Divergent changes in plasma ACTH and pituitary POMC mRNA after cortisol administration to late-gestation ovine fetus

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Jeffray, T. M., S. G. Matthews, G. L. Hammond, and J. R. G. Challis. Divergent changes in plasma ACTH and pituitary POMC mRNA after cortisol administration to late-gestation ovine fetus. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E417–E425, 1998.—Plasma concentrations of cortisol and adrenocorticotropic hormone (ACTH) rise in the late-gestation sheep fetus at approximately the same time as there is an increase in the plasma levels of corticosteroid-binding globulin (CBG). We hypothesized that intrafetal cortisol infusion during late pregnancy would stimulate an increase in fetal plasma CBG, which in turn would bind cortisol and diminish glucocorticoid-negative feedback regulation of the fetal pituitary, leading to an increase in plasma ACTH concentrations. Cortisol was infused into chronically catheterized fetal sheep beginning at 126.1 ± 0.5 days of gestation and continued for 96 h. Control fetuses were infused with saline. In cortisol-infused fetuses, the plasma cortisol concentrations rose significantly from control levels (4.4 ± 0.6 ng/ml) to 19.3 ± 3.1 ng/ml within 24 h and remained significantly elevated throughout the infusion period. Plasma immunoreactive (ir) ACTH concentrations were significantly elevated in cortisol-infused fetuses within 24–48 h and remained significantly higher than in controls throughout the 96-h experimental period. Plasma free cortisol concentrations increased 10-fold and remained significantly elevated in cortisol-infused animals, despite a rise in plasma corticosterone-binding capacity. Levels of pituitary proopiomelanocortin (POMC) mRNA in the fetal pars distalis and pars intermedia were 96% and 38% lower, respectively, after 96 h of cortisol infusion. Therefore physiological elevations of plasma cortisol, in the late-gestation ovine fetus, lead to increases in mean plasma irACTH concentrations, but this is not associated with increases in fetal pituitary POMC mRNA levels.

Corticosteroid-binding globulin; free cortisol; adrenocorticotropic hormone; proopiomelanocortin

In fetal sheep, basal plasma concentrations of immunoreactive (ir) adrenocorticotropic hormone (ACTH) and cortisol (32) and pituitary proopiomelanocortin (POMC) expression (29) rise concomitantly in late gestation. This occurs despite demonstrable negative-feedback effects of glucocorticoids on stress-induced (1) or corticotropin-releasing hormone (CRH)-stimulated pituitary-adrenal function (31), suggesting that there may be a decrease in cortisol negative feedback on the hypothalamus and pituitary at this time. Apostolakis et al. (3) previously showed that administration of cortisol to the ovine fetus at 134 days of gestation altered ACTH pulsatility, increasing ACTH pulse peak and nadir values (3). However, the mechanism whereby intrafetal cortisol has a positive effect on plasma ACTH concentrations during late gestation is not clear. Therefore we infused cortisol to fetal sheep, beginning before the prepartum rise in endogenous cortisol, in amounts that would reproduce plasma cortisol concentrations similar to those near term to determine the effects on plasma ACTH and to examine the underlying mechanisms of any changes.

The plasma concentration of corticosteroid-binding globulin (CBG), the high-affinity binding protein for cortisol, also rises during the last third of gestation (4, 7), reflecting an increase in its synthesis in the fetal liver (7). We have previously showed that CBG biosynthesis was stimulated by exogenous cortisol (5, 23) and reduced after bilateral adrenalectomy of the ovine fetus (23). It was therefore suggested that the rise in cortisol stimulated CBG and would in turn help to maintain a low free cortisol concentration in plasma despite elevations in the total (free + bound) cortisol concentration (4, 11). We reasoned that a rise in CBG would result in decreased free cortisol (11, 26), which in turn would diminish the negative-feedback effects of cortisol on the pituitary, resulting in an increase in pituitary POMC mRNA levels and ACTH output. To examine the underlying mechanisms of the ACTH response to exogenous cortisol, we measured levels of POMC mRNA in different regions of the pituitary and CBG mRNA in the liver at various times during an intrafetal cortisol infusion. Therefore the overall hypothesis of the present study was that intrafetal cortisol administration during late gestation would increase hepatic CBG synthesis and circulating CBG levels, thereby reducing free cortisol concentrations in plasma. In turn, this would reduce the negative-feedback effects of cortisol on pituitary POMC expression and irACTH output, resulting in an increase in circulating irACTH concentrations, despite elevated total cortisol concentrations in plasma. In this manner, we would approximate the plasma profiles of ACTH and cortisol observed in the fetal sheep near term.

METHODS

Animals. Surgery was performed under general anesthesia, on mixed breed ewes, at days 119–122 of gestation (full term is 145–147 days). The techniques used have been...
described previously (14). Briefly, a midline incision was
made in the ewe's lower abdomen to expose the uterus, which
was then opened, and the fetal head was exteriorized. Polyvi-
nyl catheters, filled with sterile heparinized saline, were
inserted into a fetal carotid artery and jugular vein. Cath-
eters were also placed into the amniotic cavity and a maternal
femoral vein. Uterine electromyographic (EMG) leads (Cooner
Wire, Chatsworth, CA) were attached to the uterus to monitor
myometrial electrical activity. Prophylactic antibiotics were
administered at the time of surgery and continued for 3 days
postoperatively as described previously (14).

Blood samples were collected daily, and pH, Pco2, and PO2
were measured using an ABL-5 blood gas analyzer (Radiomi-
eter, Copenhagen, Denmark). The protocols were approved by
the Animal Care Committees of St. Joseph's Health Centre,
University of Western Ontario, and University of Toronto in
accordance with the guidelines of the Canadian Council on
Animal Care.

Experimental protocols. Animals were allowed a minimum
of 5 days to recover after surgery before experimentation
began. Starting on days 124–129, fetuses received an intrave-
nous infusion of either cortisol (11β,17,21-trihydroxy-4-
pregnen-3,20-dione; Steraloids, Wilton, NH; 5 µg/min, n = 7)
or an equal volume of saline (3 ml/h, with 2%/vol ethanol, n = 6)
for 96 h. Fetal arterial blood samples (4 ml) were
collected into chilled, heparinized syringes every 8 h begin-
ing 24 h before the start of infusion and continuing through-
out the experiment. Samples were centrifuged immediately
at 1,500 g for 10 min at 4°C, and the plasma was stored at
−20°C until analysis. Additional animals were infused for 12
(n = 4) or 24 h (n = 4) to examine changes in POMC and CBG
mRNA levels associated with the initial phase of the rise in
plasma cortisol concentrations. At the conclusion of the
infusion periods (12, 24, or 96 h), the animals were killed with
an overdose of 24% pentobarbital sodium (Euthanyl, MTC
Pharmaceuticals, Cambridge, ON, Canada), and fetal tissues
were collected quickly. Fetal pituitaries were frozen on dry ice
for in situ hybridization and immunohistochemistry. A por-
tion of the right lobe of the liver was frozen rapidly in liquid
nitrogen for Northern blot analysis.

Fetal blood gases and uterine activity. Fetal blood pressure
and amniotic fluid pressure were measured continuously
using Statham pressure transducers (P23XL, Spectramed,
Oxnard, CA) and displayed on a chart recorder (model 78D,
Grass Instrument). Cortisol has previously been shown to
inhibit pro-ACTH or POMC (kindly provided by Dr. J. Schwartz,
Bowman Gray School of Medicine, Winston-Salem, NC). The
antibody recognized >95% immunoreactivity corresponding
to ACTH-(1—39) when samples of fetal sheep plasma in
normoxemia or during hypoxemia were assayed after high-
performance liquid chromatography separation of ACTH-
related peptides (12). Plasma cortisol concentrations were
quantified by RIA after extraction with diethyl ether. The
antibody characteristics and assay validation for measure-
ment of cortisol in fetal sheep plasma have been described
previously (14). The combined intra- and interassay coeffi-
cient of variation was 12%. The percentage of free cortisol in
duplicate samples of fetal plasma was measured by the
centrifugal ultrafiltration-dialysis technique developed and
described by Hammond et al. (20). All of the samples were
measured in a single assay. Fetal plasma CBG levels were
measured as corticosteroid binding capacity (CBC) deter-
mined using the saturation binding assay of Ballard et al. (4),
with modifications described previously (13). The within-
assay coefficient of variation was <1.0%.

Northern blot analysis of CBG mRNA. We previously
showed that the liver is the major site of CBG biosynthesis in
fetal sheep (7). Total cellular RNA was extracted from the
fetal liver, electrophoresed, and blotted as described previ-
ously (6). The blotting membranes were probed using an
ovine CBG cDNA (6). After autoradiographic exposure, the
blots were stripped and reprobed with a cDNA to mouse 18S
rRNA to allow correction for variations in gel loading and
transfer. The relative optical densities (ROD) were deter-
mined using computerized image analysis (Imaging Re-
search, St. Catharines, ON, Canada). Results are expressed
as the ratio of the ROD of the CBG mRNA to 18S rRNA
hybridization signals.

In situ hybridization of pituitary POMC mRNA. Frozen
pituitaries were sectioned (15-µm coronal sections) using a
crystal (Tissue-Tek, Miles Canada, Etobicoke, ON, Canada),
to poly-L-lysine-coated slides and fixed in 4% paraformaldehyde (28). Pitu-
itary sections were incubated with a 35S-labeled, 45-mer
oligonucleotide complementary to bases 711–756 of the por-
cine POMC gene (19) as previously described in detail (37).
The slides were exposed to autoradiographic film (XAR 5,
Kodak) for 2 h at room temperature to determine the hybrid-
ization signal for POMC mRNA levels in the pars intermedia
and then reexposed for 4 days to measure the levels of POMC
mRNA in the pars distalis. The two exposure times were
necessary for the hybridization signal to be within the linear
range of the film for the two regions of the pituitary. Linearity was
established by the simultaneous exposure of the film to
13C standards (27). The autoradiograms were then analyzed
using computerized image analysis (Imaging Research). Re-
results are expressed as ROD for a minimum of 15 pituitary
sections per animal. A control 45-mer sense oligonucleotide
was also synthesized, and no signal was observed when it was
hybridized with pituitary sections (10). The fetal pituitary
sections were then coated with IIFord K5 liquid emulsion,
developed and counterstained with Carazzi’s hematoxylin to
identify nuclei. The silver grain deposits were visualized
using light microscopy.

Immunohistochemistry. Immunohistochemical detection of
irACTH was performed on 15-µm frozen pituitary sections
prepared as described above for in situ hybridization. A
RESULTS

Plasma cortisol concentrations. The daily mean concentration of cortisol in fetal plasma rose during cortisol infusion from basal values of 4.4 ± 0.6 to 19.3 ± 3.1 ng/ml within 24 h (n = 6, P < 0.05; Fig. 1). The mean plasma cortisol concentration rose progressively to a maximal concentration of 38.6 ± 2.7 ng/ml, which is similar to that seen in the ovine fetus near term (32). Plasma cortisol concentrations in the control animals did not change significantly throughout the infusion period.

Fetal blood gases and uterine activity. Fetal arterial PO2, PCO2, O2 saturation, and pH were unchanged throughout the study in both cortisol- and saline-treated fetuses (Table 1). Mean arterial pressure increased significantly within the first 24 h of cortisol administration from 42.6 ± 1.0 to 48.7 ± 0.7 mmHg and remained at this level throughout the infusion period. There was no significant change in mean arterial pressure in the saline-infused fetuses (Table 1). The number of contractures or contractions per 2-h interval did not change significantly in the saline-treated fetuses. Similarly, during intrafetal cortisol infusion, uterine activity was not altered during the first 72 h (at 48–72 h, mean number of contractures = 3.4 ± 0.2 and contractions = 1.0 ± 0.2 per 2-h period). However, there was an increase in uterine contractility during the final 24 h of intrafetal cortisol infusion (Table 1).

Effects of cortisol on plasma irACTH. Plasma irACTH concentrations rose significantly in the cortisol-treated fetuses from values of 25.4 ± 2.7 pg/ml during the control period (Fig. 2) to a significantly elevated level of 37.8 ± 3.9 pg/ml at 24–48 h (Fig. 2). Fetal ACTH values remained elevated throughout the cortisol infusion period (n = 7, P < 0.05; effect of time F = 7.9 and cortisol F = 9.6; Fig. 2). In the saline-infused animals, plasma ACTH concentrations did not change significantly from control values of 27.0 ± 1.7 pg/ml (n = 6; Fig. 2).

Table 1. Fetal arterial blood gases, mean arterial pressure, and uterine activity (per 2-h period) during first 24 h before and first and last 24 h of infusion if saline or cortisol

<table>
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<tr>
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<th>Saline</th>
<th>Cortisol</th>
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<tr>
<td></td>
<td>-24 to 0h</td>
<td>0–24 h</td>
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<tr>
<td>pH</td>
<td>7.36 ± 0.01</td>
<td>7.34 ± 0.01</td>
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<tr>
<td>PO2, mmHg</td>
<td>22.2 ± 0.7</td>
<td>20.65 ± 0.9</td>
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<tr>
<td>PCO2, mmHg</td>
<td>53.5 ± 0.8</td>
<td>55.6 ± 0.9</td>
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<tr>
<td>O2 saturation, %</td>
<td>60.2 ± 2.5</td>
<td>56.6 ± 4.0</td>
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<tr>
<td>MAP, mmHg</td>
<td>40.3 ± 0.4</td>
<td>41.6 ± 0.5</td>
</tr>
<tr>
<td>Contractures, no./2 h</td>
<td>2.6 ± 0.2</td>
<td>2.7 ± 0.2</td>
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<tr>
<td>Contractions, no./2 h</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.1</td>
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Values are means ± SE; for blood gases and saline, n = 5; for cortisol, n = 7; for mean arterial pressure (MAP) and uterine activity, n = 3. Contracture, uterine activity lasting >5 min. Contraction, uterine activity lasting <1 min and >30 s. *P < 0.05.
ACTH is secreted in pulses; therefore plasma irACTH concentrations were variable between samples within individual fetuses. The maximal and minimal changes in plasma concentrations of irACTH for the three samples collected each day were calculated relative to the initial 24-h control period for individual animals. The maximal change in irACTH was significantly higher at 24–48 h of cortisol infusion and remained significantly elevated throughout the infusion compared with saline controls.

Table 2. Mean maximal change in plasma irACTH compared with average plasma irACTH concentrations during 24-h control period

<table>
<thead>
<tr>
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<th>Mean Plasma irACTH, pg/ml</th>
<th>Maximal Change in Plasma irACTH, pg/ml</th>
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<tbody>
<tr>
<td></td>
<td>n Control 0–24 h 24–48 h 48–72 h 72–96 h</td>
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<tr>
<td>Saline</td>
<td>6 27.0 ± 2.3 8.7 ± 2.0 7.7 ± 2.9 13.7 ± 5.5 11.4 ± 4.8</td>
<td></td>
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<tr>
<td>Cortisol</td>
<td>7 25.8 ± 4.1 11.1 ± 4.4 20.7 ± 6.4 23.7 ± 5.4 * 23.7 ± 4.7 *</td>
<td></td>
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Values are means ± SE. *P < 0.05. ir, Immunoreactive.
cortisol concentration did not change significantly from mean values of 0.3 ± 0.1 ng/ml (n = 3; Fig. 5B).

Effect of cortisol on levels of pituitary POMC mRNA and irACTH. Levels of POMC mRNA in the pars distalis of cortisol-treated animals were lower (Fig. 6C) than those in saline control animals (Fig. 6A) after 96 h of infusion. POMC mRNA levels in the pars intermedia were also decreased after 96 h of cortisol infusion (Fig. 6D) compared with the saline-treated controls (Fig. 6B).

POMC mRNA levels were quantified by computerized image analysis. There was no significant change in levels of POMC mRNA within the pars distalis or the pars intermedia after 12 h of cortisol treatment, but the levels had fallen to 4% of controls by 96 h (Fig. 7). High-resolution analysis using liquid silver emulsion autoradiography confirmed this finding by the near absence of silver grain deposits in the pars distalis after 96 h of cortisol infusion (Fig. 8C), compared with the abundance of silver grain deposits seen in the salinetreated fetuses (Fig. 8A). POMC mRNA levels in the pars intermedia of the cortisol-treated animals were unchanged at the end of the first 24 h of infusion, but the levels had decreased by 38% compared with saline controls after 96 h of infusion (Fig. 7). There was no apparent change in the number of silver grains deposited between groups (Fig. 8, E and G).

The intensity of irACTH peptide staining within the pars distalis did not appear different after 96 h of
cortisol treatment (Fig. 8D), compared with the saline-infused animals (Fig. 8B). However, the number of irACTH-positive corticotrophs in the pars distalis was significantly reduced (a decrease of 13.9%) from 218.6 ± 12.5 immunopositive cells per 1.7 × 10³ µm² in the saline controls to 188.2 ± 15.1 immunopositive cells per 1.7 × 10³ µm² after 96 h of cortisol infusion. There was no diminution in the number of immunopositive cells in the pars intermedia after 96 h of cortisol infusion (Fig. 8H) compared with control (Fig. 8F). Adjacent sections were incubated with antibody preabsorbed with an excess of human ACTH-(1—24), and there was no staining present (data not shown; see also Ref. 22).

DISCUSSION

We have shown that, in the late-gestation ovine fetus, mean plasma irACTH concentrations rise significantly in response to physiological elevations in plasma cortisol concentrations. However, this does not appear to be due to a decrease in cortisol negative feedback, since plasma free cortisol concentrations remained elevated despite an increase in plasma CBG levels. The high concentration of plasma free cortisol suppressed pituitary POMC mRNA levels in both the pars distalis and pars intermedia, and the number of irACTH immunopositive cells within the pituitary pars distalis was also reduced after 96 h of cortisol infusion. However, the number of immunopositive cells within the pars intermedia did not appear to be appreciably altered in the presence of elevated circulating free cortisol.

We believe that the increase in plasma irACTH predominantly reflects changes in ACTH-(1—39). The ACTH antibody used to measure fetal ACTH concentrations has been validated extensively and does not show cross-reactivity with the higher molecular weight ACTH-related peptides. We suggest that the increase in irACTH reflects an increase in ACTH output in response to the infusion of cortisol, although we cannot exclude the possibility that the metabolic clearance rate of circulatingACTH is reduced by cortisol infusion. Mean fetal arterial pressure increased by ~7 mmHg within the first 24 h of cortisol infusion and remained at this level throughout the experiment. This is similar to the increase reported during a 48-h cortisol infusion to the midgestation ovine fetus (17). However, the hypertensive effects of cortisol do not elicit an immediate ACTH response and seem very unlikely to be causal in the later rise in plasma irACTH concentrations. Although uterine activity does increase slightly within the last 24 h of cortisol infusion, this event is preceded by the ACTH rise; therefore, the initial rise in plasma irACTH cannot be labor induced. The increase in plasma irACTH at 72–96 h may be associated with an increase in uterine activity; however, there was no change in fetal arterial PO₂, which indicates that the rise in ACTH is not hypoxia mediated. In addition, there was no change in the plasma irACTH concentrations of the saline-treated fetuses, verifying that the rise in ACTH is not an effect of the sampling protocol employed.

It has previously been reported that a 96-h cortisol infusion to the ovine fetus at 134 days of gestation affects pulsatility of irACTH, increasing pulse peak and nadir (3). We examined the maximal and minimal changes in irACTH and compared these with the mean irACTH concentration during the control period. The maximal change in irACTH from the three plasma samples collected each day was significantly elevated during the cortisol infusion compared with that of control fetuses. However, we did not employ a frequent sampling protocol necessary to establish whether the cortisol infusion administered in these animals altered ACTH pulse frequency over the course of the experiment.

It has been demonstrated that plasma CBG levels rise within 2–4 days of cortisol infusion (3, 5). In the present study, plasma CBG levels rose in response to cortisol, becoming significantly greater than those in controls at 48–72 h of infusion. This was associated with an increase in hepatic CBG mRNA levels. Low-dose cortisol infusion to the ovine fetus at 100 days of gestation (for 100 h), or administration of dexamethasone at 130 days of gestation (for 96 h), increased CBG biosynthesis and secretion and also altered the pattern of CBG glycosylation (5, 6). Changes in CBG glycoforms may increase the half-life of CBG in the circulation, and this may account for the earlier rise in CBC in plasma than in steady-state levels of hepatic CBG mRNA determined in the present study.

The percentage of free cortisol in plasma rose within 8 h of cortisol infusion and then returned to control levels by 72 h of infusion. However, the absolute concentrations of free cortisol remained elevated throughout the experiment. This suggests that CBG is effective at maintaining the percentage of free cortisol in fetal circulation but does not control the absolute concentration of free cortisol during periods of rapidly increasing plasma cortisol concentrations. This reflects the CBG response to increasing plasma cortisol concentrations in the sheep fetus at term. Plasma CBG and cortisol concentrations rise in parallel during late gestation, and low free cortisol concentrations are effectively maintained until the last 5 days of pregnancy (4, 7). Although CBG did not appear to be effective in decreasing the negative-feedback effects of the rapidly increasing plasma cortisol concentrations, this does not preclude a role for circulating CBG in modifying feedback control of the prepartum rise in plasma ACTH levels. In addition, we showed earlier that the fetal sheep pituitary synthesizes CBG (7), which could alter local feedback mechanisms. However,
we did not determine changes in pituitary CBG biosynthesis in the current study. POMC mRNA levels in the pars intermedia and pars distalis were not affected by 12 h of cortisol treatment, as has been demonstrated previously (28). However, after 96 h of cortisol infusion, POMC mRNA levels were suppressed in both the pars intermedia and pars distalis. In addition, the number of irACTH-positive cells within the pars distalis was decreased after 96 h of cortisol treatment, suggesting that the elevated plasma free cortisol concentrations are exerting negative-feedback effects on the corticotrophs of the pars distalis. Nonetheless, it is possible that cortisol affects the rate of POMC translation or ACTH secretion. However, within the pars intermedia there was no apparent change in irACTH-positive cells or the intensity of irACTH staining. Pars intermedia corticotrophs from fetal sheep secrete ACTH-(1—39) in vitro (18). In addition, attenuation of the endogenous cortisol rise, by bilateral fetal adrenalectomy, does not alter the basal ACTH-(1—39) output from subsequently cultured pars intermedia cells (18). The pars intermedia may therefore provide an additional source of ACTH. In the presence of high plasma concentrations of cortisol, there may be an alteration of POMC processing within the pars intermedia, resulting in an increase in circulating ACTH-(1—39). Alternatively, the pars intermedia may secrete large molecular weight POMC products into the circulation (34), which are processed to ACTH-(1—39) under the influence of elevated cortisol at other sites.

Schwartz et al. (35) have examined the regulation of pituitary ACTH secretion in vitro and have shown that there are subpopulations of corticotrophs within the pars distalis which are differentially regulated. These are the CRH-sensitive corticotroph, which secretes ACTH in response to CRH via an increase in adenosine 3',5'-cyclic monophosphate, and the arginine vasopresin (AVP)-responsive corticotroph, which stimulates ACTH release by a second pathway likely involving inositol trisphosphate (38). In vitro studies indicate that glucocorticoids do not inhibit ACTH-(1—39) secretion and significantly increased ACTH precursor release from the AVP-responsive corticotrophs (35). A direct effect of cortisol on fetal corticotrophs has also been described. Antolovich et al. (2) characterized a change in the morphology of the corticotrophs from ovine fetuses treated with cortisol from a “fetal”- to an “adult-type” cell. This morphological change occurs normally during late gestation but did not occur in adrenalectomized fetuses (2). Histological maturation of the corticotroph may also be associated with altered processing of POMC to produce ACTH-(1—39) preferentially (2). ACTH-(1—39) is the predominant POMC product of the adult corticotroph (36). In support of this possibility, Brieu and Durand found that fetal ovine corticotroph cells treated with cortisol (4 days) in vitro decreased the output of total ACTH peptides but increased the relative proportion of immunoreactive material that coeluted with ACTH-(1—39) (9) and augmented the release of bioactive ACTH (8). The pituitaries collected for the present study were slow-frozen for in situ hybridization, and therefore the cellular morphology could not be examined effectively. It remains possible that the rise in plasma irACTH in response to the cortisol infusion could be a result of AVP-stimulated ACTH release and/or a change in the processing of POMC such that ACTH-(1—39) is preferentially released from POMC remaining in the anterior pituitary corticotrophs. Alternatively, we speculate that changes in prohormone convertase activities (39) in the pars distalis and pars intermedia of the fetal pituitary might lead to increased proportions of ACTH-(1—39) secreted or that circulating ACTH might be derived from alternate sources, including the lung and placenta (15, 16, 24). At present, the effects of cortisol on these alternative sites of POMC production and processing have yet to be elucidated.

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