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Relationships Between Plasma Cortisol, Corticosteroid-binding Globulin (CBG) and The Free Cortisol Index (FCI) In Pigs Over A 24 h Period

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Abstract: The relationship between plasma free cortisol and the free cortisol index (FCI, the ratio of cortisol to CBG) was evaluated in eight 8-wk old pigs over a 24 h period and in response to administration of saline or ACTH. A high ($p<0.001$) correlation was found between actual free cortisol and the FCI in both saline ($r = 0.73$) and ACTH ($r = 0.85$) treated pigs. A diurnal rhythm was apparent for total cortisol, free cortisol, percent free cortisol, pCBG and the FCI. Total cortisol ($p<0.05$), free cortisol ($p<0.05$) and the FCI ($p<0.01$) were elevated during the first four h following administration of ACTH. Concentrations of pCBG differed ($p<0.001$) over time and were higher ($p<0.01$) for ACTH treated pigs over the 24 h period. The results from this study affirm the contention that FCI is a suitable estimate of free cortisol in swine.

Key words: Pig * cortisol * Corticosteroid-Binding Globulin (CBG) * Free Cortisol Index (FCI)

INTRODUCTION

A circadian pattern of total cortisol is present in many species including man, horse and pig. For the pig this rhythm is characterized by peak amounts of circulating cortisol secreted in the morning with reduced levels during the afternoon and early evening [1, 2]. Cortisol exists in the bloodstream in biologically active and inactive forms. The active form consists of cortisol that is free or unbound and that which is loosely bound to albumin, thus allowing it to be biologically available to the cell. The majority of circulating cortisol is tightly bound to its specific carrier glycoprotein, Corticosteroid-Binding Globulin (CBG). The percentage of CBG-bound cortisol in circulation varies among species from 65% for swine[3] to more than 90% for humans[4]. A diurnal rhythm for CBG has been reported in both humans and rats. In humans, CBG binding capacity for cortisol paralleled oscillations for the diurnal rhythm of total cortisol with a lag time of approximately four hours[5]. No lag time between oscillations of corticosterone and CBG were observed in the circadian pattern of CBG in rats[6]. During stressful situations, cortisol levels can increase while CBG levels may increase or decrease, leaving the free cortisol fractions to be incorrectly represented. The Free Cortisol Index (FCI), a ratio of circulating total plasma cortisol to CBG, has been demonstrated in human studies to be a reliable and easy to use measure of plasma free cortisol[7, 8]. The objectives of this study were to examine the relationship between the FCI and actual levels of circulating free cortisol over a 24 h period and to document changes in plasma total cortisol, percent free cortisol, free cortisol, porcine CBG (pCBG) and FCI in response to adrenal stimulation in pigs.

MATERIALS AND METHODS

Animals and housing: Pigs (Premier x QMax 100) were weaned at 25 days of age and housed in raised pens (2.44 m x 2.44 m) with slotted floors. Animals were given *ad libtum* access to a commercial diet (3.315 kcal/kg of ME, 18% CP, 1.15% lysine; as fed basis) and water. Artificial lights were provided for 13 h, starting at 0630 h and room temperature was maintained at 25±2°C. A red light source was activated during the dark period to aid in blood sampling. After catheterization, pigs were placed individually in 0.61 m x 1.22 m pens with *ad libitum* access to feed and water. All animal procedures were reviewed and approved by The University of Tennessee Animal Care and Use Committee.

Experimental design

Catheterization: Eight 8-wk old female pigs (12.7±1.8 kg BW) from two litters were fitted with an indwelling catheter according to the procedure of Carroll *et al.*[9]. Briefly, animals were anesthetized using halothane

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administered with oxygen via a facemask attached to a closed circuit anesthesia machine. The jugular vein was located using a 21-ga access needle and a guide wire was inserted through the access needle into the jugular vein. The access needle was removed, leaving the guide wire inside the jugular vein. A polyurethane catheter (Global Veterinary Products, New Buffalo, MI, USA) was inserted over the guide wire into the jugular vein approximately 7 to 8 cm in depth and the guide wire removed. The catheter was secured at the site of insertion using tissue adhesive and sutures placed on either side of the catheter collar. A 20 cm extension was attached to the catheter and routed to the dorsal surface of the neck and secured with flexible bandaging tape. Animals were placed individually in 0.61 m x 1.22 m pens and an additional 90 cm long extension was attached to the existing catheter/extension to allow remote access for the collection of blood samples without disturbing the pigs. The entire catheterization process took approximately 20 min per animal.

Blood collection: Blood samples were collected over a 24-h period beginning approximately 5 h post insertion of the catheter (1730 h). Immediately prior to collection of each blood sample, 2 ml of fluid was drawn from the catheter and discarded. A blood sample (5 mL) was collected in a heparinized syringe (Li-Heparin LH/4.5mL, Sarstedt Monovette, Newton, NC, USA) and the catheter was flushed with 2.5 ml of 0.9% NaCl followed by 0.5 ml of heparinized saline (50 IU/ml in 0.9% NaCl). After samples were centrifuged at 1520 x g for 15 min, the plasma was allocated to 2- 1.5 mL vials (Wheaton, Millville, NJ, USA) and stored at -20°C.

Following the fourth hourly blood sampling (2030 h), four of the eight pigs were administered saline (2.5 mL 0.9% NaCl) and the remaining four pigs administered ACTH (1 IU kg^{-1} BW, 10 IU mL^{-1} in 0.9% NaCl) via the catheter. Samples were then collected at 30 min intervals for 4 h and hourly for the remainder of the 24 h period. A total of 29 blood samples were collected per animal. Hematocrit values were determined on samples collected at 1730, 0230 and 1730 h.

Plasma analysis

Cortisol: Plasma total cortisol concentration was determined by radioimmunoassay as reported previously [10]. Intra- and inter-assay CV was 3.6 and 13.5% for low (110 nmol L^{-1}), 4.4 and 15.7% for medium (325 nmol L^{-1}) and 3.3 and 12.2 % for high (772 nmol L^{-1}) cortisol standards.

Corticosteroid-Binding Globulin (CBG): The concentration of porcine Corticosteroid-Binding Globulin (pCBG) in plasma was measured by a direct ELISA as described by Roberts *et al.* [11]. Intra- and inter-assay CV of a pooled pig plasma sample was 18 and 20%, respectively.

Free cortisol index: The free cortisol index was calculated using the ratio of plasma total cortisol to pCBG concentration[7, 8].

Percent free cortisol: Percent free cortisol was determined using an ultrafiltration assay according to Lewis *et al.*[12]. Radiolabeled cortisol (0.2μCi of ^{3}H, NEN products, Boston MA, USA) in ethanol was placed into tubes (12 x 75 mm) and allowed to dry. Unknown plasma samples (1 ml) were added to each tube, vortexed and incubated in a 39°C water bath for 30 min. Duplicate fractions (40 μL) of the mixture were pipetted into vials and 4 ml of scintillation fluid (Ultima Gold XP, Packard, Meriden CT, USA) was added for determination of total radioactivity. Two 400 μL samples of the plasma containing the radiolabeled cortisol were placed in ultrafiltration devices (Ultrafree-MC 10,000 NMWL filter unit, Millipore, Billerica MA, USA) preconditioned with assay buffer (0.05 mol L^{-1} of PBS, 0.1% Tween-20 and 0.1% gelatin) and centrifuged (Eppendorf centrifuge 5415C at 16,110 x *g*) for 30 min at room temperature. Two 40 μL aliquots of the ultrafiltrate were pipetted into vials, scintillation fluid added and all vials transferred to a liquid scintillation counter (Packard Tri-Carb, 2900TR beta counter) for measurement of radioactivity. Percent free cortisol was calculated as the ratio of ultrafiltrate to total radioactivity.

Free cortisol: The concentration of free cortisol was calculated using the product of percent free cortisol and total cortisol.

Statistical analysis: Data were analyzed using the MIXED procedure of SAS (SAS Inc., v9.0 Cary, NC, USA) for a completely randomized design. Repeated measures were utilized for differences due to time. The fixed effects of treatment (saline and ACTH), time and treatment x time interaction were used to analyze for differences in total plasma cortisol, pCBG, percent free cortisol, free cortisol and FCI. Random effects were pig within treatment. The mean of samples taken prior to treatment administration was used as a baseline value when analyzing for differences over time. Significant differences were separated using Fisher's Least Significant Difference test. A regression analysis with log transformation, due to unequal variances, was used to estimate the correlation of free cortisol and FCI. Data are presented as least squares means with standard errors.

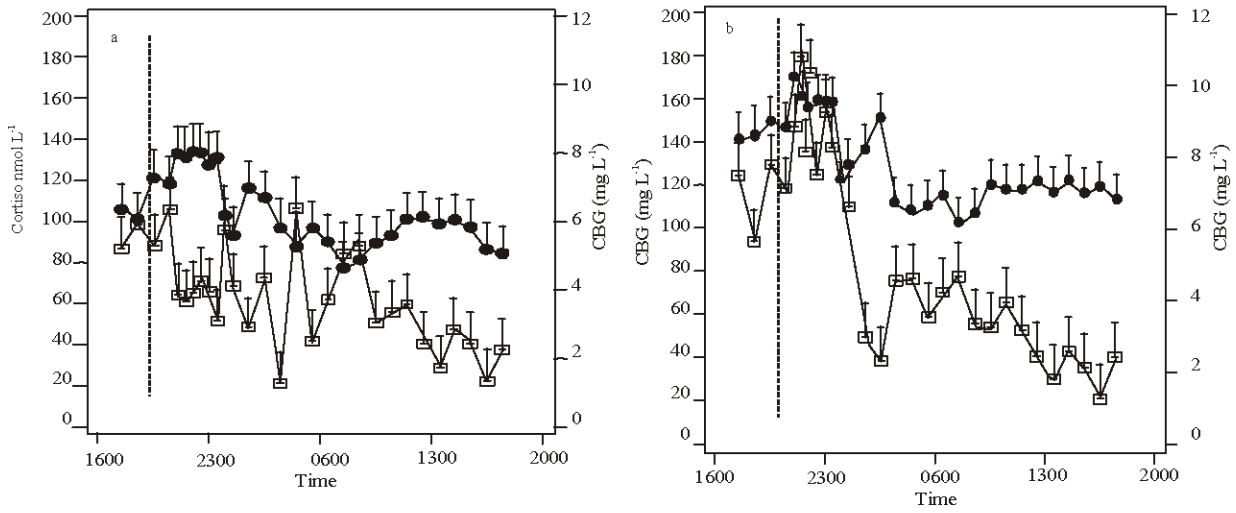

Fig. 1: Relationship between total cortisol (□) and pCBG (•) in pigs sampled over 24 h following administration of saline (a; 0.9% NaCL) or adrenocorticotrophin (b; 1 IU kg^{-1} BW). Each point represents the mean value (± S.E., n = 4). Treatment was administered immediately following 2030 h sample (vertical dotted line)

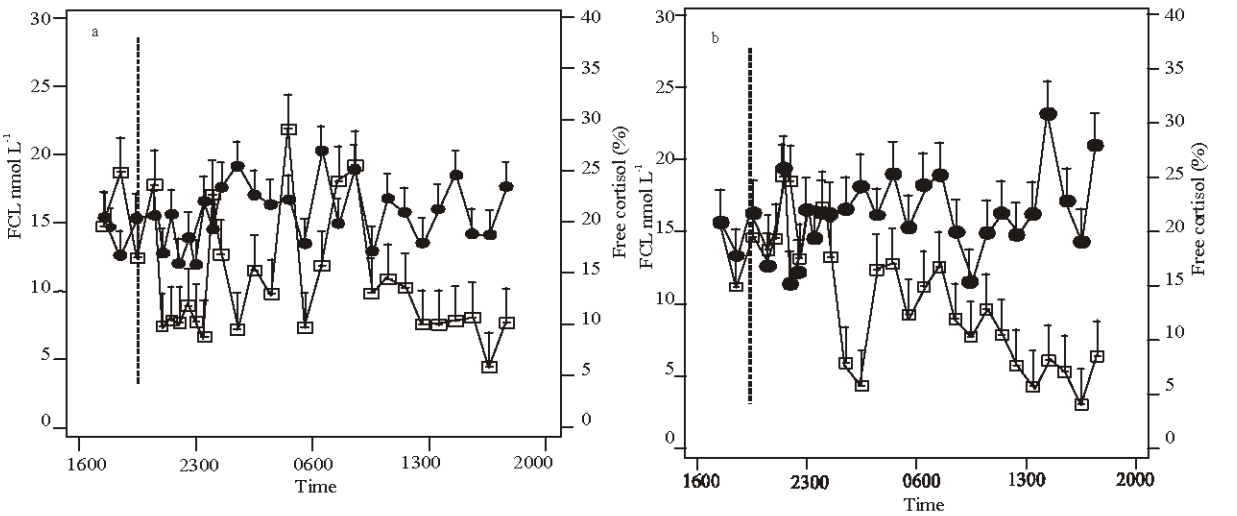

Fig. 2: Relationship between the free cortisol index (FCI; □) and free cortisol (•) in pigs sampled over 24 h following administration of saline (a; 0.9% NaCL) or adrenocorticotrophin (b; 1 IU/kg BW). Each point represents the mean value (± S.E., n = 4). Treatment was administered immediately following 2030 h sample (vertical dotted line)

RESULTS

Blood samples were successfully collected from seven of the eight pigs at each of the designated sampling times. The catheter in one of the control pigs lost patency but was reinserted; resulting in missed samples from 2200 to 2330 h. Hematocrit values at the beginning and end of the experiment were not different ($p>0.10$, data not shown).

Plasma total cortisol: Mean plasma total cortisol concentration prior to treatment was similar ($p>0.10$) between saline (94.8±19.4 nmol L^{-1}) and ACTH (116.3±19.4 nmol L^{-1}) treated pigs (Fig. 1a and b). Cortisol in saline treated pigs decreased ($p<0.001$) to 51.2 nmol L^{-1} at 2030 h and oscillated ($p<0.001$) above and below this concentration throughout the remainder of the experiment. Compared to the mean baseline value (1730–2030 h), plasma cortisol concentration in ACTH treated pigs was elevated ($p<0.001$) at 1 h, returned to baseline within 4 h and was lower ($p<0.001$) beginning 5 h post injection and remained lower for the remainder of the 24 h. Although not statistically different, total cortisol for saline treated pigs was higher than that measured for ACTH treated pigs at 0430, 0730 and 0830 h. Cortisol concentration in

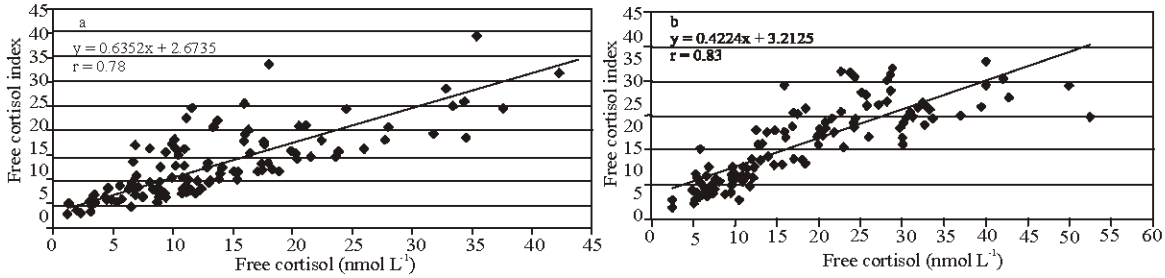

Fig. 3: Relationship between the calculated free cortisol index (total cortisol/pCBG) and free cortisol for saline (a; 0.9% NaCl; n = 4) and adrenocorticotrophin (b; 1 IU kg^{-1} BW; n = 4) treated pigs

both control and ACTH treated pigs exhibited a nadir at 1630 h.

Plasma pCBG concentration: Mean plasma concentration of pCBG was similar ($p>0.10$) among saline (6.7±0.8 mg L^{-1}) and ACTH (8.7±0.8 mg L^{-1}) treated pigs prior to treatment administration (Fig. 1a, b). For saline treated pigs, pCBG values were lower ($p<0.01$) than baseline values at 0730 and 0830 h (4.6 and 4.9±0.8 mg L^{-1}, respectively) and were similar to baseline for the remainder of the 24 h. Pigs administered ACTH exhibited lower ($p<0.001$) than baseline pCBG concentrations at 0330 h (6.7±0.8 mg L^{-1}). pCBG values returned to baseline by 0930 h and remained unchanged for the remainder of the experiment. At 0230 h, pCBG for ACTH treated pigs (9.0±0.8 mg L^{-1}) was greater ($p<0.01$) than that measured for the control pigs (6.7±0.8 mg L^{-1}). The pCBG for both saline and ACTH pigs decreased ($p<0.01$) over the 24 h. The lowest concentration of pCBG was observed at 0730 h in both control and ACTH treated animals.

Percent free cortisol: Mean percent free cortisol for saline (19.0±0.7 %) and ACTH (19.2±0.7 %) treated pigs was similar ($p>0.10$) prior to treatment administration (Fig. 2a and b). No treatment or treatment x time ($p>0.10$) effects were noted. Regardless of treatment, percent free cortisol fluctuated ($p<0.001$) over the 24 h. Morning peak values were observed in both the saline and ACTH treated pigs. The percentage of free cortisol in ACTH pigs during the morning peak was slightly higher (24.3 and 25.2±2.5%) than saline treated pigs (24.8 and 22.2±2.5%). Two afternoon peaks occurred for both saline and ACTH treated pigs at 1430 and 1730 h.

Plasma free cortisol Mean plasma free cortisol (total cortisol x percent free cortisol) was similar ($p>0.10$) for the two groups of pigs prior to treatment (18.2 and 22.4±3.1 nmol L^{-1} for control and ACTH pigs, respectively; data not shown). Following administration of treatment, free cortisol for control pigs fluctuated within the range of the baseline values with intermittent values that were lower ($p< 0.001$) than baseline over the 24 h. Free cortisol in ACTH treated pigs fluctuated in a similar fashion. Values were higher ($p<0.001$) than baseline at 2100 and 2130 h, subsequently fell below ($p<0.001$) baseline at 0130 h and remained low thereafter. Both treatment groups exhibited their lowest ($p< 0.05$) concentrations of free cortisol at 1630 h.

Free Cortisol Index (FCI): Mean FCI (total cortisol/pCBG) values were similar ($p>0.10$) for control (15.1±2.6 nmol mg^{-1}) and ACTH (13.7±2.6 nmol mg^{-1}) pigs prior to treatment administration (Fig. 2a, b). FCI for control pigs fluctuated from 4.5 to 21.9±2.5 nmol mg^{-1} over the 24 h, with lower ($p<0.001$) values persisting for 3 h following administration of saline and again during the late afternoon and evening hours. The FCI for ACTH treated pigs increased ($p<0.001$) by an average of 175% compared to that in saline treated pigs for 4 h following treatment. The FCI in pigs given ACTH was lower ($p<0.01$) than baseline from 0130 - 0230 h and from 1130 - 1730 h. Saline treated pigs exhibited peaks in FCI at 0430 and 0730 - 0830 h, while the FCI for ACTH treated pig's remained low during these same time periods.

Relationships among total cortisol, free cortisol, pCBG and FCI: A high correlation ($p<0.001$) was found between FCI and free cortisol in saline ($r = 0.78$; Fig. 3a) and ACTH ($r=0.83$; Fig. 3b) treated pigs. Plasma total cortisol was not correlated ($p>0.10$) with pCBG concentration in either saline or ACTH pigs during the pre-treatment period (1730 - 2030 h). During the 4 h following injections (2100 - 0030 h), a positive correlation ($r=0.67$; $p<0.001$) existed between plasma total cortisol and pCBG in pigs administered ACTH, while no such relationship was found in saline treated pigs. During the early morning through afternoon hours (0130 - 1730 h) a negative correlation ($r=-0.48$; $p<0.001$) was evident between

cortisol and pCBG in the ACTH but not saline treated pigs. A similar relationship ($r = -0.38$; $p < 0.001$) was found during this same period between plasma free cortisol and pCBG in ACTH treated pigs due to plasma total cortisol values being lower than baseline values and pCBG increasing slightly during this same time.

DISCUSSION

The results from the present study affirm our contention that FCI is a suitable estimate of free cortisol in swine. A better illustration of the temporal relationships between plasma total cortisol and pCBG may be assessed by the measurement of both along with the calculation of the FCI.

The diurnal pattern for plasma cortisol was evident in the present study with a morning peak occurring at 0730 - 0830 h and an afternoon trough at 1630 h. Whipp *et al.*[1] reported a morning peak in 12 to 24 wk-old-pigs at 0800 h and a trough at 1600 h, while Bottoms *et al.*[2] observed a morning peak at 1000 h and a trough at 1400 h in adult pigs. Higher cortisol values in the morning have been associated with the awakening response in diurnal animals[13]. Values for the afternoon trough were similar to those reported by Bottoms *et al.*[2] and Whipp *et al.*[1]. As reported for humans, the concentration of cortisol is typically lower during the afternoon in response to the onset of the sleep cycle[14].

Plasma pCBG concentrations for pigs in the present study were similar to those reported previously[11]. Hematocrit values did not change over the 24 h period suggesting that the decrease in pCBG concentration, as well as that of total cortisol, was not a result of changes in plasma volume. A diurnal pattern was observed for pCBG over the 24 h period with higher concentrations observed during 2100 - 2330 h and lower values evident through the rest of the morning and afternoon. Compared to previous studies, pCBG values in the present study did not parallel oscillations of cortisol like that for CBG and corticosterone in rats[6], nor was there a lag time detected between the two plasma constituents similar to that observed in humans[5].

The percentage of free cortisol as determined by a ligand-binding assay did follow a circadian pattern like that of cortisol, increasing through the night and early morning hours before a gradual decrease through the afternoon hours. This is consistent with studies by Edwards *et al.*[13] who observed an increase in free cortisol within 30 min of awakening in humans. In our study, the afternoon trough that was observed for plasma total cortisol was also present for percent free cortisol. Actual free cortisol in circulation, that which is biologically available for uptake by cells[15], is the product of plasma total cortisol and percent free cortisol. The diurnal pattern of plasma free cortisol in our study is similar to that measured in saliva of 8 d old pigs, as documented by Gallagher *et al.*[16], where a gradual decrease was observed from morning to afternoon and evening. Salivary cortisol measured every 4 h over a 24 h period in 12 to 25 wk old pigs revealed a circadian rhythm of cortisol that oscillated in a bell shape with peak values present shortly after 1200 h[17]. The amplitude of the curve decreased as pigs aged from 12 to 24 wks. Results from our study found a similar oscillating pattern. Free cortisol values fluctuated during the morning hours to reach a peak value at 0830 h, which decreased through the late morning and early afternoon, but not at a constant decline as reported previously by Ruis *et al.*[17].

The FCI, a ratio of circulating total cortisol to CBG, has been demonstrated to be a reliable and easy to use estimate of plasma free cortisol in humans[7,8]. In our study, a circadian pattern was observed in FCI, with elevated values observed during 0730 - 0830 h when pCBG was low and total cortisol concentration was high. In the afternoon hours when both plasma pCBG and cortisol were reduced, the FCI was at its lowest value. The inability to detect a relationship between plasma total cortisol and pCBG concentrations for the saline pigs may be due to the fact that FCI does not take into consideration the amount of cortisol that is loosely bound to albumin. Results of earlier research conducted in our laboratory indicated that concentrations of pCBG do not peak until 12 wk of age in swine and remain fairly constant over the next 12 wk[11]. The pigs used in the present study were 8 wk old and therefore may not have reached peak pCBG concentrations.

Injection of ACTH temporarily interrupted the circadian rhythm of plasma cortisol for 4 h before returning to values similar to control pigs. A previous study in 8 wk old pigs reported that plasma cortisol reached peak concentrations at 1 h and returned to baseline values within 2 h post injection of ACTH[18]. In our study the administration of ACTH at 2030 h resulting in elevated plasma cortisol values from 2100 to 0030 h, appeared to have suppressed the morning cortisol peak, but the afternoon trough was similar to that in control pigs. Tether-housed gilts exhibited an increase in plasma cortisol in the evening with no morning peak, resulting in a suppressed diurnal rhythm[19]. Afternoon concentrations of plasma total cortisol and percent free cortisol were elevated in gilts following restraint[20]. This restraint temporarily interrupted the diurnal rhythm of total cortisol for 4 d before returning to a pre-restraint rhythm.

Plasma pCBG concentrations in ACTH treated pigs fluctuated over time in a fashion similar to that in saline treated pigs. Armario *et al.*[21] similarly reported that CBG values in rats did not change following a single injection of ACTH. The positive relationship between plasma total

cortisol and pCBG observed following ACTH administration in our study was probably a result of increasing plasma total cortisol values and stable pCBG concentrations. The negative correlation between total cortisol and pCBG in the ACTH pigs during the early morning and afternoon hours, suggests a delayed effect of the elevated cortisol on circulating pCBG concentrations. Fleshner *et al.*[22] reported that corticosterone concentrations in rats were elevated immediately following inescapable tail shock, while it took 6.5 h to see a corresponding reduction in CBG. However, the authors concluded that the reduced CBG values could be the result of two possible mechanisms, either an increase in clearance or a decrease in CBG synthesis. Fleshner and coworkers chose to focus their discussion on the decrease in CBG synthesis.

Following injection of ACTH, percent free cortisol remained similar to that in control pigs. However, free cortisol (percent free x total cortisol) was elevated for 4 h following ACTH injection. This was due to plasma total cortisol being elevated while pCBG concentrations remained constant during the 4 h following injection. Total cortisol and actual free cortisol decreased over the remainder of the 24 h period. At 0230 h, cortisol values in the ACTH pigs were lowest while pCBG values were at their highest concentrations, resulting in a low FCI. Percent free cortisol remained stable over the 24 h relative to the amount of total and actual free cortisol in circulation. Excess cortisol may have bound to albumin and then shifted to CBG later or may have been excreted. An apparent shift in cortisol from unbound and albumin-bound to CBG bound has been documented occurring in pigs from birth to 6 wks of age[3].

The FCI was elevated for 4 h following administration of ACTH due to elevated plasma total cortisol and stable pCBG values. Once the FCI peaked in pigs administered ACTH, it then fell below baseline concentrations for 2 h before returning to values similar to that in saline treated pigs. However, the FCI in ACTH treated pigs did not increase like that in saline pigs during the morning hours. Our laboratory proposed that a decrease in CBG synthesis, corresponding to increasing cortisol levels, would effectively increase the clearance rate and thereby lower circulating cortisol concentrations[23]. This is evident in the ACTH treated pigs where the concentration of plasma total cortisol, pCBG and free cortisol fell below baseline values after increasing in response to ACTH. In a recent study, the FCI in pigs following 7 d of heat and social stress was not different compared to d 0 due to a simultaneous decrease in plasma total cortisol and pCBG concentrations[24]. Our results indicate that when pCBG concentrations remain relatively stable over a 24 h period, the FCI will change according to fluctuations in total cortisol.

The significance of the observed plasma cortisol-CBG relationship reported here and in previous studies may reside in the calculation of the FCI. Since 60% of total cortisol is bound to pCBG in the pig[3], the amount of cortisol that is available to cells may be misrepresented if the concentration of pCBG is not taken into consideration along with that of total cortisol. Therefore the FCI can provide a better understanding of temporal changes associated with the adrenal response in animals as compared to total plasma cortisol alone.

Implications

To our knowledge this is the first experiment to measure plasma total cortisol, pCBG, percent free cortisol, plasma free cortisol and FCI in swine over a 24 h period. It is also the first experiment to examine the relationship between plasma cortisol and pCBG in swine immediately following acute stimulation of the adrenal gland. By factoring in CBG values through the application of the FCI, a more accurate portrayal of the availability of circulating cortisol is possible. The calculation of the FCI illustrates the amount of free cortisol available to cells without employing time consuming and expensive analytical procedures for the actual measure of free cortisol.

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Mention of trade names or proprietary products does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

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