The effect of hypothalamo-pituitary disconnection on the renin-angiotensin system in the late-gestation fetal sheep

Kai Chen, Luke C. Carey, Jingfang Liu, Nancy K. Valego, Stephen B. Tatter, and James C. Rose. The effect of hypothalamo-pituitary disconnection on the renin-angiotensin system in the late-gestation fetal sheep. Am J Physiol Regul Integr Comp Physiol 288: R1279–R1287, 2005. First published December 16, 2004; doi:10.1152/ajpregu.00560.2004.—The activity of the renin-angiotensin system (RAS) increases significantly in the late-gestation fetal sheep. Fetal cortisol is also increased during this time, and it is thought that the increase in cortisol may modulate the RAS changes. Previous studies have examined the effects of cortisol infusion on RAS activity, but the effects of blocking the peripartum increase in cortisol concentrations on the developmental changes in the RAS are not known. Therefore, we utilized the technique of hypothalamic-pituitary disconnection (HPD), which prevents the cortisol surge from occurring, to investigate the importance of the late-gestation increase in cortisol on the ontogenic changes in RAS activity. HPD of fetal sheep was performed at 120 days of gestational age (dGA), and fetuses were delivered between 135 and 139 dGA. Control fetuses were sham operated. HPD blocked the late-gestation cortisol increase but did not alter renal renin mRNA, renal renin or prorenin protein content, nor plasma renin levels compared with sham operated. However, HPD fetuses had increased ANG II receptor subtype 1 (AT1) mRNA and protein expression in the kidney and lungs. ANG II receptor subtype 2 (AT2) expression was not altered in these tissues at either mRNA or protein level. HPD did not change AT1 or AT2 mRNA in the left ventricle but did result in decreased protein levels for both receptors. These studies demonstrate that blockade of the naturally occurring increase in fetal cortisol concentration in late gestation is associated with tissue-specific alterations in expression of AT1 and AT2 receptors. These changes may impact on fetal tissue maturation and hence have consequences in postnatal life.

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Previous studies indicate that all components of the renin-angiotensin system (RAS) in fetal sheep are detectable in the kidney at 0.27 of gestation (65). It has also been demonstrated that renin, angiotensinogen, ANG I, and ANG II are present in the fetal circulation (18, 41). During fetal development, both activity and function of the RAS change significantly. Human and animal studies reveal that renal renin mRNA and protein levels peak during the perinatal period, while there is an overall increase in RAS activity on parturition (7, 11, 20, 39, 64). It has also been noted in the sheep and horse fetus that plasma angiotensin converting enzyme (ACE) concentrations (15, 16, 37) and ANG II levels (44) significantly increase close to term.

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We chose these particular tissues as the RAS is purported to play developmental and/or functional roles in all (5, 49, 53–55).

**MATERIALS AND METHODS**

*Animal preparation and surgical procedures.* Cross-bred pregnant ewes 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 housed in individual pens with food and water provided ad libitum. After 5 days of acclimatization, surgery was performed. After surgery, ewes were returned to their pens where they remained until the fetuses were delivered. A total of 10 fetuses were used: 5 HPD (3 male, 2 female) and 5 controls (3 male, 2 female).

*Surgical preparation.* Surgeries were performed at −120 days of gestational age (dGA). Polyvinyl catheters previously filled with sterile saline were inserted into the fetal femoral arteries and veins and advanced to the descending aorta and inferior vena cava, and HPD was performed as described by Antolovich et al. (1), with slight modifications (72).

Catheters from the fetus and maternal arterial and venous catheters were exteriorized through a small incision in the maternal flank, placed into a sterile glove and protected by netting placed around the ewe’s abdomen. When a control operation was performed, all steps of the surgery were done except the median eminence and pituitary stalk were not separated, the median eminence tissue was not removed, and a piece of latex glove 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 ewe and fetus every other day to assess fetal and maternal health via blood gas and pH measurements. Fetal plasma samples were collected following 3 days of post-surgery recovery, and just prior to necropsy for measurement of cortisol.

*Renal prorenin concentration measurement.* Prorenin concentrations were determined by measuring active renin before and after treatment of kidney homogenate with bovine pancreatic trypsin at a concentration determined to yield maximum renin activation. Each lot of trypsin was tested by constructing a dose-response curve with pooled plasma or kidney homogenate. Once the optimal dose of trypsin was established for each, this dose was used for subsequent assays. Trypsin activation was at 4°C and pH 7.3 for 0.5 h. The activation was stopped by addition of trypsin inhibitor at room temperature for 15 min. The total renin concentration represented the sum of active and prorenin.

*RNA extraction.* Total tissue RNA was extracted using standard procedures recommended by the manufacturer of Trizol (Gibco BRL, Carlsbad, CA). Briefly, tissues were homogenized in Trizol reagent (50 mg tissue/1 ml Trizol) using a high-speed polytron for 30–60 s. Chloroform was added (0.2 ml/1 ml Trizol), and the mixture was incubated at room temperature for 5 min, before centrifugation at 12,000 g for 15 min at 4°C. 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) and recentrifuged at 7,500 g at 4°C for 5 min. The isopropanol was removed, and the RNA pellets were allowed to air dry and then redissolved in RNase-free water. RNA concentrations were determined by absorbance at 260 nm in a spectrophotometer. The integrity of all RNA samples was determined by electrophoresis on a 1.0% agarose gel containing 6.6% formaldehyde.

*Synthesis of antisense RNA probes.* The probe used for sheep renin mRNA was partial sheep renin cDNA from coordinates 117–983 cloned into pGEM-T easy (Promega, Madison, WI) and cut with the restriction enzyme EcoRI to linearize the plasmid in preparation for in vitro transcription. The probe used for sheep AT1 mRNA was partial sheep AT1 cDNA from coordinates 114–783 cloned into pGEM-T easy (Promega, Madison, WI) and cut with the restriction enzyme SpeI to linearize the plasmid in preparation for in vitro transcription. The probe used for sheep AT2 mRNA was partial sheep AT2 cDNA from coordinates 142–921 cloned into pT7/T3 U18 (Ambion, Austin, TX) and cut with the restriction enzyme Hind III to linearize the plasmid in preparation for in vitro transcription.

In vitro transcription was performed by adding the following items in this order (4 µl 5X transcription buffer, 2 µl 100 mM dithiothreitol, 1 µl RNasin RNase inhibitor, 4 µl ATP, GTP, and CTP mix (25 mM each), 2.4 µl 100 µM UTP, 5 µl [α-32P]UTP (3,000 Ci/mm mol; Perkin-Elmer, Boston, MA), and 1 µl SP6 (for renin) or T7 (for AT1 and AT2) polymerase) and incubating for 2 h at room temperature. One microliter RQ1 RNase-free DNase was added, and the reaction was incubated for an additional 15 min at 37°C to remove the DNA template. Unincorporated nucleotides were removed by G-50 Sephadex, RNA was precipitated with ethanol (Roche Molecular Biochemicals, Indianapolis, IN). One microliter of the purified probe was placed into a scintillation vial to determine counts per minute. Sense strand used for the standard was synthesized with linearized plasmid by in vitro transcription similar to the above; however, [α-32P]UTP and 100 µM UTP were replaced with 25 µM UTP. RNase protection assay. Renin, AT1 and AT2 mRNAs were quantified by RNase protection assay (RPA; RPA kit III; Ambion). Briefly, 20 µg total tissue RNA was mixed with 10 µl hybridization buffer and 100,000 cpm of the renin, AT1, or AT2 probe. Samples were hybridized to the probe under the conditions recommended by the manufacturer. After hybridization, the gel was dried and exposed to X-ray film.
were then heated at 95°C for 4 min and placed in a 48°C water bath for overnight hybridization. RNaseAT1 (1:150 dilution in RNase digestion buffer) was then added to the samples to digest unhybridized probe and RNA. Digestion was stopped, and the hybridized RNA was precipitated by adding RNase inactivation/precipitation buffer and incubating 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 then exposed to film (Biomax-MR, Kodak, Cealex, France) with an intensifying screen.

Immunoblotting. Western blot analysis for AT1 and AT2 was performed as previously described (51). Briefly, tissue samples were homogenized in 50 mM Tris, 10 mM EDTA, 150 mM NaCl, 0.1% (vol/vol) Tween-20, 0.1% (vol/vol) 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotonin, pH 7.5, and sonicated on ice for 15–20 s. One-milliliter aliquots were centrifuged in a microfuge at 12,000 g for 3 min, at 4°C. The pellet was discarded, and samples of supernatant were diluted in a sample buffer. Protein concentrations of samples harvested from HPD and control animals were determined using a modified Bradford method. A standard curve was produced with known concentration of bovine albumin, and the protein concentrations of samples were determined by comparing their optical density (OD) 595-nm values with values from the standard curve. Both AT1 and AT2 receptor-specific polyclonal antibodies (Santa Cruz Biotechnology, CA) have been used to detect the ovine AT1 and AT2 receptors, respectively (51). Forty micrograms of protein per lane were electrophoresed on a 12% polyacrylamide gel containing SDS for 1.5 h and then blotted onto a polyvinylidene fluoride membrane (Immobilon, Millipore, Marlborough, MA) by semidy electroblotting. The blot was blocked overnight at 4°C with 6% nonfat milk in 0.05% Tween-20 Tris-buffered saline (TTBS) and then incubated with the primary antibody using a 1:2,000 (AT1) or 1:6,000 (AT2) dilution in 6% dry milk/TTBS for 2 h at room temperature. Blots were then rinsed, washed, and incubated with a 1:4,000 dilution of monkey anti-rabbit horseradish peroxidase-conjugated antibody in 6% dry milk/TTBS for 1 h at room temperature. Binding of the secondary antibody was detected using a chemiluminescent system consisting of horseradish peroxidase-hydrogen peroxide oxidation of luminol (ECL plus, Amersham, Arlington Heights, IL). Blots were then exposed to film for 5–10 min before densitometric analysis.

Densitometry. Films were scanned and analyzed using Quantity One software (PD Imageware Systems, San Diego, CA). Sense RNA standards were used to calibrate the system for RPA data. Data were converted from optical density readings to picograms mRNA per 10 μg total RNA for RPA data. Western blot data are reported in OD units.

Data analysis. Data pertaining to renal renin mRNA, and renal active and prorenin levels were compared by Student’s t-test. All other evaluations were made using two-way ANOVA, followed by Newman-Keuls multiple comparison test. Results are expressed as means ± SE, with P < 0.05 considered significant.

RESULTS

Fetal plasma cortisol, T4 and T3 levels, and fetal health. Plasma cortisol concentrations were not different between HPD and sham-operated fetuses at 120–125 dGA. However, by 135–139 dGA, plasma cortisol concentrations were significantly elevated in control fetuses but not HPD fetuses, where 120–125 dGA levels were maintained (Fig. 1). Fetal health, as assessed by arterial blood gas and pH measurements, was normal throughout the duration of the study in both HPD and control fetuses (Table 1). Although PO2 levels in the HPD group exhibited a modest decline during gestation, the values remained in the normal range and were not different from those in the control group.
When the studies were conducted, the dose of steroid given, the different effects is not known but may relate to the gestational age. The explanation for these differences is not different from those in normal dogs (26).

There is some evidence that renin processing in the fetal kidney is influenced by the absence of the rise in fetal plasma cortisol. A small decline in the active renin concentration and increase in the prorenin concentration in the kidney of HPD animals resulted in a small decrease in the active renin-to-prorenin ratio ($P = 0.056$) compared with the ratio in intact animals. This suggests that the prenatal increase in fetal plasma cortisol may enhance the processing of prorenin to renin.

### DISCUSSION

The purpose of the present study was to determine if the naturally occurring increase in fetal plasma glucocorticoid levels close to term is responsible for some of the developmental changes observed in the RAS (7, 11, 15, 16, 20, 39, 44, 64). To study this issue, we utilized the ovine fetus in which hypothalamic communication with the pituitary was interrupted, as this manipulation abolishes the peripartum increase in fetal plasma cortisol but leaves the fetal adrenals intact. An alternative approach for blocking the prepartum elevation in plasma cortisol is fetal adrenalectomy. However, that procedure also removes a source of aldosterone and catecholamines from the circulation. Thus to avoid problematic and likely confounding issues related to replacement of these hormones, HPD was utilized. We found that HPD, while preventing the late-gestational plasma cortisol surge, did not influence renal renin mRNA expression, renal renin content, or plasma renin concentration. It seems therefore that the increases in renal renin expression and plasma renin concentrations close to term in fetal sheep are not regulated by heightened cortisol levels. In contrast, blockade of the increase in fetal plasma cortisol did alter the expression of angiotensin receptor subtypes in a tissue-specific fashion. Thus the data suggest that the natural increase in cortisol in late gestation may be an important modulator of the effects of angiotensin in the period immediately preceding the transition from fetal to newborn life.

Close to term, glucocorticoids are known to induce enzyme activity in many fetal tissues in preparation for delivery and extraterine life (16), and it has been suggested that the heightened RAS activity close to parturition may be modulated by changing plasma cortisol concentrations (15). However, there are conflicting observations in the literature concerning the effect of cortisol on renin secretion and expression, with some laboratories reporting that infusions of the steroid suppress renin (52, 69) while others note either increases or no significant change (8, 15, 68). The explanation for these different effects is not known but may relate to the gestational age when the studies were conducted, the dose of steroid given, the duration of the exposure to steroid, or a combination of these variables. However, the lack of any effect on renal renin expression and plasma renin concentration caused by blocking the age-related increase in fetal plasma cortisol demonstrates that the peripartum elevation in glucocorticoids is not a prerequisite for the maturation-related increase in renin secretion. Indeed, in both control and HPD animals, plasma renin concentrations were similar shortly after surgery, and the renin concentration increased in parallel as gestation progressed. This pattern is similar to what has been reported previously (39) and is consistent with the increased renin concentration expression in the fetal kidney in late gestation (7). The lack of effect of elevated levels of endogenous glucocorticoids on plasma renin levels in the fetus is consistent with similar observations in adult dogs with hypercortisolemia. Plasma renin activity in these animals is not different from those in normal dogs (26).

There is some evidence that renin processing in the fetal kidney is influenced by the absence of the rise in fetal plasma cortisol. A small decline in the active renin concentration and increase in the prorenin concentration in the kidney of HPD animals resulted in a small decrease in the active renin-to-prorenin ratio ($P = 0.056$) compared with the ratio in intact animals. This suggests that the prenatal increase in fetal plasma cortisol may enhance the processing of prorenin to renin.
While a number of enzymes have been implicated in this processing (10, 23, 61, 62), the influence of glucocorticoids on the conversion has not been systematically investigated. Additional work in this area is needed to firmly establish a role for the prenatal increase in fetal plasma cortisol in regulating the posttranslational processing of renin.

Renal AT1 mRNA expression is known to increase rapidly close to term and then decrease postpartum (42, 43). The fact that this increase parallels the cortisol surge suggests that cortisol may play a regulatory role. If this were the case, then HPD, in preventing the cortisol surge, would also inhibit the normal increase in AT1 receptor mRNA expression. Somewhat surprisingly, in the present studies, HPD significantly elevated AT1 mRNA levels and the corresponding protein levels in kidney and lung. Our findings therefore imply that the renal AT1 increase in late gestation is not positively regulated by cortisol. On the contrary, it would appear that cortisol acts to prevent renal AT1 receptor overexpression during this period of development.

The effect of cortisol on renal AT1 receptor expression appears to vary depending on the age at exposure. Exposure to increased cortisol levels in early gestation (~27 dGA) tends to upregulate renal AT1 expression (34), while treatment in later gestation (120 dGA) depresses AT1 mRNA expression in fetal sheep (52). Our findings are supportive of the late-gestation observations, in that by preventing the normal increase in cortisol concentrations we demonstrated uninhibited/increased renal AT1 receptor expression in HPD fetuses. It is apparent that the effect of cortisol on renal AT1 expression is developmentally regulated.

The differential effects of cortisol on renal AT1 receptor expression in early and late gestation suggest that receptor function may change as the fetus develops. In early and midgestation, before nephrogenesis is complete, ANG II is known to act via the AT1 receptor (67) as a renal growth factor, stimulating proliferation in mesangial (40) and medullary interstitial cells (31). Interestingly, overexpression of the AT1 receptor during this time results in increased kidney weight at birth, thus further emphasizing the importance of the receptor in renal development (58). In later gestation, the role of the AT1 receptor changes to that of mediating salt and water excretion by the metanephrons, thereby maintaining volume in fetal fluid compartments and ensuring normal growth and development over the last third of pregnancy (65).

It has been demonstrated that the cardiac AT1 receptor is important in mediating cardiac function and growth during the perinatal period (4, 47, 48). During fetal life, the cardiac inotropic, chronotropic, and growth-promoting effects of ANG II appear to be mediated by the cardiac AT1 receptor (47, 48). Blockade of the AT1 receptor with losartan attenuates the rapid

![Fig. 4. AT1 (A) and AT2 (B) mRNA expression in kidney, lung, and left ventricle of HPD and sham-operated fetuses. # P < 0.001, AT1 mRNA vs. AT2 mRNA; + P < 0.05, AT1 mRNA in kidney and lung vs. that in left ventricle; * P < 0.05, AT2 mRNA in lung and left ventricle vs. that in kidney; $ P < 0.05, HPD vs sham.](http://ajpregu.physiology.org/)

![Fig. 5. Effect of HPD on AT1 protein expression in kidney (A), lung (B), and left ventricle (C) of HPD and sham-operated fetuses. * P < 0.05, HPD vs sham-operated fetuses.](http://ajpregu.physiology.org/)

**Fig. 4.** AT1 (A) and AT2 (B) mRNA expression in kidney, lung, and left ventricle of HPD and sham-operated fetuses. # P < 0.001, AT1 mRNA vs. AT2 mRNA; + P < 0.05, AT1 mRNA in kidney and lung vs. that in left ventricle; * P < 0.05, AT2 mRNA in lung and left ventricle vs. that in kidney; $ P < 0.05, HPD vs sham.

**Fig. 5.** Effect of HPD on AT1 protein expression in kidney (A), lung (B), and left ventricle (C) of HPD and sham-operated fetuses. * P < 0.05, HPD vs sham-operated fetuses.
growth of the left ventricle that normally occurs in the first 3 days of life in newborn piglets (4). The effects of cortisol infusions on cardiac AT1 receptor mRNA expression have been described. Cortisol infusion at ~120 dGA significantly increases cardiac AT1 receptor expression (52). Cortisol also significantly increases the heart-to-body weight ratio, but not the kidney- or lung-to-body weight ratios in fetal sheep and rats, as well as in preterm infants (13, 27, 56). Our studies in fetal sheep show that the HPD-mediated lower cortisol levels are associated with significantly decreased cardiac AT1 receptor protein expression. This finding is consistent with those from previous studies and indicates that the inhibited fetal cardiovascular function associated with low cortisol levels may be due in part to decreased cardiac AT1 receptor expression.

The relationship between fetal lung maturation and AT1 receptor expression is, however, not so clear. It has been demonstrated that fetal sheep lung AT1 receptor mRNA levels are elevated following maternal malnutrition in early gestation, suggesting that the associated cortisol increase may be the driving factor (58). In contrast, our data (where cortisol concentrations were manipulated in late gestation) imply that lower fetal cortisol concentrations promote lung AT1 expression. These different findings imply that fetal lung AT1 receptor expression is also developmentally regulated.

In contrast to AT1, the function of the tissue AT2 receptor is not clear. In the kidney, there is evidence that the AT2 receptor may play an important role in nephrogenesis. This is suggested by the observation that AT2 receptor expression is high in interstitial cells of the cortex, and in the macula densa during the nephrogenic period (6), and by the finding that expression is very low in the third trimester, when nephrogenesis is complete (17). With respect to the effects of glucocorticoids on AT2 expression, it has been demonstrated that exposure to dexamethasone in early gestation (26 and 28 days of gestation) increases levels in the macula densa (34), while a late gestation (130 dGA) infusion of cortisol has no effect (52). Our observation that the lower cortisol concentrations in HPD fetuses did not effect renal AT2 expression close to term is in keeping with the theory that cortisol-induced changes in AT2 receptors play an important role in regulating kidney development in early to middle, but not in late, gestation when the organ has attained functionality.

The AT2 receptor is also thought to be an important factor in cell proliferation and differentiation and tissue remodeling and repair (25, 36, 63). Thus, in the present study, the tendency for AT2 expression to be higher in HPD fetal lungs might be indicative of lung immaturity, instigated by the lower prevailing plasma cortisol concentrations.

Cardiac AT2 protein was detected as two bands, at 44 and 78 kDa. In sheep, the 44-kDa band has been reported to predominate in the fetal kidney, while the larger band is more evident in the mature fetal arteries and adult adrenal (51). The finding...
that AT2 protein was unchanged by HPD at the 44-kDa band, but decreased at the 78-kDa band, suggests that hearts from HPD fetuses were less mature than those from control counterparts and implicates the lack of cortisol as a mediating factor. Changes in mRNA expression do not always translate to the protein level and vice versa. In the present study, neither AT1 nor AT2 receptor mRNA expression in left ventricle was altered by HPD; however, patterns of protein expression were. A study in sheep by Moritz and colleagues (34) demonstrated that AT1 mRNA was elevated following dexamethasone treatment, while there was no corresponding change in protein levels. Posttranscriptional regulation of the AT1 receptor may result in changes in mRNA expression that do not directly reflect changes in cell surface receptor number (73). In this situation, it is critical that expression of both mRNA and protein is quantified to facilitate complete interpretation of data.

We recognize that HPD, in addition to preventing the cortisol surge, may also affect other hormones and physiological parameters that could influence the RAS. For instance, with respect to fetal blood pressure, any HPD-associated decrease should result in increased renal and plasma renin concentrations, changes that were not observed in our study. Thyroid hormones have also been implicated in regulating renin and angiotensin receptor expression (28–30, 33, 59). However, in the present study we measured both T4 and T3 concentrations and found there to be no difference between HPD and sham-operated fetuses. Similarly, vasopressin and oxytocin have been shown to affect renin secretion in adult animals (24, 50). Because the concentration of these peptides is low in fetal sheep (35, 46), it seems unlikely that any further reduction possibly caused by HPD would alter renin secretion, and no differences in plasma renin concentration were found in the HPD animals compared with the sham-operated fetuses. Another hormone with possible effects in the RAS is growth hormone (GH). GH treatment in humans has been demonstrated to activate the RAS (22), and in rat astrocytes and GH-deficient rats increase AT1 mRNA expression and ANG II receptor density, respectively (70, 71). The effect of HPD on plasma GH concentrations in the fetal sheep has not been examined; however, evidence in adult animals suggests no significant effects are apparent (21). Thus there does not appear to be compelling evidence suggesting that other variables potentially altered by HPD would significantly influence the RAS in fetal sheep.

In summary, our studies demonstrate that HPD-induced lower plasma cortisol concentrations in fetal sheep are associated with tissue-specific alterations in expression of the AT1 and AT2 receptors at the mRNA and corresponding protein levels in kidney and lung. In contrast, HPD did not alter the peripartum increase in renin expression. These changes may influence fetal tissue maturation and hence have consequences in neonatal life. The importance of the naturally occurring increase in cortisol in regulating aspects of RAS development is emphasized by our findings.

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GRANTS

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