Ontogeny and effect of cortisol on vasopressin-1b receptor expression in anterior pituitaries of fetal sheep

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Young, Sharla F., Jennifer L. Smith, Jorge P. Figueroa, and James C. Rose. Ontogeny and effect of cortisol on vasopressin-1b receptor expression in anterior pituitaries of fetal sheep. Am J Physiol Regul Integr Comp Physiol 284: R51–R56, 2003. First published October 3, 2002; 10.1152/ajpregu.00427.2002.—Corticotroph responsiveness to arginine vasopressin (AVP) increases during late gestation in fetal sheep. The mechanism of this increase in AVP responsiveness is currently unknown but could be related to an increase in vasopressin type 1b (V1b) receptor expression in the pituitary during development. To determine if there are ontogenic changes in V1b receptor expression that may help explain the changes in ACTH responses to AVP, we studied pituitaries from three groups of fetal sheep [100, 120, or 140 days gestational age (dGA)]. V1b receptor mRNA and protein significantly decreased by 140 dGA. Peak V1b mRNA levels were detected at 100 dGA, while peak V1b protein levels were detected at 120 dGA. The reduction in V1b receptor expression in late gestation may be due to the naturally occurring peripartum increase in fetal plasma cortisol because cortisol infusion at 122–130 dGA decreased V1b receptor mRNA. Thus there is a marked decrease in the expression of the V1b receptor in the pituitary during fetal development, leaving the role of the V1b receptor in increasing AVP responsiveness uncertain.

DURING LATE GESTATION there is a marked increase in cortisol secretion by the fetal adrenal gland. This increase in plasma cortisol is necessary for the final maturation of many fetal organs, including the lungs and gastrointestinal tract (reviewed in Ref. 5), and it occurs in all species that have been studied to date, including rats, sheep, and humans. Despite the well-known importance of the cortisol surge in fetal maturation, the mechanisms underlying it are still not understood.

ACTH-(1–39) is the primary hormone that controls cortisol release in fetal and adult life. However, immunoreactive ACTH (irACTH) levels do not rise dramatically at the time of the cortisol surge (6, 12, 20, 23, 29), arguing that a simple rise in ACTH is not responsible for the increase in cortisol release. Further study into irACTH release in fetal sheep has shown that while irACTH levels may not be elevated in late gestation, there is an increase in the ratio of bioactive ACTH-(1–39) to its precursor, pro-opiomelanocortin (POMC), released from the fetal pituitary that occurs in the same time frame as the cortisol surge (1–4, 15, 26). This is important because it has been demonstrated that in fetal sheep, POMC is capable of attenuating ACTH-stimulated cortisol release (22, 25). Thus increasing the ratio of ACTH-(1–39) to POMC may enhance adrenal cortisol secretion in late-gestation fetal sheep.

Arginine vasopressin (AVP) has been shown to increase the ratio of ACTH-(1–39) to POMC released from the pituitary (1, 2, 24, 32). Because ACTH responsiveness to AVP increases in late-gestation fetal sheep (8, 17), this could be a possible explanation for the relative rise in ACTH-(1–39) compared with POMC that occurs at this time. The mechanism underlying the rise in AVP responsiveness is not known at this time but may be due to an increase in vasopressin receptor expression in the anterior pituitary. Because the V1b receptor is the only vasopressin receptor present in the anterior pituitary, the objective of this study was to measure V1b receptor mRNA and protein levels throughout the last trimester in pituitaries from fetal sheep to test the hypothesis that the rise in AVP responsiveness is due to an increase in V1b receptor levels in the anterior pituitary. We also wished to determine if the physiological increase in fetal plasma cortisol before 0.95 gestation would produce changes in V1b receptor mRNA levels similar to those found close to term (147 days gestational age (dGA)).

MATERIALS AND METHODS

Animals

Ontogeny study. Fetuses of time-dated, mixed-breed sheep (100, 120, and 140 dGA; n = 6 in each group) were studied. All animal protocols were approved by the Animal Care and Use Committee of Wake Forest University School of Medicine and were in accordance with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society. Ewes were brought into the animal facility, anesthetized with pentobarbital sodium, and sacrificed at 100, 120, and 140 dGA. The ewes were killed at the time of the cortisol surge (1–4, 15, 26).

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maintained under general anesthesia (halothane in O2). The uterus was exposed, and the fetus was removed and killed by an overdose of pentobarbital sodium administered via the umbilical vein. To remove the pituitary, the fetal scalp was incised, and the skull cap was removed. The brain was removed and the pituitary was removed from the sella turcica. The neurointermediate lobe was separated from the anterior lobe. The anterior lobe was bisected and snap-frozen in liquid nitrogen before being stored at –80°C.

Cortisol infusion study. Ten time-dated pregnant sheep were brought to the animal facility and allowed to acclimate for several days before surgery as previously described (9). Surgery was performed at 120–128 dGA. Ewes were fasted 48 h before surgery. Before surgery, anesthesia was induced with ketamine (20 mg/kg im) and maintained with halothane (1–2%) in oxygen. The surgery included placement of catheters in the femoral arteries and veins of the fetus and ewe as well as placement of an amniotic catheter. Details of the surgical procedures have been described previously (19). Animals were allowed 2 recovery days after surgery before the cortisol or saline infusion was begun. Pituitary tissue was harvested after the infusion period in the same fashion as the ontogeny study.

Amniotic Pressure Monitoring

Amniotic pressure was monitored for 1 h before beginning the infusion and for 1 h after the 5-day infusion period via the amniotic catheter using a transducer coupled to a BMP amplifier (Louisville, KY). The signal was sampled at 100 Hz, averaged, and recorded at 1-min intervals using a Packard Bell computer (Thousand Oaks, CA).

Infusion

Continuous cortisol or saline infusion via the fetal femoral venous catheter was begun after allowing 2 days of postsurgical recovery (122–130 dGA). Hydrocortisone (H4001, Sigma, St. Louis, MO) was dissolved in ethanol and diluted in sterile isotonic saline to a final concentration of 42 µg/ml. Based on the estimated fetal weight at the time of surgery, a Harvard infusion pump (Natick, MA) was used to deliver the saline (n = 5) or cortisol (0.8 µg·kg⁻¹·min⁻¹; n = 5) for 5 days via the fetal femoral venous catheter. This dose has been shown to increase plasma cortisol concentration to the physiological range seen during late gestation (31).

Blood Sample Collection

Fetal and maternal arterial blood was collected daily to measure blood gases and hormone concentrations. Blood samples were placed into tubes containing 25 µl EDTA/ml blood (1.4 mg EDTA/ml blood) and were kept on ice until centrifuged at 4°C for 15 min at 1,500 g. After centrifugation, the plasma was stored at −20°C until hormones were assayed. Blood was also collected into heparinized syringes for determination of blood gases on an ABL5 blood gas analyzer (Radiometer, Copenhagen, Denmark).

Cortisol Assay

The cortisol concentration in unextracted plasma was measured by RIA using the DSL 2000 cortisol kit from Diagnostics Systems Laboratories (Webster, TX). The inter- and intra-assay coefficients of variation were 9.04 and 9.12%, respectively.

RNA Extraction

RNA from individual anterior pituitary halves was isolated using a modification of procedures we have employed previously (9, 30). Briefly, the tissue was homogenized in Tris-buffered reagent (50 mg tissue/ml Trizol; GIBCO BRL) with a high-speed polytron for 30–60 s. Then, 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), washing, and centrifugation at 12,000 g for 10 min. The supernatant was removed, and the RNA pellet was washed once with 75% ethanol (1 ml/1 ml Trizol) and reprecipitated 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/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.

RT-PCR of Ovine V1b Receptor

RT-PCR of ovine V1b receptor was performed with Reverse Transcript Kit (Ambion, Austin, TX). For the RT reaction, 5 µg total RNA from an adult anterior pituitary was mixed with 4 µl deoxy-NTP mix (2.5 mM each), 2 µl first-strand primers (random decamers, 50 µM), and distilled H2O to a final volume of 20 µl. This reaction was mixed well, heated to 80°C for 3 min, and then placed on ice. Two microliters of 10× RT-PCR buffer [100 mM Tris·HCl (pH 8.3), 500 mM KCl, and 5 mM MgCl2], 1 µl plasmid DNase inhibitor, and 10 ng Moloney murine leukemia virus RT were then added to the reaction, mixed gently, and incubated for 1 h at 42°C. After this, the RT was inactivated by incubation at 92°C for 10 min.

For the PCR reaction, 5 µl of the RT reaction was added to 5 µl 10× RT-PCR buffer, 2.5 µl V1b receptor-specific primers (5 µM each; BVPR, 5′-GGCGATCTCGACCCG-3′; EVPR, 5′-GGGATATCCATACCTACCCGGC-3′), 34 µl H2O, and 1 U Taq DNA polymerase. The reaction was mixed, centrifuged briefly, and covered with two drops of mineral oil. After a 5-min hot start at 94°C, 30 cycles of the following protocol were employed: 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s, followed by an extension of 15 min at 72°C. The DNA fragment generated by PCR was ligated in the pSP72 vector (Promega, Madison, WI) and sequenced in the following order: 4 µl transcription buffer, 2 µl transcription buffer, 2.5 µl first-strand primers, 1 µl RNA of DNA polymerase, and incubating for 2 h at room temperature. One microliter RQ1 DNase-free DNase 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). One mi-
RNase Protection Assay

V1b receptor mRNA in total anterior pituitary RNA was quantified using an RNase protection assay (RPA) II kit (Ambion). 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), 1 mM EDTA] and V1b probe (5 × 10^6 cpm). The samples were heated at 95°C for 5 min and immediately placed in a 48°C water bath for overnight hybridization. RNase A/TK (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 was precipitated by addition of RNase inactivation/precipitation buffer with 25 mM UTP and 100 µM UTP were replaced with 25 mM UTP.

Western Immunoblotting

Western blot analysis was performed according to the method of Laemmli (13) using 8.0% SDS-PAGE. Tissue was homogenized in a buffer consisting of 50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, plus a 1:200 dilution of protease inhibitor cocktail [P8340, Sigma; contains 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane, bestatin, leupeptin, and apro tinin]. Fetal samples (45–160 mg) were placed in liquid nitrogen and crushed in a stainless steel mortar, and the powder was homogenized in 800 µl of the buffer per 100 µg 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. 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 onto 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 (5 µg/ml; Research Diagnostics) and for 1 h with horseradish peroxidase-conjugated secondary antibody (1:7,500). A positive reaction, defined as a 90-kDa band, was identified with enhanced chemiluminescence (ECL Plus, Amersham Pharmacia Biotech, Arlington Heights, IL).

Data Analysis

Densitometry. Films were scanned and analyzed using TINA software (version 2.09; Raytest). Sense RNA standards were used to calibrate the system for RPA data. Duplicates were averaged, and data were converted from optical density (OD) readings to picograms mRNA per micrograms total RNA for RPA data. OD readings from Western blots were subtracted from background and are reported as OD units. Statistical analysis. Ontogeny study data were analyzed by one-way ANOVA with Neuman-Keuls post hoc analysis where appropriate. Ontogeny data were also subjected to Pearson correlation analysis to compare V1b mRNA and protein levels. The Student’s t-test was used to analyze the cortisol infusion study. Two-way ANOVA was used to analyze the plasma cortisol data. Differences were considered significant at P < 0.05. Data are presented as means ± SE.

RESULTS

Ontogeny Study: V1b Receptor mRNA Levels

Representative gels from the V1b RPA demonstrating the V1b full-length probe, negative control, and sense standards used to establish a standard curve are shown in Fig. 1A. This standard curve is also represented graphically in Fig. 1B. Representative samples of pituitary total RNA from 100, 120, and 140 dGA fetuses hybridized with the V1b receptor probe are shown in Fig. 2A. V1b receptor mRNA levels in the anterior pituitary of fetal sheep decreased over the last third of gestation (Fig. 2B). There was a significant decrease in the amount of V1b receptor mRNA between 100 and 120 dGA (7.2 ± 1.46 and 3.1 ± 0.50 pg/µg total RNA, respectively). V1b receptor mRNA tended to decrease further at 140 dGA (2.0 ± 0.30 pg/µg total RNA); however, this was not significantly different from levels obtained from 120 dGA fetuses.
Ontogeny Study: V1b Receptor Protein Levels

The V1b antibody used in these experiments was directed against an 18-amino acid peptide mapping within the predicted first extracellular domain of the rat V1b receptor and had no significant similarity with the V1a or V2 receptor sequence. When used to blot membrane fractions from fetal sheep pituitary, a specific band was identified 90 kDa in size (Fig. 3A).

Specificity was tested by preincubating the primary antibody with an excess (30μg) of control V1b peptide (Research Diagnostics), which completely eliminated the 90-kDa band. In addition, no bands were detected in the absence of primary antibody.

The V1b receptor protein levels in the anterior pituitary of fetal sheep were highest at 120 dGA (55,982±5,634 OD units; Fig. 3B) and dropped significantly by 140 dGA (20,775±5,148 OD units). Protein levels at 140 dGA also tended to be lower than those obtained from 100 dGA fetuses (38,471±9,193 OD units), but this was not statistically different (P = 0.08). V1b receptor protein levels were not correlated with V1b receptor mRNA levels (r² = 0.06; P = 0.24; data not shown).

Cortisol Infusion Study

Detailed blood gases and cortisol levels have been previously reported (9). Briefly, blood gases were normal (pH > 7.30, PCO₂ < 55 mmHg, PO₂ > 17 mmHg) in both groups. These values did not change significantly over the infusion period. Amniotic pressure recordings did not show evidence of labor in any of the animals. Plasma cortisol levels significantly increased during the infusion period in cortisol-treated, but not saline-treated, fetuses. Mean plasma cortisol levels were 23.6±3.0 ng/ml in the cortisol-treated animals and 3.4±0.6 ng/ml in the saline-treated animals (P < 0.05). Continuous cortisol treatment appeared to decrease V1b receptor mRNA levels compared with saline infusion (0.77±0.16 vs. 1.51±0.30 pg/μg total RNA, respectively; P = 0.058; Fig. 4).

DISCUSSION

To our knowledge, this is the first study to examine the expression of the V1b receptor in the anterior pituitary as a function of gestational age in fetal sheep.

Fig. 2. A: representative RPA illustrating fetal total RNA samples incubated with V1b probe. Lanes 1 and 2, 100 day gestational age (dGA) fetal RNA; lanes 3 and 4, 120 dGA fetal RNA; lanes 5 and 6, 140 dGA fetal RNA. B: V1b receptor mRNA levels (pg/μg total RNA) obtained from anterior pituitaries in fetal sheep at 3 different gestational ages (100, 120, and 140 dGA). *P < 0.05 vs. 120 and 140 dGA groups.

Fig. 3. A: representative autoradiograms from V1b receptor Western blots. Lane 1, Magic Mark protein standard (Invitrogen); lanes 2–4, anterior pituitary membrane fractions from 100 dGA (lane 2), 120 dGA (lane 3), and 140 dGA (lane 4) fetal sheep. B: V1b receptor protein levels obtained from anterior pituitaries in fetal sheep at 3 different gestational ages (100, 120, and 140 dGA). *P < 0.05 vs. 140 dGA fetuses.

Fig. 4. V1b receptor mRNA levels obtained from fetuses infused with either saline (n = 5) or cortisol (n = 5) for 5 days beginning at 122–130 dGA.
We have demonstrated that V1b receptor mRNA and protein decrease dramatically by 140 dGA, an age at which cortisol levels are rising exponentially in the fetus. The time course of this decrease is somewhat different for V1b receptor mRNA and protein. While mRNA levels are highest at 100 dGA and decrease by 120 dGA, protein levels are highest at 120 dGA with a subsequent decrease at 140 dGA.

The decrease in V1b receptor levels at 140 dGA does not support the hypothesis that the increase in AVP responsiveness during late gestation is due to elevations in V1b receptor levels in the anterior pituitary. However, because AVP binding has not been assessed in fetal sheep, we cannot rule out the possibility that functional V1b receptors increase with gestational age. Indeed, findings in rat anterior pituitaries suggest that AVP binding and V1b receptor mRNA may be inversely related, and dexamethasone has been shown to enhance V1b receptor coupling to its second messenger, phospholipase C (18), which would be expected to increase the ability of AVP to stimulate ACTH release. Because cortisol levels increase during the same time frame as the change in corticotroph responsiveness, this could be one explanation why AVP responsiveness rises during late gestation.

It has also been shown that V1b binding decreased as a result of dexamethasone administration (18). This suggests that the decrease in V1b receptor levels we found close to term is a result of increasing cortisol levels at this time. In support of this, we have demonstrated in this study that a 5-day infusion of cortisol to levels at this time. In support of this, we have demonstrated in this study that a 5-day infusion of cortisol to cortisol levels is rising exponentially in the fetus. The time course of this decrease is somewhat different for V1b receptor protein levels seen in the anterior pituitary of only 5% of the anterior pituitary (7). Likewise, it seems unlikely that the reduction in V1b receptor mRNA and protein levels seen in fetal sheep over gestation are due simply to a reduction in the percentage of corticotrophs present in the pituitary because V1b receptor protein peaks at 120 dGA despite a decrease in the percentage of corticotrophs between 100 and 120 dGA (16.9 ± 4.6 vs. 9.4 ± 1.4%, respectively; unpublished observations).

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 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). This 90-kDa band has also been detected with an anti-ovine V1b receptor antibody in sheep anterior pituitaries (D. A. Myers, personal communication). Therefore, we feel confident that the 90-kDa band seen in the Western blots in this study is the ovine V1b receptor.

The 90-kDa band seen in the sheep anterior pituitary is also similar to other species studied. Western analysis of MCF-7 breast cancer cells demonstrated human V1b receptor protein ranged in size with bands seen at 82, 78, 42, and 35 kDa (16). The rat, mouse and human V1b receptor is 425, 421, and 424 amino acid residues, respectively (14, 21, 28). The predicted molecular mass of these receptors is ~47 kDa. The discrepancy in size between the predicted molecular mass and the molecular mass determined here and in previous studies is not likely to be due solely to glycosylation of the receptor (10). More likely, the 90-kDa molecular mass seen in this study is due to dimerization, since G protein-coupled receptors are capable of forming SDS-resistant dimers and trimers (11).

In conclusion, V1b receptor mRNA and protein levels decrease throughout late gestation in anterior pituitaries from fetal sheep while AVP responsiveness is increasing. It is likely that increasing cortisol levels contribute to this decrease. Evidence from rat anterior pituitaries suggests that the increase in AVP responsiveness may be due to an increase in V1b receptor coupling that is stimulated by increasing glucocorticoid concentrations. Therefore, cortisol and AVP may act together in a feed-forward mechanism in late-gestation fetal sheep such that AVP increases the release of bioactive ACTH relative to POMC. This then stimulates the release of cortisol, which in turn increases AVP responsiveness.
REFERENCES


