Differential effects of mineralocorticoid blockade on the hypothalamo-pituitary-adrenal axis in pregnant and nonpregnant ewes

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Lingis M, Richards EM, Keller-Wood M. Differential effects of mineralocorticoid blockade on the hypothalamo-pituitary-adrenal axis in pregnant and nonpregnant ewes. Am J Physiol Endocrinol Metab 300: E592–E599, 2011. First published January 4, 2011; doi:10.1152/ajpendo.00560.2010.—During pregnancy, plasma ACTH and cortisol are chronically increased; this appears to occur through a reset of hypothalamo-pituitary-adrenal (HPA) activity. We hypothesized that differences in mineralocorticoid receptor activity in pregnancy may alter feedback inhibition of the HPA axis. We tested the effect of MR antagonism in pregnant and nonpregnant ewes infused for 4 h with saline or the MR antagonist canrenoate. Pregnancy significantly increased plasma ACTH, cortisol, angiotensin II, and aldosterone. Infusion of canrenoate increased plasma ACTH, cortisol, and aldosterone in both pregnant and nonpregnant ewes; however, the temporal pattern of these responses differed between these two reproductive states. In nonpregnant ewes, plasma ACTH and cortisol transiently increased at 1 h of infusion, whereas in pregnant ewes the levels gradually increased and were significantly elevated from 2 to 4 h of infusion. MR blockade increased plasma aldosterone from 2 to 4 h in the pregnant ewes but only at 4 h in the nonpregnant ewes. In both pregnant and nonpregnant ewes, the increase in plasma aldosterone was significantly related to the timing and magnitude of the increase in plasma potassium. The results indicate a differential effect of MR activity in pregnant and nonpregnant ewes and suggest that the slow changes in ACTH, cortisol, and aldosterone are likely to be related to blockade of MR effects in the kidney rather than to effects of MR blockade in hippocampus or hypothalamus.

cortisol; adrenocorticotropic hormone; pregnancy; canrenoate; aldosterone

IN STUDIES OF BOTH WOMEN (8) and ewes (3), basal plasma adrenocorticotropic hormone (ACTH) and cortisol have been shown to increase during pregnancy. The increase in adrenal steroid secretion is critical for normal pregnancy. Reduction of either maternal cortisol or maternal aldosterone results in alterations in maternal and fetal physiology (19–21). We have hypothesized that there is a change in the regulation of basal ACTH in the pregnant state. Previous studies in our laboratory in adrenalectomized sheep have shown that the concentration for cortisol replacement required to normalize basal plasma ACTH is increased in pregnancy and that replacement of pregnant ewes with cortisol to plasma concentrations similar to those measured in nonpregnant ewes increases both basal and hypotension-stimulated ACTH release (23, 26). On the other hand, in studies using adrenal-intact ewes, the inhibition of ACTH produced by raising plasma cortisol above resting levels is not different during pregnancy (23). Therefore, we have hypothesized that during pregnancy the “set point” of the negative feedback regulation by cortisol is increased, and/or a greater increase in cortisol is required in the pregnant state to counteract stimuli to basal hypothalamo-pituitary-adrenal (HPA) activity occurring in the hypoadrenal condition.

The negative feedback effects of cortisol on ACTH occur through both mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). In sheep, both receptors are expressed within key regulatory areas of the HPA axis: the hypothalamus, hippocampus, brainstem, and pituitary (25, 35, 36). Activation of central and pituitary GR leads to feedback inhibition of stress-induced HPA axis activation, thereby reducing ACTH and cortisol secretion (24). In contrast, activation of the higher affinity MR, expressed at high levels in the hippocampus, is thought to inhibit the activity of the HPA axis at basal corticosteroid levels (5, 24, 33, 34). Both MR and GR in the hippocampus are thought to play an inhibitory role in regulation of the HPA axis through indirect connections to the paraventricular nucleus, such as those made through the septal nucleus of the stria terminalis (15, 16, 18). In regions such as the hypothalamus and hippocampus in which the dominant form of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) is the reductase form 11β-HSD type 1, rather than the dehydrogenase- or cortisol-inactivating form type 2, cortisol is available for binding at both MR and GR (39).

We have also found that in pregnant ewes there is increased cytosolic MR availability in the hippocampus (36). In the nonpregnant ewe, MR in the hippocampus are ~90% occupied at basal plasma levels of cortisol (35); occupancy appears to be reduced in pregnant ewes. This finding suggests reduced MR effects despite the higher plasma cortisol levels that occur with pregnancy. Reduced occupancy of MR would reduce the feedback inhibition that occurs at low levels of cortisol (0–5 nM free cortisol or 0–9 ng/ml total cortisol), thereby allowing basal HPA activity to be increased.

These studies were designed to test the hypothesis that blockade of MR will produce differential effects in pregnant and nonpregnant ewes. In studies in both humans and rats, administration of MR antagonists has been shown to increase ACTH and cortisol and to modulate responses to stimuli by corticotropin-releasing hormone and exercise (1, 2, 4, 11, 13, 17, 42, 44). We have similarly used the peripheral infusion of the MR blocker canrenoate, resulting in antagonism of both central and peripheral MR and thereby allowing for characterization of the relative importance of MR actions during pregnancy in the regulation of adrenal hormone secretion, electrolytes, and blood pressure in the ewe. We hypothesize that if reduced central MR action is at least in part responsible for elevated cortisol during pregnancy, then ACTH responses to MR blockade will be attenuated in pregnant compared with nonpregnant ewes. We also hypothesize that if peripheral MR
play a role in expansion of blood volume during pregnancy, treatment with an MR blocker would lead to augmented ACTH responses secondary to changes in blood volume, electrolytes, and/or blood pressure.

**MATERIALS AND METHODS**

**Animal use and protocols.** Animals were housed in climate controlled, individual pens located at the University of Florida Animal Care Facility; all animal use was approved by the Institutional Animal Care and Use Committee at the University of Florida. Mixed Western breed ewes, both nonpregnant (n = 6) and pregnant (n = 6, 136 ± 3 days of gestation; term is ~147 days), were used in this study. Studies were performed between September and December 2006, which is normally during the time that ewes are cycling. In all but one of the nonpregnant ewes, the plasma progesterone concentrations during the study period were consistent with luteal phase progesterone values in the ewe.

Before surgery, food was withheld from the ewe for 24 h. All surgeries were performed in the surgery suite of the Health Science Center Animal Resources Department. Animals were prepared for and underwent surgery under aseptic conditions. Animals were induced with isoflurane inhalant and maintained using either isoflurane or halothane inhalant (1–3% in oxygen). Surgery was performed for the insertion of sterile polyvinyl catheters in the femoral arteries and veins of the ewes, as described previously (3). During a 5-day recovery period, the animals received twice daily intramuscular injections of ampicillin (1 g); body temperature was also monitored twice daily.

At the end of the recovery period, each ewe was randomly assigned to one of two treatment regimens: intravenous infusion of the MR antagonist canrenoate in saline (4 mg/kg bolus followed by 1 mg/kg·h−1 infusion for 4 h) or saline at the same infusion rate (40 ml over 4 h). Two days later, the experiment was repeated for each ewe using the other of the two treatments.

Because this study focuses on basal HPA axis activity, we minimized contact with the ewes during sampling by using arterial and venous catheters for sampling and infusion, respectively, which reached outside of the sheep pens (described previously in Ref. 3). Blood samples (8 ml) were collected into EDTA-treated tubes just prior to the start of the infusion and at every hour during the infusion. Plasma was stored at −20°C for future analysis. At the time of the experiment, 1.5-ml blood samples collected into heparinized syringes were used for determination of hematocrit and plasma protein, so-

**Assays.** Plasma cortisol was determined using ELISA (Oxford Biomedical Research, Oxford, MI); the sensitivity of this assay is 0.5 ng/ml. Plasma ACTH was determined by radioimmunoassay (RIA) as described previously using an antibody to ACTH(1–39) (3); the sensitivity of this assay is 20 pg/ml. Plasma progesterone and aldosterone were determined by RIA using the 125I Coat-A-Count progesterone and aldoste-

**RESULTS**

**Plasma hormone levels.** Mean plasma progesterone levels were significantly greater in pregnant ewes, as expected (non-

**Changes in plasma cortisol mirrored the changes in plasma ACTH.** There were significant effects of canrenoate administration and of pregnancy on plasma cortisol responses to the MR antagonist (main effects of treatment and time and significant time by pregnancy status interactions). Separate analysis of the responses in nonpregnant ewes revealed that, similar to the pattern of stimulation of ACTH, cortisol exhibited an initial transient rise at 1 h and then declined to levels that were not different from levels measured at the corresponding time point during infusion of saline (Fig. 1). In contrast, during infusion of canrenoate in pregnant ewes, there was no increase in plasma cortisol by 1 h, but cortisol was increased significantly compared with saline by 2 h of can-

The plasma aldosterone response to canrenoate infusion over time also varied between pregnant and nonpregnant ewes (Fig. 2). There were main effects of time, treatment, and
pregnancy status on plasma levels of aldosterone ($P < 0.05$). Aldosterone levels in nonpregnant ewes were either at or below the lower limit of detection for this assay (12.5 pg/ml) during infusion of saline. There were also significant two- and three-way interactions (treatment by pregnancy status, time by treatment, pregnancy status by time, and time by treatment by pregnancy status; $P < 0.05$). Separate analysis of the pregnant and nonpregnant ewes revealed significant increases in aldosterone from 2 to 4 h in pregnant ewes, but not until the 4-h time point in nonpregnant ewes. Analysis within the canrenoate-treated ewes revealed that the aldosterone response to MR blockade was significantly greater for pregnant ewes than for nonpregnant ewes from 2 to 4 h of infusion.

Overall, the level of angiotensin II was significantly greater in pregnant compared with nonpregnant ewes (Fig. 2). Analysis of the response during infusion of canrenoate determined that there was a significant increase in plasma levels of angiotensin II over time; however, this increase was only significant in pregnant ewes at 3–4 h of infusion.

**Mean arterial blood pressure.** As expected, MAP was significantly lower in pregnant ewes during infusion of either saline or MR antagonist ($P < 0.05$); the average values of MAP over 4 h of saline infusion in nonpregnant and pregnant ewes were $116 \pm 4$ and $102 \pm 3$ mmHg, respectively. Overall, the apparent decrease in MAP in response to canrenoate infusion was not significant for either group (Fig. 3). However, time by group and treatment by group by time interactions were significant ($P < 0.05$); these interactions reflect the greater MAP in nonpregnant compared with pregnant ewes during both treatments and a transient increase in MAP after the start of the infusion of saline in the pregnant ewes.

**Plasma volume.** Plasma volume was significantly greater in pregnant ewes than in nonpregnant ewes, as expected (Fig. 4). Although the plasma volume tended to be lower after infusion of canrenoate in each group of ewes, these differences were not statistically significant.

**Plasma electrolyte and protein concentrations and hematocrit.** There were no overall differences in plasma potassium or sodium levels between pregnant and nonpregnant ewes. Canrenoate infusion altered plasma sodium concentration (interaction of treatment and time, $P < 0.05$; Fig. 5); however, the decrease in plasma sodium was significant in pregnant ewes only at 4 h. Plasma potassium increased in both groups during canrenoate infusion, although the time course for the rise in

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**Fig. 1.** Plasma ACTH and cortisol during infusion of saline (○, △, and dashed lines) or canrenoate (●, ▲, and solid lines) in nonpregnant ewes (○ and ●; left) and pregnant ewes (△ and ▲; right). Overall, plasma ACTH concentration was significantly greater in pregnant ewes than in nonpregnant ewes. *Significantly different from concentrations at 0 h in the same group; †significantly different from values obtained in the same group during saline infusion at the corresponding time point based on Duncan’s multiple range test ($P < 0.05$). Data are represented as means ± SE.
plasma potassium differed between groups (main effects of time and treatment, interaction of time and group, \( P < 0.05 \); Fig. 5). Canrenoate-induced increases in potassium concentration were significant by pregnancy in pregnant ewes and by pregnancy in nonpregnant ewes. The ratio of Na\(^+\) to K\(^+\) was significantly decreased during infusion of canrenoate in both pregnant and nonpregnant ewes. However, this decrease was more pronounced in the pregnant group by the end of the infusion (pregnant 4-h saline: 33.3 ± 0.7; pregnant 4-h canrenoate: 27.4 ± 0.7; nonpregnant 4-h saline: 33.2 ± 0.3; nonpregnant 4-h canrenoate: 29.2 ± 0.7; main effects of time and treatment, interaction of treatment and time, interaction of group, treatment, and time, \( P < 0.05 \)).

Plasma protein concentration was significantly lower in the pregnant ewes (Fig. 5, main effect of group, \( P < 0.05 \)). Plasma protein was significantly decreased at 4 h in infusion of saline in both pregnant and nonpregnant ewes but did not decrease in either group during infusion of canrenoate. Packed cell volume decreased significantly over time during infusion of saline in both groups (main effect of time \( P < 0.05 \); Fig. 5). This effect was attenuated by infusion with canrenoate (interaction of time and treatment, \( P < 0.05 \)).

Stepwise regression analysis revealed that, although plasma aldosterone concentrations were related to both plasma angiotensin II and plasma K\(^+\) concentrations (\( r = 0.58 \) vs. \( r = 0.22 \); \( n = 120 \)), the relationship of plasma aldosterone concentrations to plasma potassium concentrations was greater than that to plasma angiotensin II concentrations (pregnant: slope = 140.1, \( r = 0.72 \); nonpregnant: slope = 36.4, \( r = 0.66 \); \( n = 30 \)).

DISCUSSION

The responses of the nonpregnant ewes in this study are consistent with those found in human and rat studies in which...
infusion of the MR antagonist canrenoate caused elevated plasma cortisol concentrations. In nonpregnant human subjects, the increase in cortisol and ACTH in response to intravenous canrenoate administration occurred within 1 h of administration (1, 4, 42); similarly, a bolus of canrenoate increased ACTH and corticosterone concentrations in rats within 1 h of administration (2). In nonpregnant ewes, ACTH and cortisol were increased at 1 h, although neither ACTH nor cortisol was significantly increased at later time points. In contrast, the increase in ACTH and cortisol in the pregnant ewes occurred more gradually and was significant from 2 to 4 h. Thus, the differential pattern for stimulation of ACTH and cortisol by MR antagonism between pregnant and nonpregnant ewes suggests that different sites or mechanisms are involved in the stimulation of the HPA axis. We hypothesize that the differential early (0–2 h) response pattern is due, at least in part, to a change in relative importance of central and/or pituitary MR in negative feedback control of the axis in the pregnant state.

MR are most densely expressed in the dentate gyrus and cornu ammonis of the hippocampus as well as the septum, and these MR are thought to confer the inhibitory function of the hippocampus on HPA axis regulation (33). We have found previously that progesterone is a physiological ligand for MR in sheep (35) as well as in humans (37). Progesterone shows a high affinity for MR, with only weak transactivational activity (7, 32, 37, 41). In vitro characterization of progesterone has demonstrated that, like other MR antagonists, progesterone dissociates more rapidly from the MR than do agonists such as cortisol or aldosterone. The binding of antagonists, which all lack C-21 hydroxyl groups, results in a conformational change in the receptor that does not allow binding of several nuclear receptor coactivators (14) and prevents nuclear translocation (27). Therefore, the relatively high plasma progesterone concentrations in pregnancy, which are greater than plasma cortisol concentrations, would be expected to effectively antagonize cortisol occupancy of MR in the pregnant ewe. Consistent with this effect is the observation that cytosolic MR availability was increased in hippocampal cytosol preparations from pregnant ewes compared with nonpregnant ewes. This effect occurs without an increase in either MR protein or mRNA, suggesting that fewer MR are occupied in pregnant ewes. In adrenalectomized ewes chronically treated with cortisol to levels normally measured in nonpregnant ewes appears to prevent upregulation of MR protein and mRNA. We also do not find that chronic progesterone treatment or pregnancy increases MR protein, in contrast to the effect of chronic progesterone treatment in rats (9).

![Fig. 3. Mean arterial pressure (MAP) during infusion of saline (○ and △) or canrenoate (● and ▼) in nonpregnant (○ and ●; left) and pregnant (△ and ▼; right) ewes. Overall, MAP in pregnant ewes was significantly lower than in nonpregnant ewes (P < 0.05). Values are means ± SE of 5-min bins of MAP data.](image-url)

![Fig. 4. Plasma volume measurements at the end of 4-h infusion of saline (open bars) or canrenoate (filled bars) in nonpregnant (left) and pregnant (right) ewes. Plasma volume was higher in pregnant ewes than in nonpregnant ewes but was not significantly altered by canrenoate. **Significantly different from values obtained in the nonpregnant ewes during saline infusion (P < 0.05). Values are means ± SE.](image-url)
Thus, the lack of an early increase in ACTH in pregnant ewes during MR blockade could reflect the fact that the hippocampal MR are already occupied by the endogenous competitive antagonist progesterone, thereby resulting in elevated basal ACTH and cortisol. In contrast, the transient increase in ACTH and cortisol in nonpregnant ewes may reflect greater in vivo availability of these receptors, allowing competition of the canrenoate with cortisol for these sites and thereby reducing feedback through these MR. The fact that plasma ACTH concentrations return to basal levels by 2 h of MR blockade in the nonpregnant ewes may reflect the classical delayed feedback effect at GR in pituitary and hypothalamus by these relatively high levels of cortisol (24). This transient increase in both ACTH and cortisol during infusion of canrenoate to nonpregnant ewes is different from the pattern seen in nonpregnant women with a similar infusion of canrenoate (1). However, the increment in ACTH and cortisol at 1 h was also greater in the nonpregnant ewe than in women when compared relative to the maximal adrenal production rate and free cortisol concentrations for that species, and therefore, it is more likely to exert a delayed negative feedback effect. The women were studied near the circadian nadir in HPA activity and after 6 h of fasting. Although in sheep there is no true circadian rhythm in ACTH or cortisol (3), it is not clear whether there are diurnal variations in responses to either stimulation or cortisol feedback activity that could explain these apparent between-species differences.

The delayed increase in plasma ACTH and cortisol in the pregnant ewes is not consistent with the rapid inhibitory effects of MR shown in nonpregnant rats within 23 min of bolus injection of a high dose of canrenoate (2) and in humans within 25 min to 1 h of a bolus and infusion paradigm similar to that used in these studies (1, 4). This delayed increase in HPA axis activity may instead represent a response to other stimuli. The timing of the canrenoate-induced increase in plasma ACTH in the pregnant ewes is similar to that of plasma potassium and plasma aldosterone concentrations. This suggests that the delayed increase in ACTH is stimulated by a drive to maintain
blood volume in the face of MR blockade. It has been hypothesized that pregnancy is perceived as a chronic “underfill” state (38), resulting in a chronic drive to defend the expanded blood volume. Although the decrease in plasma volume was not significant, there was a significant decrease in the plasma Na⁺ to K⁺ ratio during infusion of canrenoate. Interestingly, this decrease was significantly greater in the pregnant ewes, suggesting a more important role for renal MR in basal electrolyte balance during pregnancy. In human pregnancy, plasma aldosterone is elevated and is thought to contribute to the expansion of blood volume through actions mediated by MR. In rats, plasma aldosterone is similarly increased, and the increase in plasma volume in late gestation is associated with an increase in renal epithelial sodium channel (ENaC)-α expression and ENaC activity; the natriuretic effect of an inhibitor of ENaC activity is abolished by the MR blocker eplerenone (43). Blockade of aldosterone action by spironolactone did not alter net sodium retention or potassium excretion in late pregnancy in rats, although plasma potassium was increased and fetal number decreased (10). These results suggest that, although sodium excretion in the pregnant rat may involve factors other than MR, MR are important for normal adaptations in late gestation. Administration of MR antagonists is contraindicated in human pregnancy, and thus we do not know the relative role of MR in ACTH or volume homeostasis in normal human pregnancy.

Although progesterone is an effective MR antagonist in vitro (37) and appears to antagonize binding of cortisol and corticosterone in hippocampus (7, 36, 41), evidence suggests that only very high progesterone concentrations antagonize renal actions of aldosterone and that even the physiologically high circulating progesterone concentrations of pregnancy do not effectively antagonize the actions of aldosterone at renal MR (31). The antagonist action of progesterone on MR appears to be mitigated by other factors in the kidney; the kidney has high activity of 3 β-hydroxysteroid dehydrogenase type 2 and 17α-hydroxylase, enzymes that metabolize progesterone (6, 29), and progesterone also appears to inhibit 11βHSD2 activity in the kidney (30), potentially allowing cortisol to bind to renal MR. Therefore, in the pregnant state, the relatively high aldosterone concentration, unopposed by progesterone, results in greater MR-mediated effects in the kidney. The importance of these actions of aldosterone in the pregnant state is revealed by the profound changes in plasma electrolytes with antagonism by canrenoate. The results are also consistent with previous findings in our laboratory that the elevated basal corticosteroid concentrations of pregnancy contribute to maternal volume expansion and that lower than normal levels of aldosterone or cortisol in pregnancy disrupt maternal and fetal homeostasis (19). However, we cannot rule out the possibility that MR antagonism at nonrenal sites contributes to the increase in potassium, aldosterone, ACTH, and cortisol at 3–4 h, since central mineralocorticoid administration can affect cardiovascular end points (reviewed in Ref. 12).

The importance of aldosterone in the pregnant ewe is underscored by the dramatic increase in plasma aldosterone that results from infusion of canrenoate, which increased plasma aldosterone sevenfold over basal concentrations. The increase in plasma aldosterone was more than threefold greater in the pregnant ewes compared with the nonpregnant ewes. The increase in plasma aldosterone and plasma K⁺ during infusion of canrenoate in the ewes is in contrast to the studies in women in which neither aldosterone nor potassium significantly changed with canrenoate infusion (1, 4). It is possible that sheep differ from humans in the sensitivity of potassium excretion to MR action; however, it is also likely that the reproductive state of the subjects contributes to the observed aldosterone responses. The women were studied in the follicular phase of the menstrual cycle, whereas five of the six nonpregnant ewes were studied in the luteal phase of the estrus cycle. There was a significant correlation of the plasma aldosterone at 4 h of canrenoate with plasma progesterone concentrations (r = 0.78, P < 0.003). It has been found that women on high-sodium diets have greater increases in aldosterone during infusion of angiotensin II in the luteal phase compared with the follicular phase of the menstrual cycle (40). Aldosterone secretion from isolated zona glomerulosa cells was also stimulated by addition of progesterone to the culture media. Thus it is likely that the pregnant ewes have a greater stimulus to aldosterone secretion secondary to two factors, the rise in plasma potassium with renal MR blockade and augmentation by progesterone of potassium-stimulated aldosterone secretion. Our data suggest that the complex interactions of progesterone and corticosteroids should be considered in understanding the adaptations of the brain, pituitary, adrenal, and kidney to the pregnant state.

Overall, these results support the hypothesis that the role of MR in feedback inhibition of HPA axis activity is blunted in pregnant ewes relative to nonpregnant ewes. However, the differential HPA axis responses during the second half of the infusion likely reflect an increase in relative importance of corticosteroids, particularly aldosterone, in homeostatic mechanisms maintaining hemodynamic end points such as maternal plasma volume and blood pressure in pregnancy.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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