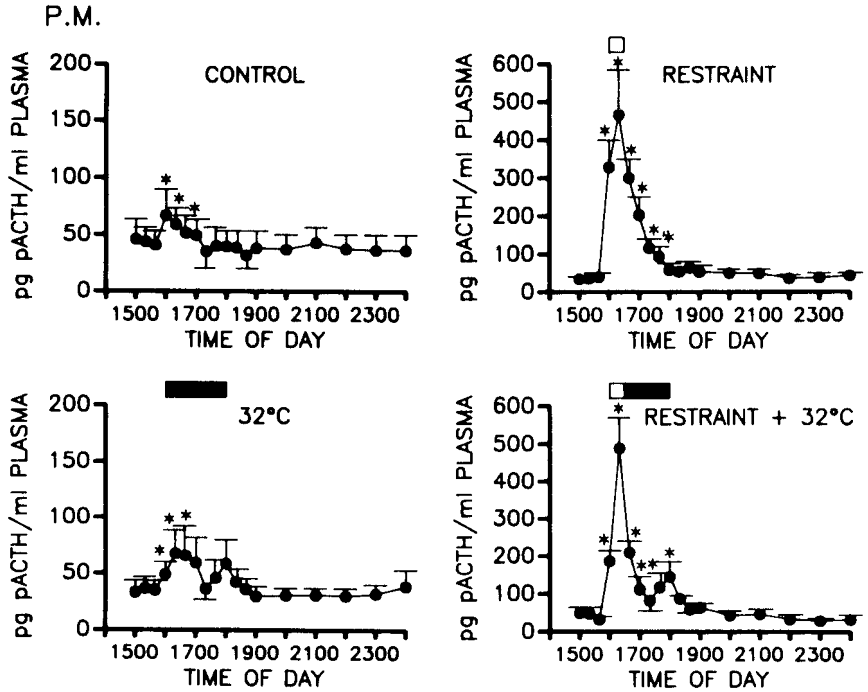

Figure 5. Plasma immunoreactive pACTH concentrations in control or afternoon (PM) stressor-treated barrows. Control and restraint animals were maintained at 20°C. One group of five barrows was placed in a restraining cage for a 20-min period (1600–1620 hr) as indicated by the open bar. A second group of five barrows was subjected to 32°C for 2 hr (1600–1800 hr) as indicated by the solid bar. A third group of four barrows was concomitantly subjected to restraint (1600–1620 hr) and to 32°C (1600–1800 hr). Blood samples were obtained via jugular catheter and at 20-min (1500–1900 hr) or hourly (2000–2400 hr) intervals. Each datum point represents the mean ± SE of four to five barrows. * Indicates means which are significantly different from pretreatment basal levels as determined by the Student-Newman-Keuls test or by preplanned linear orthogonal combinations ($P < 0.05$). Statistics computed on log-transformed data. Note that the scales of ordinates for control and 32°C are larger than for restraint and restraint + 32°C.

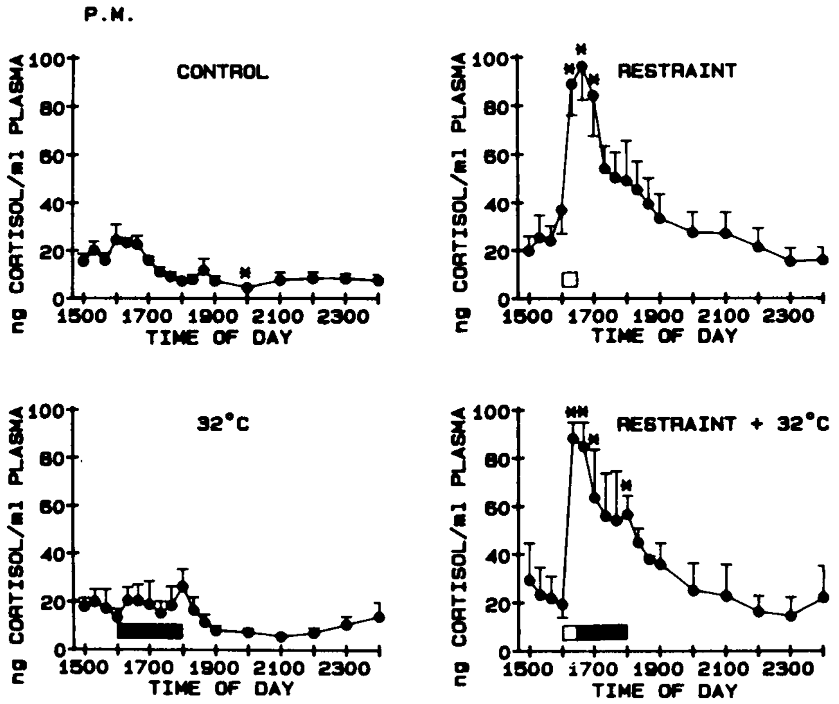

Figure 6. Plasma cortisol concentrations in control of afternoon (PM) stressor-treated barrows. Experimental conditions, sample sizes, statistical procedures, and significance indicators are identical to those described in the legend to Figure 5.

were evaluated: the maximum hormonal concentration for each animal irrespective of the point in time at which it occurred, the maximum change in hormonal concentration above baseline levels, and the integrated hormonal response above baseline levels for the 4-hr period after treatment initiation.

Baseline pACTH (the average of the three pretreatment plasma pACTH concentrations) were not significantly different between AM and PM treatment groups and averaged 41.4 ± 4.9 pg/ml. Basal plasma cortisol concentrations, however, were elevated 57% ($P < 0.05$) in the AM compared with PM when all treatment groups were combined (31.7 ± 3.1 vs 20.9 ± 1.8 ng/ml).

Exposure to 32°C was always associated with statistically similar response parameters in the AM and PM for both hormones. Although cortisol parameters for this treatment never differed ($P > 0.05$) from control values, maximum concentrations (226 ± 107 pg/ml) and changes in concentrations (173 ± 93 pg/ml) for pACTH were above control values (50 ± 7 and 11 ± 7 pg/ml, respectively; $P < 0.05$) in the AM only.

When comparing restraint alone with restraint + 32°C maximum hormonal concentrations, maximum changes in concentration and integrated responses were similar ($P > 0.05$) for both pACTH and cortisol. Furthermore, these parameters were above control values ($P < 0.05$), with the exception of the AM integrated cortisol response to restraint, and did not exhibit AM-PM differences. Such comparisons of response-associated parameters indicate that AM-PM differences in pACTH response to restraint + 32°C (compare Figs. 3 and 5) were in the nature of the response (two peaks vs one peak) and not in the overall magnitude of the response.

Discussion

There are several important observations to be obtained from data contained in this article which have not been previously reported for pigs. First, the data suggest the presence in castrated male pigs of diurnal changes in plasma pACTH and cortisol concentrations. At time periods measured, maximum concentrations occurred most frequently in the morning prior to or soon after lights-on and hence the beginning of activity. Highest plasma cortisol concentrations in the morning (AM) have also been reported for boars (5), ovariectomized gilts (7), and intact gilts (37). There have been no previous reports concerning diurnal changes in plasma ACTH in pigs.

It was readily apparent that for both pACTH and cortisol there was considerable variability among animals and within animals on different days. The apparent absence of diurnal hormonal changes in some barrows, and the apparent shifting of the occurrence of the peaks, may simply reflect the inability of the sampling frequency used to detect the occurrence of the true peaks. For the control animals of the acute stressor study, the absence of plasma measures between 2400 and 0700 hr may explain the apparent absence of diurnal changes in plasma ACTH. Interestingly, these data are similar to those reported for intact rats wherein circadian changes in plasma ACTH are modest, and some studies have been unable to detect circadian fluctuations concomitant with marked changes in plasma corticosterone (20, 38). Undoubtedly, additional studies with more frequent sample collections will have to be conducted to substantiate the diurnal hormonal changes suggested in the current studies.

Second, since in this study neither plasma NE nor E differed among hours, it can be concluded that elevated plasma pACTH and cortisol during the early morning hours did not represent a stress response to the presence of personnel in the chambers and the blood sampling procedure. The data also indicate the absence of diurnal rhythms of these two hormones in barrows at the time intervals measured. In humans NE (39), but not E (40), appears to have a significant diurnal rhythm, whereas the presence or absence of such rhythms have not been heretofore reported for pigs.

Third, there were consistent and significant increases in the ratio cortisol/$\log_{10}$ pACTH between 1900 hr and 0700 hr in unstressed pigs. Such information suggests that in the AM a given amount of pACTH elicits a greater adrenal cortisol secretion than in the PM. This hypothesis, however, is only one of several potential explanations and is made with the assumption of constant metabolic clearance rates and volumes of distribution for ACTH and cortisol. Such differences in adrenal responsiveness to ACTH have been reported for rats (12, 16, 17) and dogs (21), and in rats it was quite unlikely these results could be accounted for by circadian changes in metabolic clearance rates or volumes of distribution of either ACTH or corticosterone (17, 22).

This potential AM-PM difference in adrenal responsiveness to ACTH in *unstressed* pigs was not, however, evident in *stressed* pigs wherein neither pACTH nor cortisol responses to 32°C, restraint, or restraint + 32°C differed between AM and PM. Considerable evidence has accrued in mice (4, 14), rats (12, 16, 17, 20, 22), dogs (21, 23), and humans (24) for diurnal changes in adrenal responsiveness to ACTH. Hence, at the time periods tested, pigs do not appear to demonstrate changes in adrenal responsiveness to stressors. However, more divergent time periods need to be compared before statements concerning the absolute absence of diurnal changes in adrenal responsiveness can be made.

Fourth, the current studies were unable to demonstrate convincing AM-PM differences in the pACTH responses to stressors. Nevertheless, during application of 32°C for 2 hr, response-associated parameters for pACTH were elevated above respective controls in the AM, but not PM, in the absence of changes in plasma cortisol. Hence, with this treatment there is a modest indication that in the AM the hypothalamo-hypophyseal axis is more sensitive to mild stimulation than in the PM. Lack of AM-PM differences in pACTH responses to stronger stimuli may reflect the negative feedback inhibition of cortisol on pACTH which might mask inherent AM-PM differences in pituitary responsiveness. Such relationships have been convincingly demonstrated in rats (16).

Although somewhat controversial as to the time of greatest responsiveness, evidence exists with rats for diurnal changes in pituitary responsiveness to exogenous CRF or stressors (12, 16, 17). However, no such AM-PM differences were evident in plasma ACTH responses to CRF in humans (24) or to a hemorrhage stressor in dogs (23).

An additional difference in the AM-PM pACTH response to stressors was evidenced by the significantly different character of the AM-PM pACTH responses to restraint + 32°C. The second significant increase in plasma pACTH, which occurred at 1800 hr, may indicate that the initial 20-min restraint stressor facilitated the hypothalamo-hypophyseal axis in a manner which allowed it to respond more strongly to 32°C. There is considerable persuasive evidence for the response to an initial stressor facilitating the release of ACTH in response to a subsequent stressor (41–45). In the current study this apparent potentiation of the pACTH response occurred only in the AM.

Finally, the slight but statistically significant, when compared with pretreatment levels, increases in plasma pACTH in response to 32°C are noteworthy. These minor changes in plasma pACTH (maximum changes of 173 ± 93 pg/ml, AM; 70 ± 27 pg/ml, PM) were not sufficient, apparently, to surpass the threshold of excitation for the adrenal cortex, as has been previously reported for pigs subjected to induced frustration (46). Interestingly, moderate increases in plasma prolactin, a recognized hormonal indicator of stress in many species (47), did occur in these same pigs (26). Such data not only indicate an inability to always use plasma cortisol as an index of changes in plasma ACTH, but also further substantiate the need of multiple measures to evaluate accurately the stress response in animals.

In summary, the current studies suggest the presence of a diurnal rhythm in plasma cortisol in castrated boars and are the first direct measurements suggesting a diurnal pACTH rhythm in pigs. Strong and mild stressors applied in both the AM and PM did not reveal quantitative AM-PM differences in plasma pACTH or cortisol responses at the time intervals tested. Such information is useful since it indicates that stressors can be applied to pigs between 0800 and 1800 hr without concern for time-associated differences in the ACTH or cortisol responses. The ratio cortisol/$\log_{10}$ pACTH in unstressed barrows was significantly greater at 0700 hr than at 1900 hr in separate studies. One interpretation of such results is that there is an enhanced adrenal responsiveness to pACTH at 0700 hr in unstressed pigs. The PM plasma pACTH responses to restraint + 32°C suggest the ability of one stressor to potentiate the pACTH response to a second stressor in pigs. Finally, no diurnal changes in plasma epinephrine or norepinephrine were detected.

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