Infusion of ACTH stimulates expression of adrenal ACTH receptor and steroidogenic acute regulatory protein mRNA in fetal sheep

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Submitted 23 November 2005; accepted in final form 7 February 2006

Carey, Luke C., Yixin Su, Nancy K. Valego, and James C. Rose. Infusion of ACTH stimulates expression of adrenal ACTH receptor (ACTH-R) and steroid acute regulatory protein (StAR) mRNA in fetal sheep. Am J Physiol Endocrinol Metab 291: E214–E220, 2006—The late-gestation plasma cortisol surge in the sheep fetus is critical for stimulating organ development and parturition. Increased adrenal responsiveness, however, is one of the key reasons for the surge; hence, the underlying mechanisms are not fully understood. Our recent studies suggest that ACTH-mediated increased expression of ACTH receptor (ACTH-R) and steroid acute regulatory protein (StAR) may play a role in enhancing responsiveness. We examined effects of ACTH infusion in fetal sheep on mRNA expression of these two mediators of adrenal responsiveness and assessed the functional consequences of this treatment in vitro. Fetuses of ~118 and 138 days of gestational age (dGA) were infused with ACTH-(1–24) for 24 h. Controls received saline infusion. Arterial blood was sampled throughout the infusion. Adrenals were isolated and analyzed for ACTH-R and StAR mRNA, or cells were cultured for 48 h. Cells were stimulated with ACTH, and medium was collected for cortisol measurement. Fetal plasma ACTH and cortisol concentrations increased over the infusion period in both groups. ACTH-R mRNA levels were significantly higher in ACTH-infused fetuses in both the 118 and 138 dGA groups. StAR mRNA increased significantly in both the 118 and 138 dGA groups. Adrenal cells from ACTH-infused fetuses were significantly more responsive to ACTH stimulation in terms of cortisone secretion than those from saline-infused controls. These findings strongly suggest that ACTH regulates expression of its own receptor and that increases in receptor mRNA are positively correlated with plasma ACTH levels, adrenal ACTH-R mRNA and protein expression, and, indeed, adrenal responsiveness. We also demonstrated that ACTH-R mRNA levels and adrenal responsiveness could be restored by ACTH treatment of adrenal cells in vitro. In addition, our group has found a strong positive correlation between plasma bioactive ACTH concentrations and fetal adrenal ACTH-R mRNA expression. These findings strongly suggest that ACTH regulates expression of its own receptor and that increases in receptor mRNA are positively related to increased cortisol secretion.

Although the importance of ACTH in regulating ACTH-R expression is, to our knowledge, yet to be examined in fetal sheep, controversy exists regarding its impact on ACTH expression. Findings from numerous in vitro studies support the notion that ACTH is a positive regulator of ACTH-R mRNA expression. Conversely, the two in vivo studies undertaken to date have not demonstrated a positive influence of ACTH on receptor expression in the fetus (6). Recently, our investigative group (48) reported that HPD at around 120 days of gestational age (dGA) prevented not only the late-gestation cortisol surge but also the increase in fetal plasma ACTH levels, adrenal ACTH-R mRNA and protein expression, and, indeed, adrenal responsiveness. Two important effectors to this end, the adrenocorticotropic hormone receptor (ACTH-R, specifically known as the melanocortin-2 receptor) and steroid acute regulatory protein (StAR), exhibit ontogenic increases in expression that occur in parallel to the cortisol surge (8). The ACTH-R is a seven-transmembrane-spanning G protein-coupled receptor located in the zona fasciculata of adrenal gland, whereas StAR transports the steroid precursor cholesterol from the outer to the inner mitochondrial membrane for further processing. Fetal plasma ACTH concentrations also increase significantly in late gestation (7, 26, 29), suggesting that ACTH may be the driving force behind the changes in ACTH-R and StAR expression.

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(low: 118 dGA; high: 138 dGA) with the expectation that a more pronounced response would be evident in the older age group. Considering the importance of StAR in facilitating steroidogenesis, StAR mRNA levels also were assessed. Furthermore, we determined whether increases in mRNA levels had any functional significance by examining the responsiveness of adrenocortical cells isolated from the fetuses to ACTH.

**MATERIALS AND METHODS**

**Animals and surgery.** Time-dated, mixed breed, pregnant sheep were obtained ~4–5 days before surgery from local suppliers and housed in straw-lined pens with ad libitum access to food and water. The sheep were divided into two groups, ages ~112 and 132 dGA at the time of surgery, respectively. Surgery was performed under general anesthesia, using aseptic techniques to insert fetal femoral arterial and venous and amniotic catheters. Bolus gentamicin (80 mg; Abbott Laboratories, North Chicago, IL) and ampicillin (500 mg; American Pharmaceutical Partners, Schaumburg, IL) in isotonic saline were administered to the ewe for 2 days after surgery. All procedures were approved by the Wake Forest University Animal Care and Use Committee.

**ACTH infusion.** Approximately 5 days after surgery, fetuses were intravenously infused with ACTH1–24 (Cortrosyn; Organon, West Orange, NJ; n = 8 and 7 for the 118 and 138 dGA groups, respectively) in saline at a dose intended to deliver ~0.5 μg·kg⁻¹·h⁻¹ over 24 h. This dose was calculated with regard to expected fetal weight for the particular gestational age. Control fetuses were infused with saline only (n = 6 and 7 for the 118 and 138 dGA groups, respectively). In the case of twin pregnancies (the majority, n = 14 of 20), one fetus received ACTH whereas the other served as a control. All singleton data and twin data were pooled. Fetal blood samples (3 ml) were taken at 0, 6, and 24 h for measurement of plasma ACTH and cortisol, gases (O₂ and CO₂, pH, and hematocrit). Blood gases and pH were determined using an ABL5 blood gas analyzer (Radiometer, Copenhagen, Denmark). Fetal blood pressure, heart rate, and amniotic fluid pressure were recorded to monitor fetal health (Digi-Med; Micro-Med Enterprises, Tustin, CA) during the first 6 h and the final hour of the infusion period.

After the infusion was complete, both ewe and fetus(es) were killed by pentobarbital sodium overdose (85 mg/kg iv), and fetal adrenals were removed and immediately processed for cell culture or snap frozen for later ACTH-R and StAR mRNA analysis. Fetuses and data were obtained from corresponding saline-infused controls, whereas there was a tendency for adrenal weights to be greater after ACTH infusion (118 dGA Saline 6 118.2 mg/kg, 118 dGA ACTH 6 122.5 mg/kg, 138 dGA Saline 7 138.0 mg/kg, 138 dGA ACTH 7 142.5 mg/kg). Fetal body weight was not affected by ACTH infusion (Table 1). Fetal body weight was not affected by ACTH infusion (Table 1). Adrenals from 138 dGA ACTH-infused fetuses were significantly heavier than those from corresponding saline-infused controls, whereas there was a tendency for adrenal weights to be greater after ACTH infusion.

**RESULTS**

**ACTH dose and body and adrenal weights.** The actual dose of ACTH received (calculated postmortem) was similar between the groups, although somewhat lower than the desired 0.5 μg·kg⁻¹·h⁻¹ (Table 1). Fetal body weight was not affected by ACTH infusion (Table 1). Adrenals from 138 dGA ACTH-infused fetuses were significantly heavier than those from corresponding saline-infused controls, whereas there was a tendency for adrenal weights to be greater after ACTH infusion.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number</th>
<th>Age, days</th>
<th>Weight, kg</th>
<th>Adrenal Weight, mg</th>
<th>Adrenal/Body Weight Ratio, mg/kg</th>
<th>Actual ACTH Dose, μg·kg⁻¹·h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>118 dGA</td>
<td>6</td>
<td>118.2 ± 1.5</td>
<td>2.09 ± 0.19</td>
<td>279 ± 22</td>
<td>142 ± 24</td>
<td>NA</td>
</tr>
<tr>
<td>Saline</td>
<td>8</td>
<td>117.4 ± 1.3</td>
<td>2.15 ± 0.13</td>
<td>324 ± 27</td>
<td>154 ± 14</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>ACTH</td>
<td>7</td>
<td>138.0 ± 0.7</td>
<td>4.79 ± 0.40</td>
<td>559 ± 90</td>
<td>130 ± 18</td>
<td>NA</td>
</tr>
<tr>
<td>138 dGA</td>
<td>7</td>
<td>137.3 ± 0.7</td>
<td>4.36 ± 0.20</td>
<td>835 ± 110*</td>
<td>177 ± 18</td>
<td>0.41 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE. dGA, days of gestational age. *Significant difference between 138dGA ACTH and saline-infused groups, P < 0.05.
infusion in 118 dGA fetuses (Table 1). There were no significant differences in adrenal-to-body weight ratio (Table 1).

**Fetal health, heart rate, and blood pressure.** Within both the 118 and 138 dGA groups, there were no differences in blood gases, pH, or hematocrit over the experimental period following the ACTH infusion. Values fell within the following ranges: pH, 7.32–7.35 (118 dGA) or 7.33–7.35 (138 dGA); hematocrit: 26.5–31.6 (118 dGA) or 31.6–36.7 (138 dGA); \( P_{O_2} \), 19.7–20.6 (118 dGA) or 17.0–18.9 mmHg (138 dGA); and \( P_{CO_2} \), 43.7–51.5 (118 dGA) or 45.9–50.4 mmHg (138 dGA). Similarly, neither heart rate (data not shown) nor blood pressure (baseline values were 45.2 ± 6.4 and 54.3 ± 6.4 mmHg in 118 and 138 dGA fetuses, respectively) in any of the treatment groups changed significantly over the infusion period.

**Plasma ACTH and cortisol concentrations.** For plasma ACTH concentrations there were effects of time (F = 7.39, \( P < 0.01 \)) and treatment (F = 11.16, \( P < 0.01 \)), whereas for cortisol concentrations there were effects of time (F = 31.92, \( P < 0.0001 \)), treatment (F = 5.25, \( P < 0.05 \)), and fetal age (F = 31.10, \( P < 0.0001 \)).

There were no differences in basal plasma ACTH concentrations between groups, whereas basal plasma cortisol concentrations were significantly higher in 138 dGA fetuses (Fig. 1). In both 118 and 138 dGA ACTH-infused fetuses, plasma ACTH concentrations were significantly elevated from basal levels at 6 h but were not any higher at 24 h. In contrast, plasma cortisol levels were further elevated at 24 h (Fig. 1). Neither ACTH nor cortisol concentrations changed significantly in response to saline infusion in either age group.

**Adrenal ACTH-R and StAR mRNA.** For adrenal ACTH-R mRNA expression there were effects of both treatment (F = 22.9, \( P < 0.001 \)) and age (F = 48.0, \( P < 0.001 \)). The same was true for adrenal StAR mRNA expression (treatment: F = 11.3, \( P < 0.01 \); fetal age: F = 37.2, \( P < 0.001 \)).

Expression levels of both ACTH-R and StAR mRNA were significantly increased after ACTH infusion in both the 118 and 138 dGA groups (Fig. 2). ACTH-R and StAR mRNA levels were significantly higher in 138 compared with 118 dGA adrenals from saline-infused fetuses. Representative gels for ACTH-R and StAR mRNA are presented in Fig. 3.

**Cell cortisol secretion data.** In all unstimulated/control groups, cortisol was below the limit of assay detection. There were overall effects of both treatment (F = 10.8, \( P < 0.01 \)) and age (F = 34.6, \( P < 0.001 \)) with regard to ACTH-induced cortisol secretion from adrenocortical cells. The pattern of cortisol secretion following ACTH stimulation was consistent between the two age cohorts, with higher secretory responses evident in cells isolated from ACTH-infused fetuses (Fig. 4).

**Discussion**

Our novel findings in this study indicate that ACTH can upregulate mRNA expression of its receptor and StAR in the fetal sheep adrenal in vivo. Furthermore, we have demonstrated that this can occur in fetuses of different gestational age (118 and 138 days) and is accompanied by increased adrenal responsiveness.

In adrenals isolated from 118 and 138 dGA fetuses, we found that expression of both ACTH-R and StAR mRNA was markedly increased after 24-h infusion of ACTH. Although others have examined the influence of ACTH infusion on ACTH-R mRNA levels, this is the first study to report the effects of increased plasma ACTH concentrations on StAR mRNA expression in the fetal sheep adrenal. In accordance with this regulatory influence of ACTH on StAR expression, there have been studies conducted using other adult animal models (i.e., rats and hamsters) demonstrating that acute ACTH treatment increases expression of StAR mRNA (3, 16, 25). The induction of StAR gene expression by ACTH is predominately mediated by cAMP generated after ACTH ligand-receptor binding, which in turn facilitates the phosphorylation of various transcription factors (45).

With respect to ACTH-R mRNA expression, we are aware of only one other study comparable to our own in which measurements were made after 24 h of ACTH infusion (6). In
that study, infusion of ACTH-(1–24) at a dose of 0.5 μg/h to 126–127 dGA fetuses was found to have no effect on expression of ACTH-R mRNA. As in our study, ACTH-R mRNA was quantified using RPA, leaving only the dose, timing, and other differences in procedure to explain the disparity. One such technical difference that may have had an impact was our choice to restrict the analysis to adrenocortical tissue rather than utilizing the whole adrenal. This may have enhanced our ability to detect a treatment effect. Another investigation undertaken by Simmonds et al. (44), in which fetuses were infused for a maximum of 18 days (from 115 dGA) at 14.7 pmol·kg⁻¹·h⁻¹, also failed to demonstrate an increase in expression of ACTH-R mRNA. The relatively low sensitivity of the slot blot technique used for quantifying ACTH-R mRNA in this study may have precluded the detection of a treatment effect. Others (14, 17, 37, 50), including our group (49), have reported the presence of multiple bands with the use of Northern blot analysis for ACTH-R mRNA, with a major band at 3.6 kb, whereas Simmonds et al. were only able to detect one band at 4.0 kb. Their inability to detect all the bands usually found to hybridize with the probe also may be a factor underlying the difference between reports.

A report by Fraser et al. (17) is consistent with our findings. They examined plasma ACTH and ACTH-R mRNA changes following hypoxemia in sheep fetuses. After 48 h of hypoxia, plasma ACTH concentrations had increased from 20 to >350 pg/ml in 132–134 dGA fetuses. Corresponding ACTH-R mRNA expression was increased by ~40% over normoxic controls. In addition, our group found a strong positive relationship between plasma ACTH-(1–39) concentrations and adrenocortical ACTH-R mRNA levels in fetal sheep (49),
which is consistent with the in vivo effect in this study of ACTH on expression of its receptor.

Our findings with regard to ACTH-induced ACTH-R and StAR mRNA upregulation are supported by a number of in vitro studies. For example, Lebrethon et al. (24) noted that ACTH-R mRNA in cultured human fetal and adult adrenal cells was significantly increased after incubation with ACTH. Penhoat et al. (35) reported similar findings using adult bovine adrenal fasciculata-reticularis cells, as did Mountjoy et al. (30) with mouse and human adrenocortical carcinoma cell lines. Heightened expression of StAR mRNA following ACTH exposure also has been demonstrated in cultured adult bovine and human adrenocortical cells (23, 27, 31).

The fact that we were able to demonstrate upregulated expression of ACTH-R and StAR mRNA in fetuses from two distinct age cohorts is particularly important and provides strong evidence for ACTH being a primary mediator of the change in adrenal responsiveness. It also suggests that either 1) the effects of ACTH are not limited to a critical window of development, or 2) the critical window is large. That these changes also have functional consequences is also a crucial finding, as evident in our in vitro studies showing a positive effect of ACTH on adrenal responsiveness.

Although we did not have sufficient tissue to measure either ACTH-R or StAR protein expression in this investigation, both the mRNA and steroid secretion findings strongly suggest that they would have been accordingly upregulated. In support of this assertion, our group recently published work demonstrating in late-gestation fetuses that changes in ACTH-R mRNA expression were accompanied by similar directional and proportional changes in protein expression (48). Furthermore, in earlier work (49), our group found that there was an excellent correlation between ACTH-R mRNA expression and ACTH-R binding capacity, which in turn suggests a correlation between receptor mRNA and protein levels. With regard to StAR, Coulter et al. (8) reported that both StAR mRNA and protein expression increased in an ontogenic manner in late-gestation sheep fetuses and that parallel postpartum decreases were evident. We also have found that changes in StAR mRNA reflect changes in protein expression in adrenals from HPD fetuses (46).

As previously mentioned, the ACTH-(1–24) infusion dose used in our studies was designed to increase plasma ACTH concentrations to those seen in the near-term fetus. There have been numerous studies published in which plasma-immunoreactive ACTH concentrations in near-term control fetuses were in excess of 200 pg/ml (20, 38–40, 52, 54). Hence, we feel that the mean peak concentrations attained in the present study (over 200 pg/ml) are certainly within the physiological range. It should be noted that in all of the studies cited (as in ours), measurements were made using RIA.

There were no within-age group differences in basal plasma ACTH or cortisol concentrations in the present study. This is consistent with findings from a previous study in which Schwartz and Rose (43) showed that fetal plasma cortisol levels in twins are similar until 2 days before the onset of labor at term. These data suggest that it is feasible to utilize twins fetuses in experimental designs in which one fetus receives treatment and the other serves as a control until just before parturition, when differences in plasma cortisol may be apparent. The possibility that twins may show a slight delay in adrenal maturation (15) did not appear to affect our results, because we observed similar responses to ACTH infusion at two different gestational ages.

Aside from ACTH-R and StAR mRNA, other factors in the ACTH signal cascade are likely to have been altered by ACTH infusion in the present study. For instance, Tangelakis et al. (47) reported that mRNA expression of both 17α-hydroxylase and cholesterol side-chain cleavage enzyme were significantly elevated after infusion of ACTH, whereas in the studies of Carter et al. (6) and Simmonds et al. (44), changes in steroidogenic enzymes were evident after infusion. In addition, the G protein coupled to the ACTH-R, adenylate cyclase, and cAMP also have been shown to be positively regulated by ACTH (12, 13, 28).

Although not statistically significant, there was a definite trend for ACTH-infused fetuses to have larger adrenals relative to body weight. This was particularly evident in the 138 dGA group. Other studies have noted direct or indirect effects of ACTH on adrenal weight. For instance, Carter et al. (5) found that adrenocortical weight was increased in 105–112 dGA fetuses after 24 h of ACTH infusion. Warnes et al. (50) demonstrated that fetal sheep infused with metyrapone (a competitive inhibitor of 11β-hydroxylase) for 15 days had plasma ACTH concentrations at 140 dGA that were almost double those in control (157 vs. 81 pg/ml) and that corresponding adrenal weights also were approximately double (830 vs. 430 mg). The importance of ACTH in promoting adrenal growth is further emphasized in the fetal sheep HPD model, in which our group has demonstrated that lower prevailing bioactive plasma ACTH concentrations are associated with decreased adrenal weights close to term. A number of other studies have also found HPD fetuses to have lower adrenal weights (9, 36, 42). The answer as to whether this increased fetal adrenal weight following ACTH infusion is a consequence of hypertrophy or hyperplasia is unknown (in our study, we did not have sufficient tissue to perform histology); however, early work in adult rats suggests that ACTH exerts a hypertrophic effect (10).

The late-gestation surge in plasma glucocorticoid levels is a critical event with respect to preparation for birth in all mammals in which it has been examined. The precise mechanisms underlying this phenomenon are currently the subject of investigation, with a number of mediating factors likely to be involved. In this study we examined the importance of plasma ACTH in regulating two key mediators of adrenal responsiveness, ACTH-R and StAR mRNA, and we report for the first time that ACTH upregulates expression of both in fetal sheep of different gestational ages. Significantly, we found that these in vivo changes had functional consequences in vitro, with adrenocortical cells isolated from ACTH-infused fetuses exhibiting heightened responsiveness. Our findings are supportive of the hypothesis that ACTH acts in vivo at the adrenal to upregulate expression of the ACTH-R and StAR. In the late-gestation fetus, these changes are likely to be important contributory mediators of increased fetal adrenal responsiveness and, hence, the cortisol surge.

GRANTS
This research was supported by National Institute of Child Health and Human Development Grant HD-11210.
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