Effects of various doses of ovine corticotrophin–releasing hormone on plasma and saliva cortisol concentrations in horses

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Objective—To compare the effects of IV administration of various doses of ovine corticotrophin–releasing hormone (oCRH) on plasma and saliva cortisol concentrations in healthy horses and determine whether an oCRH challenge test protocol is valid for use in adult horses.

Animals—24 healthy Warmblood horses.

Procedures—Each horse received oCRH in saline (0.9% NaCl) via IV administration at a dose of 0 (control treatment), 0.01, 0.1, or 1.0 µg/kg (6 horses/group). Jugular blood and saliva samples were collected simultaneously 15 minutes before and immediately prior to injection (baseline); data from these samples were pooled to provide basal values. Subsequently, 14 postinjection blood and saliva samples were both collected within a 210-minute period. Cortisol concentrations in all samples were assessed via a solid-phase radioimmunoassay.

Results—All doses of oCRH induced significant increases from baseline in both plasma and salivary cortisol concentrations. Compared with the smaller doses of oCRH, the 1.0 µg/kg dose of oCRH induced significantly greater plasma cortisol concentrations. A relationship (r = 0.518) between basal cortisol concentrations in plasma and saliva was detected.

Conclusions and Clinical Relevance—For use as a CRH challenge test in adult horses, a protocol involving IV administration of a dose of at least 0.01 µg of oCRH/kg and postinjection collection of blood samples from 10 to 180 minutes and saliva samples from 20 to 50 minutes for assessment of plasma and saliva cortisol concentrations should be sufficient. Application of such a test might be helpful to detect states of chronic activation of the hypothalamo-pituitary-adrenocortical axis at the hypothalamic level. (*Am J Vet Res* 2009;70:361–364)

The HPA axis is a major component of neuroendocrine responses to stressful events. The primary adaptive response to physiologic or psychologic stress in all mammals involves activation of this axis. As a consequence, CRH is released from the hypothalamus. Corticotrophin-releasing hormone acts locally on the anterior lobe of the pituitary gland, inducing the release of ACTH, which in turn enters the systemic circulation and stimulates the adrenal cortices to synthesize and release cortisol.[1] In situations of chronic stress, the pituitary gland response to endogenous CRH and the adrenal gland response to ACTH are expected to be decreased.[2] Stress has been defined as any event that results in increased activity of the HPA axis and a subsequent increase in plasma corticosteroid concentration.[3] In plasma, cortisol is largely bound to corticosteroid-binding globulin; in horses, the concentration of this globulin is quite low.[4] As a consequence of this binding, measurement of only total plasma cortisol concentration may not accurately reflect changes in the HPA axis. Thus, preference should be given to measurement of free cortisol, which can be achieved via assessment of cortisol concentration in saliva.[5] Besides the ACTH challenge test, results of a CRH challenge test can be helpful to detect states of chronic activation of the HPA axis attributable to external stressors at the level of the hypothalamus.[6] It has been stated that the CRH challenge test is not recommended for differentiating primary (ie, at the adrenocortical level) from secondary (ie, at the hypophyseal level) adrenal gland insufficiency but may be useful for differentiating secondary from tertiary (ie, at the hypothalamic

Abbreviations

CI	Confidence interval
CRH	Corticotrophin-releasing hormone
HPA	Hypothalamo-pituitary-adrenocortical
oCRH	Ovine corticotrophin–releasing hormone

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level) adrenal gland insufficiency.[7] To our knowledge, information regarding the optimal dose of CRH to induce cortisol release from the HPA axis in horses is lacking. The purpose of the study reported here was to compare the effects of IV administration of oCRH at doses of 0, 0.01, 0.1, and 1 µg/kg on both plasma and saliva cortisol concentrations in healthy horses and determine whether an oCRH challenge test protocol is valid in adult horses.

Materials and Methods

Horses—The Committee on Animal Welfare of Veterinary Medicine, Utrecht University, approved the study, which was performed during January through March 2002. Twenty-four healthy Dutch Warmblood horses (16 mares and 8 geldings) from the Utrecht University herd were included in the study. All horses were kept under similar environmental conditions with regard to housing and feeding and were accustomed to frequent handling. The age of the horses ranged from 4 to 19 years (mean ± SD age, 11 ± 5 years), and weight ranged from 492 to 724 kg (mean weight, 603 ± 60 kg).

Procedures—Horses were randomly allocated to 1 of 4 treatment groups (6 horses/group). In each group, horses received an IV injection of oCRH[a] at a dose of 0 (control treatment), 0.01, 0.1, or 1 µg/kg. Saline (0.9% NaCl) solution was added to the oCRH treatments so that the injection volume (5 mL) was constant among groups. The control treatment consisted of an equivalent volume of saline solution.

The day before a challenge was performed in a given horse, a jugular vein catheter[b] was inserted. No food was withheld from the horses prior to the challenge test. On the day of the challenge, a blood sample and a saliva sample were obtained from each horse 15 minutes before and immediately prior to (baseline) administration of the allocated treatment (designated as –15 and 0 minutes, respectively). Each treatment was administered between 8:00 and 8:20 AM (immediately after sample collection at 0 minutes). Blood and saliva samples were simultaneously collected at 10, 20, 30, 40, 50, 60, 70, 80, 90, 105, 120, 150, 180, and 210 minutes after injection. Data obtained from the samples collected at –15 and 0 minutes were used to provide basal plasma and saliva cortisol values.

At each time point before and after injection in each horse, a blood sample (5 mL) was collected from the jugular vein catheter into a vacuum tube[c] containing EDTA. At the same time points, cotton balls[d] held with 25-cm-long forceps were used to obtain saliva from various locations in the oral cavity. Depending on the amount of saliva present, 10 to 30 cotton balls were used for each sample collection. Immediately after collection, as many as 5 cotton balls were placed in a 10-mL syringe and saliva was extracted by depression of the plunger. The saliva and blood samples were centrifuged at 23,000 × *g* for 5 minutes, and the supernatant was separated and stored at –20°C until assayed. In all samples, cortisol concentration was measured by use of a commercially available solid-phase radioimmunoassay kit[e] that was validated for measurement of total plasma cortisol (bound and free) and saliva cortisol concentrations in horses, as described previously.[8]

Statistical analysis—Analyses were performed by use of a statistical software program.[f] A 1-way ANOVA was used to determine whether plasma or saliva cortisol concentrations induced by the various doses of oCRH differed significantly at each time point. Within each treatment group, a paired *t* test was performed to evaluate whether there was a significant difference in plasma or saliva cortisol concentration from baseline at any time point. Normality of the data was analyzed by use of the normal probability-probability plot with the Blom method on data transformed into natural logarithms and the Kolmogorov-Smirnov test; accepting or rejecting normal distribution was the null hypothesis. Scatter diagrams were plotted, and the strength of the linear association between basal plasma and salivary cortisol concentrations was assessed by obtaining the correlation coefficient (r) and testing whether it was different from zero by use of the 2-tailed Pearson product moment correlation test. All data are expressed as mean ± SD. Values of $P < 0.05$ were considered significant.

Results

No adverse effects were detected after administration of oCRH at any dose in any horse. Overall, 381 plasma and 288 saliva samples were available for analysis; fewer saliva samples were collected because of dryness of the mouth in some horses at some time points.

Both basal plasma and salivary cortisol concentrations (derived from data obtained at –15 and 0 minutes) were normally distributed. The overall mean basal plasma cortisol concentration was 231 ± 66.6 nmol/L (95% CI, 98.0 to 364 nmol/L; range, 91.6 to 396 nmol/L; n = 45), and the overall mean basal salivary cortisol concentration was 3.2 ± 1.5 nmol/L (95% CI, 0.26 to 6.2 nmol/L; range, 1.5 to 7.1 nmol/L; 36). The relationship between basal plasma and salivary cortisol concentrations was significant (r = 0.518 and P = 0.001; n = 35). Relative to the basal plasma cortisol concentration, the mean basal salivary cortisol concentration was 1.5 ± 0.52% (95% CI, 0.43% to 2.5%; range, 0.46% to 2.5% as determined on the basis of 35 basal values). There were dose-dependent increases in both plasma and saliva cortisol concentrations following oCRH administration (**Figure 1**). All doses of oCRH induced significant increases in plasma and salivary cortisol concentrations from baseline (0-minute) values. Compared with baseline values, peak cortisol concentrations following administration of the 0.01 µg/kg dose of oCRH were approximately 1.5 times as great in plasma and approximately 5 times as great in saliva. In all 3 dose groups, plasma cortisol concentrations were significantly increased at 20 minutes after administration of oCRH, compared with baseline values. The 0.1 and 1.0 µg/kg doses of oCRH each resulted in a more persistent increase in cortisol concentration that remained significantly different from baseline values throughout almost the entire 210-minute period. Compared with the 0.01 and 0.1 µg/kg doses of oCRH, the 1.0 µg/kg dose of oCRH induced significantly greater plasma and saliva cortisol concentrations.

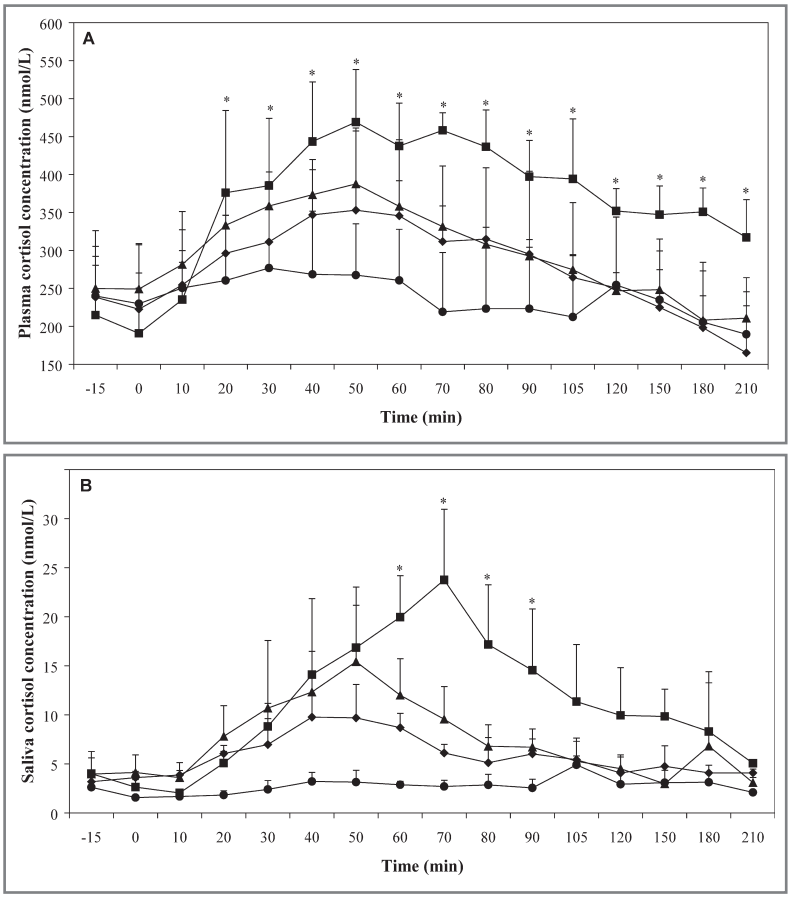

Figure 1—Mean ± SD plasma (A) and saliva (B) cortisol concentrations in 24 horses before and after IV administration of oCRH at a dose of 0 (control treatment; circles), 0.01 (diamonds), 0.1 (triangles), or 1.0 (squares) µg/kg (6 horses/group). Blood and saliva samples were collected 15 minutes and immediately before the injection (0 minutes) and at intervals during a postinjection 210-minute period. *At this time point, the value for the group that received the 1.0 µg/kg dose of oCRH was significantly ($P < 0.05$) greater than the value for the group that received the 0.1 µg/kg dose of oCRH.

Discussion

Several types of CRH that are derived from different species (eg, human, rat, bovine, and ovine types) are available. Although human and equine CRH share identical amino acid sequences, preference was given to oCRH over human CRH for use in the present study. That decision was made because the similarity between the biologically active parts of the equine and nonequine molecules was described by Livesey et al[9] on the basis of data obtained by use of oCRH and because in the report of a study by Alexander et al[10] in which administration of human CRH to horses was investigated, the dose in terms of body weight (ie, per kilogram basis) was not specified. Furthermore, although the amino acid sequence of equine CRH is apparently identical to those of human and rat CRH,[9] posttranslational modifications cannot be ruled out.

Similar to an ACTH challenge test, a CRH challenge test can be helpful to detect states of chronic activation of the HPA axis as a result of external stressors.[6] For example, chronic social stress in horses was associated with a poor fractional change in pituitary venous concentrations of ACTH following administration of 2 µg of human CRH.[10] In addition, social stress in horses was also associated with decreased capacity of corticosteroid-binding globulin and increased free plasma cortisol concentrations. To accurately assess adrenal axis status in horses, it has been proposed that it is essential to monitor the binding capacity of corticosteroid-binding globulin and evaluate free cortisol concentrations in addition to total cortisol concentrations.[5] Because only unbound cortisol can diffuse into saliva, the saliva cortisol concentration reflects the plasma free cortisol concentration.[11] Whether total or free cortisol concentration in plasma is more highly correlated with cortisol concentration in saliva needs further investigation. Although it might be questioned whether total cortisol concentration adequately reflects the free cortisol status in horses because of the low plasma concentration of corticosteroid-binding globulin in that species, Alexander and Irvine[5] determined that assessment of free cortisol concentration in plasma in horses undergoing social stress is essential for diagnostic decision making. It has been reported[9] that IV administration of approximately 1 µg of oCRH/kg causes a significant increase in mean pituitary ACTH secretion rate in pituitary venous effluent from unanesthetized horses. However, to the authors' knowledge, CRH challenge studies[12–14] have been performed in several species (eg, rats, cattle, and pigs) but not in horses. Furthermore, information regarding the effect of CRH on cortisol release in horses is also lacking. On the basis of the data obtained in the present study, the oCRH threshold dose to induce the cortisol response in horses (ie, significant increase in plasma cortisol concentration from preinjection baseline value) seems to be as low as 0.01 µg/kg. This threshold dose is comparable to that in humans (oCRH threshold dose, 0.01 to 0.03 µg/kg).[15] Calves appear to be less sensitive to CRH, compared with humans and horses (bovine CRH threshold dose, 0.1 µg/kg).[6] The peak plasma cortisol concentration in horses in the present study was similar to that achieved in humans (353 vs 331 nmol/L, respectively) following oCRH administration. On the basis of the results of the analysis of plasma and saliva samples at various time points in our study, it is evident that it takes some time for cortisol to diffuse into saliva. In the present study, the relationship between basal plasma and salivary cortisol concentrations was evaluated; the correlation coefficient was lower than that determined in other studies[8,11] in horses, which remains unexplained. Nevertheless, the findings of our study indicated that plasma and saliva cortisol concentrations increase in horses in response to administration of exogenous oCRH. To perform a challenge test with oCRH in adult horses, it appears appropriate to collect blood samples for assessment of plasma cortisol concentration

immediately before and at 30 minutes after IV injection of oCRH and to collect saliva samples for assessment of saliva cortisol concentration immediately before and at 70 minutes after IV injection of oCRH.

a. Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands.
b. Mila International Inc, Erlanger, Ky.
c. Venoject, Terumo, Leuven, Belgium.
d. Medium cotton balls, Professional Medical Products, Greenwood, SC.
e. Count-A-Coat Cortisol, Diagnostic Products Corp, Los Angeles, Calif.
f. SPSS, version 12.0 for Windows, SPSS Inc, Chicago, Ill.

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