The role of hypothalamic input on corticotroph maturation in fetal sheep

Sharla F. Young,1 Stephen B. Tatter,2 Nancy K. Valego,1 Jorge P. Figueroa,3 Jalonda Thompson,4 and James C. Rose1,3

Departments of 1Physiology/Pharmacology, 2Obstetrics/Gynecology, and 3Neurosurgery, Excellence in Cardiovascular Sciences Summer Program, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157

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Young, Sharla F., Stephen B. Tatter, Nancy K. Valego, Jorge P. Figueroa, Jalonda Thompson, and James C. Rose. The role of hypothalamic input on corticotroph maturation in fetal sheep. Am J Physiol Regul Integr Comp Physiol 284: R1621–R1630, 2003. First published February 27, 2003; 10.1152/ajpregu.00572.2002.—Corticotropin-releasing hormone receptor type 1 (CRH-R1) expression1 and vasopressin type 1b (V1b) receptor protein decrease in late-gestation fetal sheep. Because hypothalamo-pituitary disconnection (HPD) has been demonstrated to prevent the morphological maturation of corticotrophs, we hypothesized that hypothalamic input is necessary for the maturational changes in CRH-R1 and V1b receptor levels. We measured CRH-R1 and V1b receptor expression in the anterior pituitaries of fetuses at 140 days gestational age (dGA) that underwent HPD or sham surgery at 120 dGA. CRH-R1 mRNA decreased similarly in HPD and sham-operated fetuses compared with 120 dGA naive fetuses. However, CRH-R1 protein levels were elevated in HPD fetuses compared with sham and were not different from 120 dGA values. V1b protein levels decreased similarly in HPD and sham-operated fetuses compared with 120 dGA naive fetuses. We conclude that hypothalamic input to the pituitary is necessary for the decrease in CRH-R1 receptor protein levels in late-gestation fetal sheep. However, hypothalamic input is not necessary for the decrease in V1b receptor expression seen in late gestation.

corticotropin-releasing hormone; arginine vasopressin; corticotropin-releasing hormone receptor; vasopressin receptor; hypothalamo-pituitary disconnection

CORTICOTROPHS UNDERGO A TRANSITION from being predominately corticotropin-releasing hormone (CRH) responsive in early gestation to being predominately AVP-responsive in late gestation (5, 9, 13, 23, 25, 26). It is possible that this change in responsiveness is because of changes in CRH receptor type 1 (CRH-R1) and vasopressin type 1b (V1b) receptor expression in late gestation, since CRH-R1 and V1b receptor expression decreases in late gestation (11, 12, 18, 42).

During the transition in corticotroph responsiveness, bioactive ACTH increases (2, 5, 7, 20, 33–35). This increase in ACTH bioactivity may stimulate the peripartum increase in plasma cortisol, an event that is important for the maturation of several fetal organ systems in various species and for the initiation of parturition in sheep. Because AVP is known to increase the bioactivity of ACTH in fetal sheep (34, 43), it is possible that the increase in AVP responsiveness of corticotrophs in late gestation is responsible for the cascade of events leading to the cortisol surge. However, the mechanism controlling this transition in responsiveness is still unknown.

Hypothalamic input (in the form of CRH and AVP) and cortisol have been shown to inhibit the CRH response and decrease CRH-R1 receptor levels in corticotrophs in adult animals (28, 37, 38). However, cortisol does not inhibit the AVP response in adults (29), and it is unknown whether hypothalamic input modulates V1b receptor levels in fetal life. Hypothalamo-pituitary disconnection (HPD) in fetal sheep performed at ~120 days gestational age (dGA; term = 147 dGA) prevents both the cortisol surge and morphological changes in corticotrophs (2). However, the effect of HPD on the transition in corticotroph responsiveness has yet to be determined. Based on previous findings, we hypothesize that HPD will arrest the developmental changes in CRH-R1 and V1b receptor expression in the fetal pituitary. Therefore, the goal of the current study is to determine if interruption of communication between the hypothalamus and pituitary changes CRH and AVP receptor expression and responsiveness in the late-gestation fetal sheep.

MATERIALS AND METHODS

Animals

Cross-bred pregnant ewes (twin and singleton pregnancy) with known insemination dates were obtained from a local supplier. All procedures were approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee. Ewes were group-housed in individual pens...
with food and water provided ad libitum. After 5 days of acclimation, surgery was performed. After surgery, ewes were returned to their pens where they remained until fetuses were killed.

**Surgery**

Surgery was performed at ~120 dGA. Polyvinyl catheters previously filled with sterile saline were inserted in the fetal carotid artery and jugular vein and advanced to the ascending aorta and superior vena cava. HPD (n = 15) was performed similar to the procedure described by Antolovich et al. (1) and others, with modifications. Briefly, the fetal head was stabilized, and the skin and nasal bone were opened at the midline. A lateral incision was also made, and a flap of nasal bone reflected, exposing the nasal cavity. The turbinate bones were removed bluntly. Next, using an operating microscope and a hand-held drill, the surgeon drilled through the ethmoid and presphenoid bones just below the cranial fossa. Bleeding was controlled with the use of peroxide solution and gel foam. The tunnel was extended until the cartilage septum between the presphenoid and basiphenoid bone was observed. The optic chiasm was located, and the dura covering it was opened. The optic chiasm was divided in the midline and reflected, and the stalk median eminence was exposed. The median eminence and pituitary stalk were separated. A small piece of latex was placed between the hypotalamic tissue and pituitary. The tunnel in the bone was packed with gel foam. Next, the skin was sutured, and the head was returned to the uterus. The uterine incision was closed in layers. The catheters from the fetus and maternal femoral arterial and venous catheters were exteriorized through a small incision in the maternal flank, placed in a sterile glove and protected by netting placed around the ewe’s abdomen. When a sham surgery was performed (n = 12), all steps of the surgery were performed except the median eminence and pituitary stalk were not separated. A piece of latex was placed near the intact pituitary stalk. Gentamicin and ampicillin were administered to the ewe at the time of surgery and for the next 3 days through the maternal venous catheter. Blood samples were taken from both fetal and maternal arterial catheters every other day to assess plasma cortisol levels, as described below. Blood was also collected in heparinized syringes for determination of blood gases on anABL5 blood gas analyzer (Radiometer, Copenhagen, Denmark).

**Plasma Cortisol Measurement**

Plasma was isolated by centrifugation of the blood samples and stored at −20°C until assayed. Plasma cortisol levels were determined using a commercial RIA kit obtained from Diagnostic Systems Laboratories (Webster, TX).

**Tissue Collection**

Fetuses were killed at either 136–139 dGA (HPD and sham) or 120 dGA (naive) by an overdose of pentobarbital sodium. Pituitaries were collected individually, the neurointermediate lobe was removed, and the anterior lobe was bisected. One-half of the anterior lobe was placed in ice-cold HEPES dissociation buffer and dispersed immediately for use in the cell immunoblotting procedure. The other one-half of the anterior lobe was flash-frozen in liquid nitrogen and stored at −80°C for later use in either the RNase protection assay (RPA) or Western blotting. Additionally, to increase protein yield per pituitary, both halves of the anterior pituitary were used in the cell immunoblotting procedure. Plasma Cortisol was measured using a commercial RIA kit. Tissue Collection

**Cell Immunoblotting**

Cell immunoblotting was performed as described previously (26, 41), with minor modifications, to determine individual corticotroph responsiveness to CRH and AVP. Briefly, anterior pituitary cells from individual anterior pituitaries (15,000/0 μl) were placed in droplets of serum-free media (DMEM-F-12–0.2% polyep) on Immobilon-P membranes (Millipore, Bedford, MA). After allowing cells to settle to the membrane for 15 min at 37°C, 10 μl of treatment were added to the droplet. The different treatments consisted of vehicle (0.05 M Tris/NaCl; pH 7.4) or maximally stimulating concentrations of ovine CRH (10 nM; Sigma) or AVP (100 nM). All treatments were performed in triplicate for a total of nine immunoblots per anterior pituitary. Cells were then incubated for an additional 2 h at 37°C. After treatment, media were removed, and cells were fixed with 2.5% glutaraldehyde (Sigma) in PBS for 1 h at room temperature. The glutaraldehyde was subsequently washed off with Tris/NaCl buffer three times, and immunoblots were then incubated overnight at 37°C with an antibody against ACTH (1:1,000 in Tris/NaCl buffer plus 5% normal goat serum and 0.1% BSA; Sigma). Immunoblots were rinsed three times with Tris/NaCl buffer and then incubated with a secondary antibody [1:500 goat anti-rabbit IgG-horseradish peroxidase (Sigma) in Tris/NaCl buffer plus 1% BSA] for 2 h at room temperature. After being washed with Tris/NaCl buffer two times, immunoblots were stained with diaminobenzidine (Sigma) to detect immunoreactivity and were counterstained with hematoxylin. After being stained, the membrane was mounted on a microscope slide, and a glass cover-slip was mounted over it.

**ACTH antibody.** The ACTH antibody used in these experiments was raised in our laboratory as previously described (32). On a molar basis, it shows 100% cross-reactivity with ovine ACTH-(1–39), human ACTH-(1–39), and ACTH-(6–24). It also reacts ~90% with ACTH-(1–24) and <1% with ACTH-(1–17) or human ACTH-(18–39). It does not cross-react with ACTH-(1–10), ACTH-(1–10) amide, ACTH-(4–11), ACTH-(11–19), ACTH-(11–24), or ACTH-(25–39) fragments of the ACTH(1–39) peptide. The antibody also recognizes high-molecular mass forms (>12.5 kDa) of ACTH-like material obtained from gel exclusion chromatography (Sephadex G-50) of fetal sheep pituitary extracts (10). Because of the multiple forms of ACTH recognized by this antibody, positive staining with it is referred to as immunoreactive (ir) ACTH.

**Cell dispersion.** Anterior pituitary halves (HPD: n = 10; sham: n = 7; 120 dGA: n = 8) were minced individually and placed in 0.04% collagenase II (Worthington Biochemical, Freehold, NJ) in HEPES dissociation buffer. DNase I (150 units; Sigma Chemicals, St. Louis, MO) was added, and tubes were rocked gently at 37°C for 2 h. The reaction was stopped by the addition of complete medium (consisting of DMEM plus Ham’s F-12 medium (1:1) and charcoal-stripped FCS (10%); Gibco-BRL, Carlsbad, CA). Anterior pituitary cells were purified from red blood cells and noncellular debris by layering on a Histopaque (Sigma)-40.5% Percoll (Sigma) gradient. The cells at the Histopaque-Percoll interface were removed and washed three times with complete medium. Cell viability was determined by trypan blue exclusion.

**Immunoblotting.** A cell immunoblotting procedure was performed as described previously (26, 41), with minor modifications, to determine individual corticotroph responsiveness to CRH and AVP. Briefly, anterior pituitary cells from individual anterior pituitaries (15,000/0 μl) were placed in droplets of serum-free medium (DMEM-F-12–0.2% polyep) on Immobilon-P membranes (Millipore, Bedford, MA). After allowing cells to settle to the membrane for 15 min at 37°C, 10 μl of treatment were added to the droplet. The different treatments consisted of vehicle (0.05 M Tris/NaCl; pH 7.4) or maximally stimulating concentrations of ovine CRH (10 nM; Sigma) or AVP (100 nM). All treatments were performed in triplicate for a total of nine immunoblots per anterior pituitary. Cells were then incubated for an additional 2 h at 37°C. After treatment, media were removed, and cells were fixed with 2.5% glutaraldehyde (Sigma) in PBS for 1 h at room temperature. The glutaraldehyde was subsequently washed off with Tris/NaCl buffer three times, and immunoblots were then incubated overnight at 37°C with an antibody against ACTH (1:1,000 in Tris/NaCl buffer plus 5% normal goat serum and 0.1% BSA; Sigma). Immunoblots were rinsed three times with Tris/NaCl buffer and then incubated with a secondary antibody [1:500 goat anti-rabbit IgG-horseradish peroxidase (Sigma) in Tris/NaCl buffer plus 1% BSA] for 2 h at room temperature. After being washed with Tris/NaCl buffer two times, immunoblots were stained with diaminobenzidine (Sigma) to detect immunoreactivity and were counterstained with hematoxylin. After being stained, the membrane was mounted on a microscope slide, and a glass cover-slip was mounted over it.
The procedures utilized have been described in more detail elsewhere (11, 41, 42).

**RNA extraction**. RNA from individual anterior pituitary halves (HPD and 120 dGA; n = 6; sham: n = 5) was isolated. Briefly, the tissue was homogenized in TRIzol reagent (50 mg tissue/1 ml TRIzol; GIBCO-BRL) with a high-speed Polytron for 30–60 s. Next, chloroform was added (0.2 ml/1 ml TRIzol), the mixture was incubated for 3 min, and samples were centrifuged at 12,000 g for 15 min. The aqueous phase was transferred to a fresh tube, and the RNA was precipitated by the addition of isopropanol (0.5 ml/1 ml TRIzol), mixing, and centrifugation at 12,000 g for 10 min. The supernatant was removed, and the RNA pellet was washed one time with 75% ethanol (1 ml/1 ml TRIzol) and recentrifuged at 7,500 g for 5 min. The ethanol was removed, and the RNA pellets were allowed to air-dry and then redissolved in RNase-free water (250 μl/1 ml TRIzol). RNA concentrations were determined by absorbance at 260 nm in a spectrophotometer. The integrity of all RNA samples was determined by electrophoresis in 1.5% agarose gels containing 6.6% formaldehyde.

**Synthesis of antisense RNA probes.** Both CRH-R1 receptor and V1b receptor probes were synthesized by following an in vitro transcription protocol described by Promega (Madison, WI). Briefly, plasmids (pSP72; Promega) containing either ovine CRH-R1 receptor cDNA (corresponding to 211–615 bp) or ovine V1b receptor cDNA (corresponding to 1–615 bp) were linearized with EcoRI. The in vitro transcription reaction was performed by the addition of 4 μl of 5× transcription buffer, 2 μl of 100 mM dithiothreitol, 1 μl RNAin RNase inhibitor, 4 μl ATP, GTP, and CTP mix (25 mM each), 2.4 μl of 100 μM UTP, 5 μl [α-32P]UTP (3,000 Ci/mmol; NEN Life Science Products, Boston, MA), and 1 μl SP6 polymerase (in this order) and incubation for 2 h at room temperature. RQ1 RNase-free DNase (1 μl) was added to the reaction and incubated for an additional 15 min at 37°C to remove the DNA template. Unincorporated nucleotides were removed with a G-50 Sephadex column (Roche Molecular Biochemicals, Indianapolis, IN). Purified probe (1 μl) was placed in a scintillation vial to determine the counts per minute. Sense strand RNA for use as standards was synthesized by linearizing the plasmids with BamHI followed by in vitro transcription similar to above; however, [α-32P]UTP and 100 μM UTP were replaced with 25 μM UTP.

**RPA.** CRH-R1 receptor and V1b receptor mRNA in the anterior pituitary was quantified using an RPA II kit (Ambion, Austin, TX). Briefly, sample RNA from individual anterior pituitaries (10 μg; assay performed in duplicate) and standards ranging from 0.5 to 50 pg were mixed with 20 μl hybridization buffer [80% deionized formamide, 100 mM sodium citrate (pH 6.4), and 1 mM EDTA] and CRH-R1 and V1b probes (7.5 × 104 and 5 × 104 counts/min, respectively). The samples were heated at 95°C for 5 min and immediately placed in a 48°C water bath for overnight hybridization. RNase A/T1 (1:80 dilution in RNase digestion buffer) was then added to the samples to digest unhybridized probe and RNA. Digestion was stopped and hybridized RNA precipitated by addition of RNase inactivation/precipitation buffer and incubation for 30 min at −20°C. Hybridized RNA was pelleted by centrifugation at 14,000 g for 15 min. Samples were then run on a 5% polyacrylamide/8 M urea denaturing gel at 250 V for 1 h. Gels were exposed to film (Biomax-MR; Kodak) with an intensifying screen for 17 h (CRH-R1) or 4 days (V1b) at −80°C.

**Western Blotting.** CRH-R1 and V1b receptor antibodies. The polyclonal CRH-R1 antibody used in the current study was developed by Rockland Immunochemical (Gilbertsville, PA). Rabbits were inoculated with a 15-amino acid (AA) peptide corresponding to 102–116 AA of the ovine CRH-R1 sequence (22). This region is postulated to be located in the first extracellular domain of the receptor. Specific bands were seen at ~180, 118, and 40 kDa, which were absent or markedly reduced when membranes were incubated with preimmune serum, CRH-R1 antisera in the presence of excess peptide antigen (300 μg; see Fig. 3A), or in the absence of primary antibody. In addition, an anti-rat CRH-R1 receptor antibody was also used to confirm the results seen with the ovine CRH-R1 antibody. The anti-rat CRH-R1 antibody was developed in the laboratory of Dr. Greti Aguilera (National Institutes of Health, Bethesda, MD). Bands of the same size were obtained using both antibodies.

The polyclonal V1b antibody used in the current study was purchased from Research Diagnostics (Flanders, NJ). It was developed against an 18-AA peptide located in the putative first extracellular domain of the rat V1b sequence. It has been characterized previously by the manufacturing company and does not recognize the V1a or V2 receptors. The specificity of this antibody was tested by incubating membranes with V1b antibody in the presence of excess peptide antigen (30 μg; purchased from Research Diagnostics; see Fig. 5A) and by incubating membranes in the absence of primary antibody. A specific band was detected at 90 kDa.

**Membrane isolation and Western blotting.** Western blot analysis was performed according to the method of Laemmli (16) using 8.0% SDS-PAGE (n = 6 for all groups). Tissue was homogenized in a buffer consisting of 50 mM Tris-hydrochloric acid, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, plus a 1:200 dilution of protease inhibitor cocktail (P8340, contains 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane, bestatin, leupeptin, and aprotinin; Sigma). Fetal samples (35–125 mg) were placed in liquid nitrogen and crushed in a stainless steel mortar, and the powder was homogenized in 800 μl of the buffer/100 mg tissue with a Tissue Tearor (BioSpec Products, Bartlesville, OK). The homogenate was centrifuged at 2,000 g for 8 min to remove cellular debris, at 10,000 g for 20 min to remove mitochondrial debris, and then at 100,000 g for 1 h to pellet the membrane fractions. The supernatant was removed, and the pellet was resuspended in 140 μl buffer (described above). The protein concentration was measured with the bicinchoninic acid method, using albumin as the standard (Pierce Chemical, Rockford, IL). Protein aliquots (90 μg) were mixed 1:4 in loading buffer, separated in 8% tricine gels (Novex, San Diego, CA), and blotted on polyvinylidene fluoride membranes (Immobilon; Millipore, Marlborough, MA) by semidy electroblotting. Blots were blocked overnight at 4°C with 6% dry nonfat milk, rinsed with Tris-buffered saline/0.05% Tween 20, and incubated for 2 h at room temperature with primary antibody (CRH-R1: 1:1,000; V1b: 5 μg/ml) and for 1 h with horseradish peroxidase-conjugated secondary antibody (1:7,500). A positive reaction, defined as a band of 90 kDa for V1b receptor and 180, 118, and 40 kDa for CRH-R1 receptor, was identified with enhanced chemiluminescence (ECL Plus; Amer sham Pharmaic Biotech, Arlington Heights, IL).
RESULTS

Confirmation of HPD

In addition to visual confirmation at the time of necropsy, plasma cortisol levels were measured to confirm that the HPD was performed successfully. The cortisol surge normally seen in late gestation is absent in HPD fetuses (Fig. 1). Plasma cortisol levels at 122–126 dGA were not different between HPD and sham-operated groups (3.8 ± 0.67 vs. 4.4 ± 0.66 ng/mL, respectively). However, at 136–139 dGA, values from sham-operated fetuses were elevated significantly compared with HPD fetuses (26.3 ± 4.91 vs. 2.7 ± 0.71, respectively). Fetal health, as assessed by blood gas parameters, was normal throughout the duration of the study in both HPD and sham-operated fetuses (Table 1).

Corticotroph Populations in the Anterior Pituitary

HPD had a profound effect on the percentage of corticotrophs present in the pituitary (Fig. 2). Anterior pituitaries from HPD fetuses at 140 dGA contained more than two times the percentage of irACTH positive cells (24.9 ± 3.67%) than sham-operated fetuses at 140 dGA (9.7 ± 2.03%) and naive 120 dGA fetuses (9.4 ± 1.40%). AVP treatment for 2 h in vitro also caused a significant increase in the percentage of corticotrophs in all three groups (Fig. 2). On the other hand, CRH treatment for 2 h in vitro had no effect on the percentage of corticotrophs present in the anterior pituitary. Treatment of cells adhered to the Immobilon membrane for 2 h with CRH and AVP resulted in inconsistent secretory responses (data not shown).

CRH-R1 Receptor Expression

Three bands were identified in the Western blots as CRH-R1 receptor protein with molecular masses of 40, 118, and 180 kDa (Fig. 3). Sham-operated fetuses tended to have less CRH-R1 receptor protein (40-kDa band) than HPD or 120 dGA fetuses (sham: 35,556 ± 11,395 OD units; HPD: 57,757 ± 10,446 OD units; 120 dGA: 62,009 ± 7,925 OD units; ANOVA: P = 0.06; Fig. 4D). No significant differences were seen between the three groups in the 180- and 118-kDa bands (Fig. 4, B and C). Anterior pituitaries from both HPD (0.56 ± 0.18 pg/µg) and sham-operated (0.48 ± 0.10 pg/µg) fetuses contained significantly less CRH-R1 receptor mRNA compared with naive 120 dGA fetuses (1.86 ± 0.31 pg/µg total RNA; Fig. 4A). However, CRH-R1 receptor mRNA levels in anterior pituitaries from HPD fetuses were not significantly different from sham-operated fetuses. The sham operation did not significantly change the mRNA or protein levels of CRH-R1 receptor compared with age-matched, nonoperated controls (P > 0.2; data not shown).

V1b Receptor Expression

One band was identified in the Western blots as V1b receptor protein with a molecular mass of 90 kDa (Fig. 5). V1b receptor protein levels were not different between HPD and sham-operated fetuses (31,555 ± 9,193 vs. 24,884 ± 5,228 OD units, respectively; Fig
6B), but both were lower than the levels in the pituitaries of the 120 dGA fetuses (55,982 ± 5,635 OD units). V1b receptor mRNA levels from HPD fetuses were not significantly different from sham-operated animals (1.88 ± 0.33 vs. 2.01 ± 0.30 pg/µg total RNA, respectively; Fig. 6A). V1b receptor mRNA levels from naive 120 dGA fetuses (3.10 ± 0.50 pg/µg total RNA) tended to be higher than HPD and sham-operated 140 dGA fetuses, although this was not significant. The sham operation did not significantly change the mRNA or protein levels of V1b receptor compared with age-matched, nonoperated controls (P > 0.2; data not shown).

**DISCUSSION**

To our knowledge, this is the first study to examine the role of hypothalamic input on CRH-R1 and V1b receptor expression in late-gestation fetal sheep. V1b receptor protein levels significantly decreased with increasing gestational age. This decrease in V1b receptor protein was not prevented by HPD. CRH-R1 receptor mRNA and protein also decreased (P < 0.05 and P = 0.06, respectively) with increasing gestational age, consistent with the decrease in CRH responsiveness in late gestation. This decrease in CRH receptor protein levels was prevented by HPD, although the decrease in CRH-R1 mRNA was not. Therefore, we conclude that hypothalamic input to the pituitary does not appear to be necessary for the decrease in V1b receptor expression seen in late gestation. Hypothalamic input is also not essential for the decrease in CRH-R1 mRNA but appears to be necessary for the decrease in CRH-R1 receptor protein levels in late-gestation fetal sheep.

The decrease in CRH-R1 mRNA, CRH-R1 protein, and V1b protein seen during late gestation was determined by comparing naive 120 dGA fetuses and sham-operated 140 dGA fetuses. We are aware that interpretation of these results could be complicated by potential changes in the measured variables produced by surgery. However, we have determined that the sham surgery does not affect levels of CRH-R1 and V1b mRNA and protein by comparing anterior pituitaries from sham-operated fetuses with age-matched, nonoperated fetuses (data not shown). Therefore, we feel confident that the age-related changes reported here are an accurate representation of normal fetal development.

Fetal blood gases and pH were normal in both HPD and sham-operated fetuses within 2 days after surgery until the time of necropsy. Plasma cortisol levels were also normal in sham-operated fetuses, which demonstrated a significant increase in plasma cortisol levels at 136–139 dGA. However, in HPD fetuses, there was no increase in plasma cortisol in late gestation. This is consistent with other studies in HPD fetuses that show no change in plasma cortisol levels, even 8 days beyond normal gestation (155–156 dGA; see Refs. 2–4 and 25) and suggests that the prepartum increase in fetal plasma cortisol requires communication between the hypothalamus and pituitary. In addition, fetal sheep infused with a CRH antagonist, antalarmin, from 130 to 140 dGA displayed a similar hormonal profile to HPD fetuses, suggesting that specifically the lack of
CRH disrupts normal hypothalamus-pituitary-adrenal axis maturation (40).

We saw an increase in the percentage of corticotrophs in the anterior pituitary in HPD fetuses compared with both sham-operated and 120 dGA naive fetuses. This increase in the percentage of corticotrophs in HPD fetuses has also been shown previously (2). In addition, there was an effect of 2 h AVP treatment to increase the percentage of irACTH positive cells in the anterior pituitary. However, AVP treatment did not increase the percentage of proopiomelanocortin positive cells (39); thus, AVP appears to increase proopiomelanocortin processing to ACTH in the fetal pituitary.

In the current study, we identified three molecular sizes of the ovine CRH-R1 receptor (40, 118, and 180 kDa) using an ovine CRH-R1 antibody and a rat CRH-R1 antibody. The 40-kDa band detected in the current study is similar to the predicted molecular mass of the deglycosylated CRH-R1 receptor (~45 kDa; see Ref. 8) and is therefore likely to be physiologically relevant. A 40-kDa band has also been detected in pituitary extracts from rats (6). Previous work using an antibody directed against 335–346 AA of the rat CRH-R1 sequence also produced three specific bands (200, 118, and 70 kDa) in fetal sheep anterior pituitaries that differed slightly from the molecular masses detected here (11). Multiple molecular masses similar to the range seen in fetal sheep have been reported in other species. For example, molecular masses ranging from 115 to 40 kDa have been reported in the rat (6).

There are many possible explanations for the differences in molecular masses observed on Western blots by investigators in the field. Differences in the sequence chosen for production of the CRH-R1 antibody could affect its protein recognition. Other differences could simply be because of differences in molecular mass markers used or could be because of differences in the membrane preparation technique used. Because there are multiple splice variants of the CRH-R1 receptor detected in humans and mice (27) and one splice variant so far detected in sheep (22), this could also account for multiple molecular masses. It is also possible that the large-molecular-mass proteins seen are the result of dimer- and trimerization, since G protein-coupled receptors have been demonstrated to form SDS-resistant dimers and trimers (15).

We interpret the decline in receptor mRNA and in the 40-kDa protein as indicating that CRH-R1 receptor expression decreased in late gestation in the sham-operated fetuses. We acknowledge that the P value for the difference in protein is 0.06, but the data from sham-operated fetuses are similar to data obtained from nonoperated, age-matched fetuses and are consistent with previous reports of declining CRH-R1 receptor expression in late-gestation fetal sheep (11). Therefore, the normal decrease in CRH responsiveness in late gestation may be a consequence of reduced CRH-R1 receptor expression.

Studies in vivo have indicated that this decrease in CRH responsiveness is prevented by HPD. A comparison of the irACTH response to CRH infusion in intact and HPD fetuses demonstrated that CRH responsiveness decreased in intact fetuses between <128 and >138 dGA. This decrease in CRH responsiveness did not occur in HPD fetuses between <128 and >138 dGA, with HPD fetuses demonstrating increased plasma ACTH concentrations in response to CRH infusion at >138 dGA compared with intact fetuses of the same age (25). Similar to the effect of HPD on CRH responsiveness, we found that HPD prevented the normal decrease in CRH-R1 receptor protein levels, strengthening the suggestion that CRH responsive-
ness in late-gestation fetal sheep is mediated by a decline in CRH-R1 receptor expression in the pituitary.

Additional studies have examined the role of CRH and AVP input to the pituitary by lesioning the source of CRH and AVP, the paraventricular nucleus of the hypothalamus. Paraventricular nucleus lesions performed in fetal sheep at ~120 dGA prevented the normal decrease in CRH binding in pituitaries collected at ~140 dGA (21), consistent with the lack of a decline in CRH-R1 receptor protein found in HPD fetuses. Lesioning the paraventricular nucleus of fetal sheep also abolishes the prepartum increase in plasma cortisol, similar to HPD (19). This strengthens the argument that hypothalamic input to the pituitary and/or increasing cortisol is necessary for the normal decline in CRH-R1 receptor expression.

Despite the effect of HPD on CRH-R1 receptor protein levels, there was no effect of HPD on CRH-R1 receptor mRNA. This lack of correlation between CRH-R1 mRNA and protein levels has been demonstrated previously (30) and adds to the increasing evidence that mRNA levels are not always an accurate indicator of protein levels. This discord between mRNA and protein could be the result of many factors, including differences in mRNA and protein half-lives and/or differences in efficiency of mRNA translation.

The mRNA and protein sequence for the ovine V1b receptor has not yet been published. Therefore, for these studies, a fragment of ovine V1b receptor cDNA was used to measure mRNA levels by RPA, and a commercially available V1b receptor antibody directed against the rat V1b receptor was used for Western blotting. The homology between the rat and sheep V1b receptor AA sequence is unknown at this time. The anti-rat V1b antibody reacted with a 90-kDa protein from membrane fractions of fetal sheep anterior pituitaries. This band was completely eliminated by preincubation of the primary antibody with the peptide antigen supplied by the synthesizing company (Research Diagnostics) and was also not present in nonmembrane protein fractions (supernatant from 100,000 g spin). A 90-kDa band has also been detected with an ovine V1b receptor antibody in sheep anterior pituitaries (D. A. Myers, personal communication) and is also similar to the 82-kDa band seen in human MCF7 breast cancer cells (24). Therefore, we feel confident that the 90-kDa band seen in the Western blots in this study represents the ovine V1b receptor.

Unlike the discord seen with CRH-R1 mRNA and protein, V1b receptor mRNA and protein correlated

Fig. 4. CRH-R1 receptor mRNA (A) and protein levels (B-D) in the anterior pituitary of HPD (n = 6), sham-operated (n = 5), and 120 dGA (n = 6) fetal sheep. B: 180-kDa CRH-R1 protein. C: 118-kDa CRH-R1 protein. D: 40-kDa CRH-R1 protein. OD, optical density. A: *P < 0.05 vs. HPD and sham. D: *P = 0.06 vs. HPD and 120 dGA.
with each other with peak levels at 120 dGA, which decreased in both HPD and sham-operated fetuses at 140 dGA. Therefore, HPD did not block the decline in V1b receptor expression seen in late gestation. This suggests that hypothalamic input to the pituitary and increasing cortisol levels are not necessary for normal V1b receptor expression in fetal sheep.

The decrease in V1b receptor expression in late gestation is in contrast to the increase in AVP responsiveness that occurs at the same time (26). However, findings in adult rat anterior pituitaries suggest that AVP binding and V1b receptor mRNA may be inversely related (29). Thus it is possible that, even though V1b receptor mRNA decreases in late gestation, there could be an increase in AVP binding. In addition, glucocorticoids have been shown to increase V1b receptor coupling to its second messenger, phospholipase C, in rats (29). Therefore, the increase in fetal plasma cortisol that normally occurs close to term may enhance receptor coupling and thereby promote the increase in AVP responsiveness in late gestation in spite of a reduction in receptor expression. The effect of CRH and AVP on irACTH secretion collected from the cell immunoblots performed in this study was variable and therefore difficult to interpret. Additional in vivo and in vitro studies are needed to establish the mechanism by which AVP responsiveness changes in fetal pituitaries during development.

In conclusion, CRH-R1 mRNA levels decrease in late-gestation fetal sheep, and this decrease is not dependent on hypothalamic input. The decrease also occurs in the absence of increasing cortisol levels, since cortisol levels do not increase in HPD fetuses. Therefore, it appears that control of CRH-R1 mRNA is mediated by an intrapituitary factor or an unknown non-hypothalamic factor. V1b receptor protein levels follow the same pattern as CRH-R1 mRNA, suggesting that it may be controlled in the same fashion. On the other hand, the naturally occurring decline in CRH-R1 protein levels was prevented by HPD. This suggests that hypothalamic input and/or increasing plasma cortisol is necessary for the normal decrease in CRH-R1 receptor levels in late gestation.

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