Cortisol infusion depresses the ratio of bioactive to immunoreactive ACTH in adrenalectomized sheep fetuses

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Zehnder, Timothy J., Nancy K. Valego, Jeffrey Schwartz, Jennifer Green, and James C. Rose. Cortisol infusion depresses the ratio of bioactive to immunoreactive ACTH in adrenalectomized sheep fetuses. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E391–E396, 1998.—We examined the effects of exogenous cortisol on plasma immunoreactive adrenocorticotropic hormone (iACTH), bioactive ACTH (bACTH), and ACTH-(1—39) in nine adrenalectomized fetuses at 126–130 and 136–140 days of gestation. Fetuses received 4 h of cortisol (2 µg·kg⁻¹·min⁻¹) or saline infusions on consecutive days. Blood was obtained before and at intervals during infusions. Arterial blood gases and hemocrits were normal and did not change with age. Plasma cortisol did not change during saline infusions but increased significantly (range 30–70 ng/ml) during cortisol infusions. Basal plasma iACTH, bACTH, ACTH-(1—39), and bACTH-to-iACTH and ACTH-(1—39)-to-iACTH ratios were significantly higher in the older fetuses. Cortisol infusions decreased plasma iACTH, bACTH, and ACTH-(1—39) in both groups, and the suppression as a percent of the baseline was similar. The bACTH-to-iACTH ratio declined to the same level at 126–130 (0.201 ± 0.040 to 0.051 ± 0.002) and 136–140 (0.389 ± 0.088 to 0.046 ± 0.002) days of gestation. These data suggest that physiological concentrations of cortisol selectively inhibit bACTH secretion, and the ACTH response to cortisol inhibition is not different between 126 and 140 days of gestation in adrenalectomized sheep fetuses.

IN THE SHEEP FETUS, maturation of the hypothalamic-pituitary-adrenal axis is necessary for organ maturation, the response to stress, and the onset of parturition (6). Adrenocorticotropic hormone (ACTH), secreted by anterior pituitary corticotrophs, plays an integral role in this axis by inducing the synthesis and release of cortisol from the adrenal cortex. Plasma concentrations of ACTH are regulated positively by the hypothalamic hormones corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) and negatively by cortisol. ACTH-(1—39) is synthesized as part of larger precursor molecules (7). Two posttranslational processing steps of pro-opiomelanocortin are necessary for the production of ACTH-(1—39). ACTH-(1—39) and its precursors [the latter have been reported to have weak steroidogenic activity (8)] represent immunoreactive ACTH (iACTH) secretory products of the anterior pituitary. Because ACTH antibodies bind to specific amino acid sequences of the ACTH-(1—39) molecule, radioimmunoassays (RIAs) for ACTH can react with both ACTH-(1—39) and its higher molecular weight precursors. Given the differences in bioactivity of ACTH and its precursors, changes in iACTH may or may not reflect identical changes in bioactivity.

There is increasing evidence of differential regulation of the secretion of bioactive ACTH (bACTH), iACTH, ACTH-(1—39), and ACTH precursors during basal and stimulated conditions. ACTH-(1—39) is the most potent known activator of adrenal steroidogenesis, whereas the ACTH precursors have been shown to block this effect at the fetal adrenal (21). Experiments in humans (10), dogs (8), and sheep (3, 28) have shown that the basal secretion of iACTH consists predominantly of ACTH precursors that contain relatively little ACTH bioactivity. In contrast, various stressors not only increase plasma iACTH but also increase the bACTH-to-iACTH (8, 10, 28) and ACTH-(1—39)-to-iACTH precursor ratios [bACTH/iACTH and ACTH-(1—39)/ACTH precursors] (3, 28). In the developing sheep fetus it is also particularly interesting that the ACTH-(1—39)/ACTH precursors under basal conditions increases as a function of age over the last third of gestation (3).

Recent findings have shown that the secretion of bACTH and ACTH-(1—39) appears to be relatively more sensitive to negative steroid feedback than is secretion of iACTH and ACTH precursors. We have observed that a chronic elevation of plasma cortisol attenuated the iACTH and abolished the bACTH responses to hemorrhage in 0.70 gestation sheep fetuses (28). We have also observed that in fetuses with higher concentrations of plasma cortisol, the bACTH and ACTH-(1—39) responses to both CRF and AVP were reduced, whereas the iACTH and ACTH precursor responses were unchanged (29). These results were consistent with the finding that dexamethasone, a potent synthetic corticosteroid, inhibited the CRF- and AVP-induced pituitary secretion of ACTH-(1—39) but had little or no effect on the secretory responses of ACTH precursors in vitro (20).

The primary goal of this investigation was to test the hypothesis that an acute elevation of plasma cortisol would have a greater negative feedback effect on plasma bACTH than plasma iACTH, thereby causing a reduction in the plasma bACTH/iACTH. We tested this hypothesis in adrenalectomized sheep fetuses to begin with a situation in which plasma iACTH and bACTH would be elevated. We also measured ACTH-(1—39) to better characterize the nature of the plasma peptides constituting iACTH. Furthermore, because there are some data that suggest the sensitivity of ACTH secre-
tion to steroid feedback is reduced near term, we studied the same fetuses at 128–131 (0.70) and 136–140 (0.95) days of gestation to determine whether the inhibitory effects of cortisol are diminished close to term (15, 26, 27).

METHODS

Animal preparation. In a longitudinal study, fetuses of nine time-dated sheep of mixed breeds were all studied twice at 126–130 and at 136–140 days of gestation (term is about 145 days). The ewes were kept in portable carts and given food and water ad libitum. One day before surgery (120 ± 0.5 days of gestation) food and water were removed. The surgery included bilateral adrenalectomy of the fetus and the placement of catheters in the femoral arteries and veins of the fetus and ewe. Details of the surgical procedures have been described (17). Because the dose of cortisol was relative to weight, fetal weight was estimated at the time of each infusion and was based on previous data (11). All procedures were approved by our Institutional Animal Care and Use Committee.

Infusion. Each study was performed at the two ages. On two consecutive days the fetus received either a 4-h infusion of 2 µg·kg⁻¹·min⁻¹ cortisol (11,17,19,21-trihydroxy-4-pregnene-3,20-dione) or isonotic saline (room temperature) in random order at an infusion rate of 1.0 ml/h. Based on previous work this dose of cortisol has been shown to increase plasma cortisol within a physiological range.

Blood collection. Arterial blood (6–8 ml) was obtained before (0) and 30, 60, 120, 180, and 240 min after the infusions began to measure blood gases, hematocrit, and hormone concentrations. After each sample an equivalent volume of saline was given as volume replacement, and at the end of the experiment 40 ml of maternal blood were infused into the fetus. Blood was collected in sterile syringes with 0.70 ml used for the measurement of blood gases and pH, whereas the remainder was transferred to chilled polystyrene test tubes with EDTA (1.4 mg EDTA/ml blood) and centrifuged (3,000 revolutions/min) for 10 min at 4°C for the measurement of plasma hormones. Plasma was then collected and stored at −20°C.

RIAs. Cortisol in plasma and incubation medium was measured by RIA as previously described (19). The tracer [1,2,6,7-³H]cortisol was supplied by Amersham, and the antibody [antigen: cortisol-3–21,21-trihydroxy-4-pregnene-3,20-dione] was supplied by ICN (Costa Mesa, CA). Total iACTH was measured by RIA as previously described (18). We define iACTH as that material obtained from plasma that: (a) binds to antibody [antigen: cortisol-3–21,21-trihydroxy-4-pregnene-3,20-dione] and dissociates the binding of tracer quantities of ¹²⁵I-labeled ACTH extracted from plasma with each assay was 65 ± 3%. Details of these extraction procedures have been recorded (4, 5).

Bioassay. We define bACTH as that material obtained from plasma extracts that stimulates the secretion of cortisol from dispersed, cultured adult sheep adrenocortical cells. Bioactivity is compared with known concentrations of synthetic ACTH-(1–39) in the same assay. Adrenocortical cells were cultured and equilibrated to serum-free conditions for 3 days and then replicate wells were exposed to either standard ACTH or various concentrations or a sample of plasma extract (same volume). After 2 h the media were recovered, and cortisol secreted into the media was measured by RIA. The minimum effective concentration of bACTH was 2 pg/ml, and all data were corrected for recovery. Details of the bioassay procedures have been described (4, 5, 21).

IRMA. Because saline had no inhibitory effects on plasma iACTH and bACTH, ACTH-(1–39) was measured by a two-site IRMA only in the cortisol-infused groups. The IRMA for ACTH-(1–39) uses two radiolabeled monoclonal antibodies: one is directed at the sequence ACTH-(10–18) and the other is directed at the sequence ACTH-(25–39). The IRMA is specific for ACTH-(1–39). At concentrations present in fetal sheep plasma, cross-reactivity with ACTH precursors is negligible. Details of the IRMA procedures and validation for use in sheep plasma have been given (2, 23).

Statistical analysis. Changes in hormone concentrations, blood gases, and hematocrits were analyzed by the analysis of variance. Newman-Keuls test was used for post hoc analyses. Regression analysis was used to compute the slope of the lines describing the relationship between the change in cortisol and the percent change in ACTH between 0 and 120 min for animals at both gestational age ranges. Student’s t-test was then used to compare the slopes at the two age ranges. Differences were significant when P < 0.05. Data are presented as means ± SE.

RESULTS

Blood gases, pH, and hematocrit. Arterial blood gases, pH, and hematocrit were not significantly different between the age groups. The means for both groups were pH7.32 ± 0.01, PO2 22.0 ± 2.0 mmHg, PCO2 53.0 ± 4.0 mmHg, and hematocrit 37.0 ± 2.0%. In each age group there were small, yet significant, increases (37.4 ± 2.0 to 38.6 ± 2.0%) in hematocrit in the cortisol-infused groups and decreases in PO2 (21.3 ± 2 to 20.5 ± 2 mmHg) in the saline-infused group.

Plasma cortisol. Basal plasma concentrations of cortisol were not significantly different between the two age groups and did not significantly change during the saline infusions (Table 1). Plasma cortisol concentrations significantly increased during the cortisol infusions at all time points in each age group and were not significantly different between the age groups at 0, 30, 60, or 120 min, but were higher at 180 and 240 min in the older animals.

Plasma iACTH by RIA. Plasma concentrations of iACTH were significantly greater in the older than younger group (F = 8.4, P < 0.02) at 0 (i.e., basal) and 60 min (Fig. 1, top). In both age ranges, plasma concentrations of iACTH significantly decreased during the cortisol infusion (F = 16, P < 0.01). In contrast, plasma concentrations of iACTH did not significantly change at either age during the saline infusion with the exception of an increase observed at 240 min (not shown).
Plasma bACTH by bioassay. Basal plasma bACTH concentrations were significantly higher in the older than younger group (F = 7.1; P < 0.04; Fig. 1, middle). Plasma concentrations of bACTH significantly decreased (F = 15.1, P < 0.01) during the cortisol infusion but did not significantly change in either age group during the saline infusion.

Plasma ACTH-(1—39) by IRMA. Basal plasma ACTH-(1—39) concentrations were significantly higher in the older than younger group (F = 6.8; P < 0.05; Fig. 1, bottom). Plasma concentrations of ACTH-(1—39) significantly decreased during the cortisol infusion in each age group (F = 13.2, P < 0.01).

Percent suppression of iACTH, bACTH, and ACTH-(1—39). Relative to basal concentrations, plasma iACTH, bACTH, and ACTH-(1—39) were significantly reduced in each age group (F = 58.7, P < 0.01). Furthermore, there were no significant differences between the age groups in the percent reductions in iACTH, bACTH, and ACTH-(1—39).

Correlations among iACTH, bACTH, and ACTH-(1—39). The correlations for iACTH-ACTH-(1—39) and bACTH-ACTH-(1—39) were 0.79. The correlation for iACTH-bACTH was 0.69.

Plasma bACTH/iACTH and ACTH-(1—39)/iACTH. Basal plasma bACTH/iACTH was significantly higher in the older than younger group (F = 7.7; P < 0.02; Fig.

### Table 1. Plasma concentrations of cortisol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time, min</th>
<th>Cortisol, ng/ml</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0.70 (126-130 days)</td>
<td>0.95 (136-140 days)</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2.9 ± 0.8</td>
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<tr>
<td></td>
<td>60</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>3.1 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>3.7 ± 1.1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0</td>
<td>3.2 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>14.3 ± 4.8*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>25.5 ± 4.9*</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>32.5 ± 8.7*</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>37.3 ± 10.2*</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>36.6 ± 8.7*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9 sheep fetuses. *Significantly different from 0 min. †Significantly different from 0.70.

Plasma bACTH by bioassay. Basal plasma bACTH concentrations were significantly higher in the older than younger group (F = 7.1; P < 0.04; Fig. 1, middle). Plasma concentrations of bACTH significantly decreased (F = 15.1, P < 0.01) during the cortisol infusion but did not significantly change in either age group during the saline infusion.
2). Plasma bACTH/iACTH and ACTH-(1—39)/iACTH significantly decreased during the cortisol infusion at each age (F = 9.8, P < 0.01).

DISCUSSION

To our knowledge this is the first study that measures the bioactivity of ACTH in fetal plasma after adrenalectomy and tests the hypothesis that an acute elevation of plasma cortisol has a greater negative feedback effect on plasma bACTH than plasma iACTH, thereby causing a reduction in plasma bACTH/iACTH. We tested this hypothesis in bilaterally adrenalectomized sheep fetuses because we anticipated marked elevations in both resting plasma iACTH and bACTH. The results of this study supported our hypothesis as well as our expectations regarding resting plasma iACTH and bACTH. Thus the present study suggests that secretion of the different molecular weight forms of ACTH may be subject to differential influence by exogenous cortisol in adrenalectomized sheep fetuses, thereby resulting in alterations of the ratios of bioactive to immunoreactive ACTH and total immunoreactive to ACTH-(1—39) in the plasma.

Several studies have shown that the secretion of bACTH appears to be more sensitive to negative steroid feedback than the secretion of iACTH. In intact fetuses at 0.70 gestation, a 6-day infusion of cortisol that increased basal plasma cortisol concentrations from about 3 to 21 ng/ml attenuated the iACTH but abolished the bACTH responses to hemorrhage (28). Other experiments have compared responses at two gestational ages with different background levels of cortisol. In older (0.92 gestation) intact fetuses with basal plasma concentrations of cortisol ranging from about 30 to 40 ng/ml, the bACTH and ACTH-(1—39) responses to CRF and AVP were reduced, whereas the iACTH and ACTH precursor responses were unchanged compared with responses in younger (0.70 gestation) intact fetuses with basal plasma cortisol concentrations ranging from 3 to 7 ng/ml (29). In another study, the percentage of low molecular weight ACTH in plasma was found to be higher (48%) in adrenalectomized than intact fetuses (27%) between 126 and 139 days of gestation (16), again suggesting a selective inhibition of ACTH-(1—39) by adrenal steroids. In the present study a 4-h infusion of cortisol that increased plasma cortisol from about 4 to 60 ng/ml led to greater and more rapid reductions in bACTH than iACTH. Taken together these studies suggest that the secretion of bACTH is more sensitive to physiological increments in cortisol than iACTH in both intact and adrenalectomized sheep fetuses.

In this study adrenalectomized sheep were used to test the effects of cortisol against a background of elevated ACTH. In intact term fetal sheep plasma ACTH concentrations are low relative to the magnitude of ongoing pulsatile fluctuations (1, 12). After adrenalectomy, ACTH levels are higher and the effects of cortisol more likely to be pronounced and measurable.

The quicker decrease in plasma bACTH than iACTH may be due to the relative activity of cortisol on different cells. In rat pituitaries there are reports of heterogeneity among individual corticotrophs with respect to the presence of unprocessed pro-opiomelanocortin relative to fully processed ACTH in secretory granules (22). In adult sheep pituitary cells dexamethasone inhibits the ACTH-(1—39) secretory response to vasopressin in some but not all classes of corticotrophs (20). A preferential action of glucocorticoids on cells that contain relatively more ACTH-(1—39) would thus be expected to decrease bACTH concentration more than iACTH. In this regard, a decrease in the ACTH-(1—39)/ACTH precursors secreted in response to glucocorticoids has been demonstrated in vitro using slices of fetal sheep pituitary (14). Another possible explanation for the more rapid decrease in bACTH involves clearance from the plasma rather than, or in addition to, effects on secretion. If bACTH (probably mostly ACTH-(1—39)) is degraded or excreted faster than other immunoreactive forms of ACTH in fetal sheep, as in rats (2), then cortisol could produce the results reported here.

It is important to note that several investigators have reported that at increasing gestational ages there are increases in the percentage of low molecular weight ACTH and ACTH-(1—39) in fetal sheep plasma during both unstimulated and stimulated conditions (3—5, 12, 13). In light of the finding that ACTH-(1—39)/ACTH precursors secreted by fetal sheep pituitary slices in-
crease with age, the changes in plasma ACTH-(1—39) and ACTH precursors likely reflect the physiological maturation of the pituitary. In a recent study we did not observe age-related increments in the basal bACTH/iACTH in intact fetal sheep (29). However, we noticed an interesting difference between the earlier study (intact fetuses) and the present study (adrenalectomized fetuses). Compared with intact fetuses, the basal bACTH/iACTH and ACTH-(1—39)/iACTH were significantly greater at both ages in the adrenalectomized fetuses. This suggests that the rising concentrations of plasma cortisol during the last third of gestation are necessary for maintaining the low bACTH/iACTH in fetal plasma under basal conditions.

Another aim of this investigation was to determine if the degree of suppression of plasma bACTH and iACTH by cortisol changes between 126–130 and 136–140 days of gestation in adrenalectomized fetuses. Based on previous work in adrenalectomized fetuses, we anticipated that basal concentrations of iACTH and bACTH would increase with age (24). As expected, the basal concentrations of bACTH and iACTH were higher in the older group. Therefore, we expressed the negative effects of cortisol on bACTH and iACTH by measuring the suppression of bACTH and iACTH relative to their basal concentrations. Although significantly higher concentrations of plasma cortisol were measured in the older fetuses at 180 and 240 min (probably because of an overestimate of growth), the suppression (as a percent of basal value) of bACTH and iACTH was very similar in both groups when plasma cortisol concentrations were not significantly different (i.e., at 30, 60, and 120 min) between groups. Furthermore, when comparing the percent suppression of bACTH or iACTH vs. the absolute changes in cortisol, the two age groups were not significantly different (P > 0.8). Thus the ability of increase in plasma cortisol to suppress bACTH and iACTH levels does not appear to change between 126 and 140 days of gestation in adrenalectomized sheep fetuses.

However, there are data suggesting that the sensitivity of ACTH secretion to steroid feedback is reduced in intact sheep fetuses late in gestation. For example, cortisol infusions did not inhibit basal or stress-induced increments in ACTH levels between 132 and 142 days of gestation (26, 27), whereas between 117 and 131 days of gestation the fetal ACTH responses to stress were blocked (25). Also, plasma iACTH concentrations do not decline despite exponential increments in endogenous plasma cortisol in late gestation (6). Furthermore, the negative effects of dexamethasone implants adjacent to the paraventricular nucleus on steady-state concentrations of corticotropin-releasing hormone and proopiomelanocortin mRNA appear to be attenuated in late-term fetuses (15). Thus some data suggest that both the hypothalamus and pituitary may exhibit reduced sensitivity to glucocorticoids in late gestation, but the specific mechanisms are unknown. If there is, in fact, a reduced sensitivity to cortisol feedback in late gestation, then the results of this present study indicate that the reduced sensitivity is dependent on the presence of the fetal adrenal glands.

Finally, this is the first study that measured the correlations among bACTH, iACTH, and ACTH-(1—39). The correlations suggest that although ACTH measurements by RIA, bioassay, and IRMA are not quantitatively identical, they do reflect similar qualitative changes.

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