A Role for Mineralocorticoid Receptors in the Physiology of the Ovine Fetus: Effects on ACTH and Lung Liquid Composition

MAUREEN KELLER-WOOD, CHARLES E. WOOD, JARRET MCCARTNEY, NATHAN M. JESSE, AND DANA PERRONE

*Departments of Pharmacodynamics [M.K.-W., J.M., D.P.], Physiology and Functional Genomics [C.E.W.], and Pediatrics [N.M.J.]
University of Florida, Gainesville, Florida 32610*

ABSTRACT: In the human and ovine fetus, the presence of 11β -hydroxysteroid dehydrogenase 1 allows cortisol and other corticosteroids to act at mineralocorticoid receptors (MRs) in lung and brain. To test the physiologic role of MRs in the late gestation fetus, fetal lambs were infused with a specific MR antagonist for 12 h. Infusion of the MR antagonist significantly increased plasma ACTH and cortisol concentrations. Infusion of the MR antagonist also significantly increased fetal P_{CO_2} and hematocrit, and decreased fetal pH, but did not alter fetal heart rate or blood pressure. Infusion of the MR antagonist altered the ratio of Na^+ to K^+ in lung fluid but did not alter the rate of production of lung liquid or the expression of the epithelial sodium channel α or of the Na,K ATPase $\alpha 1$ in lung. These results suggest that corticosteroids act at MR to regulate ACTH and blood volume and modulate lung fluid composition in the fetus, but basal levels of corticosteroids do not alter lung liquid production rate through effects on MR. (*Pediatr Res* 69: 491–496, 2011)

Corticosteroids exert important maturational and activation effects in fetal tissues at term, including influencing the maturation of the liver, lungs, pancreas, and gastrointestinal tract, and effects on the brain, adrenal medulla, and adrenal cortex (1). In the course of normal gestation, these effects are stimulated by the increase in fetal adrenal secretion of cortisol, which precedes delivery. These effects can be induced through glucocorticoid receptor (GR) activation by synthetic glucocorticoids (2–6).

Before the time of fetal adrenal maturation, fetal cortisol levels are appreciably lower and are derived primarily from transplacental passage of maternally produced cortisol (7,8). These low plasma cortisol concentrations are within the range expected to cause greater occupancy and action *via* the mineralocorticoid receptor (MR) than GR (9). In humans and sheep, MR are expressed before birth (10–12) and in fetal lung, hypothalamus, and hippocampus, the reductase activity of 11β hydroxysteroid dehydrogenase 1 (11β HSD1) predominates over the cortisol-inactivating dehydrogenase activity of 11β HSD2 (13). Therefore, cortisol and the classical mineralocorticoids aldosterone and 11-deoxycortisol could bind to MR in the ovine fetal brain and lung and exert physiologic effects before the time of the periparturient surge in cortisol production by the fetal adrenal. We hypothesize

that low levels of corticosteroids do have effects in the fetus; in the late gestation, fetal heart MRs are involved in the enlargement produced by cortisol (14). These studies were designed to test the hypothesis that endogenous corticosteroids act at MR to regulate ACTH and lung liquid composition or production in the late gestation fetus at an age at which MR are abundantly expressed in both hypothalamus and lung (10,11).

METHODS

Experimental protocol. These experiments were approved by the University of Florida IACUC. Ewes with singleton fetuses (113–122 d gestation) were prepared with catheters in the fetal tibial arteries, saphenous veins, amniotic space, and maternal femoral arteries and veins. For sampling of lung liquid, two nonocclusive catheters were placed in the fetal trachea, one advanced toward the lung and one toward the larynx (15,16); these were joined to form a circuit to allow free flow of fluid before the lung liquid sampling period.

Ewes were treated with ampicillin (750 mg Polyflex; Fort Dodge, IA), and body temperature was monitored for 5 d postoperatively. Animals were subsequently studied during an 18-h period, ending on 120–132 d gestation (126 ± 2 ; fetal body weight 2.96 ± 0.17 kg). Maternal and fetal blood samples for blood gases, plasma hormones, electrolytes, and osmolality were collected before the start of the fetal i.v. infusion of MR antagonist (RU26752; Sigma Chemical Co.-Aldrich, St. Louis, MO; 1.68 mg/h in 30% ethanol-70% saline, $n = 8$) or control infusion (saline, $n = 4$ and ethanol in saline, $n = 4$). The infusions were delivered at 0.57 mL/h beginning at 1800 h, 6 h before “0” time, to allow for the dead space of the fetal venous catheter (3.1 mL). The dose of RU26752 (20 mg/12 h) was chosen as 20-fold excess relative to cortisol, assuming maternal cortisol production of 1 mg/kg/d and 1.4% maternal-to-fetal transfer (7). This dose is 4- to 5-fold the dose of spironolactone used to block infused aldosterone in the fetus (17); spironolactone and RU26752 have similar inhibitory concentrations at MR (18). The timing was chosen based on preliminary studies and an expected half-life of 1–2 h of the drug. The total dose of ethanol infused was 2 mL/12 h. Fetal ACTH does not increase after infusion of 0.75 g/kg/h ethanol (19), and 0.5 g/kg/8 h ethanol was shown not to alter blood gases in the fetus (20). Infusion of ethanol in our study produced similar responses as infusion of saline alone.

After 8 h of infusion, ewes were loaded into a metabolic cart; one end of the tracheal catheter was attached to a sterile reservoir to allow sampling of lung liquid volume. Lung liquid production from 10 to 12 h was determined by dye dilution using blue dextran as previously described (15,16). Dye dilution was calculated in samples (0.5 mL) collected at 10-min intervals; additional fluid (0.3 mL) was collected each 30 min for measurement of electrolyte concentrations. The initial volume of lung fluid was determined from the dilution of the dye in the 10 h sample; lung liquid volume at each time was calculated from the dye absorbance at 620 nm taking into account the volume of dye injected, dye concentration, and the sample volumes. The

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Correspondence: Maureen Keller-Wood, Ph.D., Department of Pharmacodynamics, University of Florida, Box 100487, Gainesville, FL 32610; e-mail: kellerwd@cop.ufl.edu

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Abbreviations: CRH, corticotropin releasing hormone; ENaC, epithelial sodium channel; GR, glucocorticoid receptor; HPA, hypothalamo-pituitary-adrenal; MR, mineralocorticoid receptor; PCV, packed cell volume; POMC, proopiomelanocortin; PVN, paraventricular nucleus; SGK, serum and glucocorticoid kinase

rate of lung liquid production was calculated as the slope of the volume *versus* time relationship for each fetus.

Fetal blood pressure, amniotic pressure, and heart rate were measured from 9–12 h of infusion (Lab View; National Instruments, Austin, TX). Fetal arterial blood samples were collected every 30 min from 9 to 12 h (0.75 mL) for measurement of fetal PO_2 , PCO_2 , pH (Radiometer ABL77; Copenhagen, Denmark), osmolality (Precision Instruments Inc, Natick, MA), and electrolytes (Roche 9180 analyzer). Packed cell volume (PCV) was also measured (International Equipment, Needham, MA). Blood (5.5 mL) for hormone analysis was collected at 9, 10, 11, and 12 h from the fetus (total volume of blood 27.5 mL over 3 h) and at 10 and 12 h from the ewe (EDTA Vacutainer tubes; BD, Franklin Lakes, NJ); aliquots of plasma were frozen for subsequent analysis of hormone concentrations. Plasma ACTH was measured by RIA (16). Plasma cortisol was measured using ELISA (Oxford Biomedical Research Inc, Oxford, MI). Plasma aldosterone was measured by RIA (Diagnostic Products, Los Angeles, CA).

One fetus became severely hypoxic and died during the last hour of MR antagonist infusion; data from that fetus were excluded from the analysis. Data from one severely hypoxic fetus in the MR antagonist group and two fetuses in the control group (multiple $\text{PO}_2 < 16$ mm Hg before infusion) were also excluded from analysis. In two fetuses, one per group, lung liquid did not properly mix with dye, and lung liquid production data were also excluded from analysis. Therefore, the sample sizes for lung liquid analysis are five per group, whereas the sample sizes for plasma hormone, plasma electrolytes, and blood gas data are six per group.

Gene expression after infusion of MR antagonist. Immediately after the 12 h sample, the ewe was killed (Virbac Inc, Fort Dodge, TX), and fetal tissues (hypothalamus, pituitary, and lung) were removed and frozen in liquid nitrogen. RNA was extracted from lung, a hemisected hypothalamus, and whole pituitary; real-time PCR was performed (10,11). MR, GR, proopiomelanocortin (POMC), the MR target genes (21,22), sodium-potassium ATPase $\alpha 1$ (Na,K ATPase $\alpha 1$), the epithelial sodium channel (ENaC α), and serum and glucocorticoid-regulated kinase1 (SGK1) mRNA were measured in lung ($n = 5$ /group). MR, GR, corticotropin-releasing hormone (CRH), AVP, and β -actin mRNA were measured in hypothalamus, and MR, GR, POMC, and β -actin mRNA were measured in pituitary ($n = 6$ /group). All samples for each gene, including no template control, were run on the same plate. The expression of each gene was quantified relative to the expression of β -actin in the same sample and analyzed by the ΔCt method. ΔCt values were used for statistical analysis. Fold changes were calculated as $2^{\Delta\Delta\text{Ct}}$ using the mean value of the control group.

ENaC, Na,KATPase, and SGK protein in lung. The expression of the ENaC α and SGK1 proteins was quantified in whole cell and membrane-enriched fractions prepared from fetal lungs using previously described immunoblot techniques and validated antibodies (ENaC α , 3464, 1:100; Ab-Cam, Cambridge, MA; Na,K ATPase, MA3-929 1:10,000; Affinity BioReagents, Golden, CO; SGK1, sc-15885, 1:1000; Santa Cruz Bioreagents, Santa Cruz, CA; β -actin, A-5441, 1:20000; Sigma Chemical Co.-Aldrich) (11). Single aliquots of each sample were run in gels containing samples from both groups; all blots for a given protein were run and developed simultaneously using the same reagents. The blots were analyzed with a Bio-Rad Chemi-Doc system and Quantity One software (Bio-Rad, Hercules, CA); the ENaC α , Na,K ATPase $\alpha 1$ (100 μg protein/sample) and SGK1 (90 μg protein/sample) density results were expressed as the OD units normalized to the corresponding OD of the β -actin band on the same immunoblot.

Immunohistochemistry of MR and GR in fetal hypothalamus. Sections of fetal hypothalamus collected in a previous study from four control fetuses of 122–128 d were used to determine whether MR and GR colocalize in the fetal hypothalamus. Hypothalami were collected in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm . Sections were subjected to antigen retrieval (microwaving in sodium citrate buffer 0.1 M), blocked with blocking buffer (5% milk, 5% goat serum, 0.2% SDS in 0.05 M Tris pH 7.6, for 1 h), and incubated with GR primary antibody (M20, sc-1002, 1:100; Santa Cruz Bioreagents) in blocking buffer for 1 h, then with goat anti-rabbit IgG coupled to Alexa-Fluor 544 (1:500; Invitrogen, Carlsbad, CA) for 1 h. Slides were reblocked and incubated with anti-MR primary antibody (M1-18 6G1, 1:40; Elise Gomez-Sanchez; Ref. 23) overnight at 4°C in blocking buffer with 0.05% Tween 20 (Sigma Chemical Co. Aldrich), then with secondary antibody (goat anti-mouse IgG coupled to AlexaFluor 488; 1:500; Invitrogen). Sections treated with all reagents except primary antibodies were used as negative control; positive controls were fetal hippocampal sections from the same fetuses. Sections were viewed and photographed using an Olympus BX41 system microscope with DP-71 camera and DP-BSW Olympus imaging software (Olympus America Inc., Center Valley, PA).

Data analysis. Changes in plasma ACTH, cortisol, aldosterone, blood gases, PCV, plasma and lung fluid electrolytes, and lung liquid volume over time were analyzed by two-way ANOVA corrected for repeated measures

over time. Differences in gene and protein expression, initial lung liquid volume at 10 h, and lung fluid ratio of Na to K were analyzed by *t* test. Differences in the rate of lung liquid production (mL/h) were analyzed by Mann-Whitney *U* test as this variable was not normally distributed. The criteria for significance for all tests were $p < 0.05$.

RESULTS

Effects on the hypothalamo-pituitary-adrenal axis. Treatment with an MR antagonist significantly increased ACTH and cortisol (Fig. 1). Plasma ACTH was significantly greater in MR antagonist-treated fetuses than in control fetuses at 12 h. In addition, in the MR antagonist-treated fetuses, ACTH concentrations at 12 h were greater than in the same fetuses before treatment. In contrast, there was no significant increase in plasma ACTH in the control fetuses. Similarly, plasma cortisol concentrations at 11–12 h were greater in the MR antagonist-infused fetuses than in control fetuses, and cortisol increased significantly over time in the fetuses treated with MR antagonist, but not in control fetuses. There were no differences in plasma aldosterone concentrations in the fetuses treated with the MR blocker compared with the control fetuses (control fetuses: 137 ± 33 pg/mL pretreatment and 191 ± 34 pg/mL at 12 h; MR antagonist-treated fetuses: 189 ± 32 pg/mL pretreatment and 221 ± 32 pg/mL at 12 h).

Expression of CRH in the hypothalamus was not significantly changed by administration of the MR antagonist, nor was expression of POMC, MR or GR in the pituitary (Fig. 2). However, there was a significant decrease in GR and AVP mRNAs in the hypothalamus of MR antagonist-treated fetuses (Fig. 2). Immunofluorescence staining of hypothalamus showed coexpression of MR and GR in cells, these were primarily localized to the periventricular area of the paraventricular nucleus (PVN; Fig. 3).

Figure 1. Plasma ACTH (A) and cortisol (B) concentrations in control fetuses (○) and fetuses infused with MR antagonist, RU 26752 (●). Data are shown as mean $\pm$ SEM; * indicates values in the treated fetuses greater than those in the control group at the same time; † indicates values different from 0 h.

Figure 2. Gene expression in hypothalamus (A–D) and pituitary (E–G) in control fetuses (□) and fetuses infused with MR antagonist (■). (A and E) GR; (B and F) MR; (C) CRH; (D) AVP; (G) POMC; * indicates different from control. Data are mean fold changes $\pm$ SEM relative to the mean in the control group.

Figure 3. Localization of GR (red) and MR (green) in PVN (A–D) and hippocampus of the fetus. Nuclei are stained with blue with DAPI. Examples of colocalization of MR and GR (yellow or orange) are indicated with white arrows. Inset in (A) is from a control section with application of all reagents except the primary antibodies. Bars represent 200 μ m for A–C and 100 μ m for D and E.

Several factors related to fetal homeostasis were also changed in the MR antagonist-treated fetuses (Fig. 4). P_{CO_2} was significantly increased, and pH was significantly decreased in fetuses after 11.5 and 12 h of MR antagonist infusion compared with control fetuses. PCV was significantly increased at 9–12 h of infusion of MR antagonist, although the MR antagonist did not significantly alter either fetal blood pressure or heart rate (blood pressure at 9–12 h in control: 43.7 ± 2.6 mm Hg compared with MR antagonist: 44.8 ± 1.9 mm Hg; heart rate at 9–12 h in control: 167.7 ± 1.7 min^{−1} versus MR antagonist: 156.1 ± 6.5 min^{−1}). Infusion of the MR antagonist did not alter fetal plasma electrolytes or plasma Na/K ratio.

Effects on fetal lung. Lung liquid production over time was not altered in fetuses infused with RU26752 (Fig. 1); the rate of lung liquid production (slope of the volume versus time relationship) was also not altered (control: 27.0 ± 11.4 mL/h; MR antagonist: 16.8 ± 4.5 mL/h). However, the electrolyte content of lung liquid was significantly altered by infusion of

the MR antagonist (Fig. 5 and Table 1). The ratio of Na⁺ to K⁺ in lung liquid was significantly greater in the fetuses treated with MR antagonist. Lung liquid osmolality was not significantly different in MR antagonist-infused fetuses. There were no significant effects of MR antagonist on expression of any genes measured in fetal lung, including MR, GR, POMC, ENaC α , Na,K ATPase α 1, or SGK1, nor were whole cell content of ENaC α , Na,K ATPase α 1, or SGK1 protein, or membrane content of ENaC α proteins altered by MR antagonist infusion (Table 1).

DISCUSSION

The results demonstrate actions of endogenous corticosteroids at MRs in fetal sheep. Infusion of a MR antagonist increased plasma ACTH and cortisol, suggesting a role of MR in determining hypothalamo-pituitary-adrenal (HPA) activity in the fetus. Infusion of a MR antagonist did not alter basal lung liquid production, but did increase the Na/K ratio in lung liquid, suggesting that corticosteroids play a role in the composition of lung liquid.

MR antagonists have been shown to increase ACTH concentrations in rats (24) and humans (25,26), indicating that MR are involved in negative feedback control of ACTH. Presence of a similar negative feedback by MR in the fetus is consistent with the expression of MR in the ovine fetus in brain areas involved in regulation of the HPA, including the hippocampus, hypothalamus, and brainstem (10).

There was no change in either pituitary or pulmonary expression of POMC, nor were hypothalamic CRH or AVP mRNAs increased. However, we cannot rule out the possibility that small changes in expression in a discrete subgroup of cells were diluted in our analysis. It is also likely that the acute nature of the stimulus to ACTH increased hormone secretion without altering transcription. Although 6 h of hypoxemia stimulated CRH mRNA (27), more acute hypoxia stimulates ACTH without increasing CRH mRNA in hypothalamus (unpublished results, Wood laboratory).

Figure 4. Plasma sodium (A), potassium (B), PCV (C), PO₂ (D), PCO₂ (E), and pH (F) values in control fetuses (○) and fetuses infused with MR antagonist (●). Data as given in Fig. 1.

Figure 5. Lung liquid volume (A) and lung liquid production (B) in control fetuses (○) and fetuses infused with MR antagonist (●). Data as given in Fig. 1.

Table 1. Effects of MR antagonist treatment on the fetal lung

	Control fetuses	MR antagonist-treated fetuses
Lung liquid production (mL/2 h)	27.0 ± 11.4	16.8 ± 3.8
Lung liquid Na/K ratio	30.2 ± 1.7	34.7 ± 1.6*
Lung ENaCa protein		
Whole cell homogenate	0.11 ± 0.05	0.07 ± 0.03
Lung ENaCa protein		
Membrane fraction	0.38 ± 0.06	0.41 ± 0.05
Lung Na,K ATPase α1 protein		
Whole cell homogenate	1.40 ± 0.23	1.33 ± 0.17
Lung Na,K ATPase α1 protein		
Membrane fraction	5.82 ± 0.62	5.09 ± 0.47
Lung SGK protein	0.74 ± 0.18	0.87 ± 0.09
Lung ENaCa mRNA	1.08 ± 0.17	1.37 ± 0.38
Lung SGK1 mRNA	1.06 ± 0.16	0.98 ± 0.20
Lung Na,K ATPase α1 mRNA	1.12 ± 0.25	1.36 ± 0.21
Lung GR mRNA	1.07 ± 0.17	0.88 ± 0.22
Lung POMC mRNA	1.07 ± 0.20	1.60 ± 1.00

Data are expressed as mean ± SEM. Values for protein are expressed as optical density relative to that of β-actin. Gene expression is expressed as fold changes relative to the mean for the control fetuses.
* Value different than control fetuses.

GR expression in the hypothalamus was decreased in the fetuses after MR antagonist treatment, suggesting the possibility that increased ACTH could result from decreased feed-

back at GR and MR. We found appreciable MR expression in the late gestation ovine fetal hypothalamus and MR and GR protein coexpressed within parts of the PVN; there is also colocalization of MR and GR in parvocellular neurons of the adult rat PVN (28). It is known that chronic increases in cortisol cause down-regulation of GR; however, GR down-regulation has generally been shown to occur with chronic and sustained high levels of cortisol. The modest, acute changes in cortisol in the current studies would not be expected to alter GR expression; physiologic increases in cortisol do not seem to alter pituitary GR expression in the late gestation fetal sheep (29), and there is no decrease in hypothalamic GR expression at term (10). The unexpected decrease in GR may contribute to decreased feedback and increase HPA activity.

Our data also suggest that the MR blockade increased stimuli of ACTH. Both fetal PCV and fetal Pco₂ significantly increased and pH decreased after 10–12 h of MR antagonist infusion. Acidemia and hypercapnia stimulate ACTH secretion in late gestation fetuses, although the magnitude of the changes in Pco₂ and pH which alters ACTH is greater than the change in this study (30,31). The changes in blood gases are also reminiscent of the changes during a progressive hemorrhage in late gestation fetal sheep (32). However, in contrast to progressive hemorrhage, the increase in ACTH occurs with relatively modest blood withdrawal (<10% of blood volume), without changes in blood pressure or heart rate. Using the PCV values to calculate changes in blood volume (33) and assuming an initial blood volume of 110–160 mL/kg (16,33), we calculated significant differences in the change in blood volume in the control *versus* MR blocker-treated fetuses at 11 h (Δvol of + 8–10 mL/kg in control *versus* –3 to –5 mL/kg after MR blocker). Although actions *via* MR in the fetal cardiovascular system have not been characterized, our data suggest that there may be changes in placental blood flow in the fetus during MR blockade, which result in an exaggerated hypercapnic response to modest blood withdrawal. MR are expressed in fetal brainstem (10), suggesting that MR blockade could alter responses to volume loss. If this is the case, then the increase in ACTH and cortisol may occur

secondary to changes in blood flow and/or regulation of fluid balance, rather than simply by diminution of cortisol feedback. We cannot distinguish the relative importance of these effects in these studies.

Plasma aldosterone concentration did not change in fetal plasma during infusion of MR antagonist. However, plasma aldosterone concentration is not an index of MR blockade; MR do not directly alter renin or angiotensin secretion, and even infusion of a high dose of aldosterone does not alter plasma sodium in the fetus (34). In the fetus, plasma renin and angiotensin levels are high, but the fetal adrenal is relatively unresponsive to increases in angiotensin (35).

We found that AVP expression was decreased in the hypothalamus after administration of the MR antagonist. Increased cortisol levels suppress plasma AVP (36). In fetal sheep, cortisol decreased AVP mRNA in the parvocellular, although not the magnocellular, PVN (37). The reduction in AVP expression may therefore be secondary to the increased cortisol concentrations at 10–12 h.

Our results suggest that MR effects in the fetal lung are relatively minor compared with the GR-mediated effects at term (38). The endogenous levels of cortisol in the 120–130 d fetus may not be sufficient to activate gene transcription or MR may not regulate these genes in the fetal lung. In the late gestation fetus, MR-mediated actions have been demonstrated (17,34,39). In the postnatal kidney, effects of aldosterone are thought to be mediated by induction of SGK, ENaC, and Na,K ATPase (21,40). However, the mechanism of MR action in the lung may differ from that in the kidney, as aldosterone does not seem to affect SGK in the adult lung (22). MR and GR activate gene transcription through glucocorticoid response elements, which have been identified in the ENaC α promoter (41). Synthetic glucocorticoids cause a robust induction of ENaC and Na,K ATPase (42–44). However, for other genes, cortisol binding at MR induces less transcriptional activation than does aldosterone binding to MR or cortisol binding to GR (45), consistent with an absence of corticosteroid effect on “classical” MR-induced genes in fetal lung. The effect on Na/K ratio in lung liquid suggests the possibility of a non-genomic MR effect on sodium conductance in the fetal lung. Absence of MR in knockout mice results in decreased sodium conductance without decreasing ENaC expression (46). Our results support a physiologic role of MR in altering composition of the fluid secreted as lung liquid without a significant effect on ENaC transcription.

Our results demonstrate that then normal low level of fetal corticosteroids act at MR in the ovine fetus and play a role in ACTH responses to mild stress. This effect limits the increase in fetal ACTH in the period before the fetal adrenal secretes appreciable quantities of cortisol. The results also suggest that MR play a role in fluid balance and lung liquid composition in fetal life. The fetal HPA axis itself is stimulated by changes in fluid homeostasis and changes in blood gases and contributes to fetal homeostatic responses to hypercapnia and to volume perturbations. Corticosteroid action at MR, therefore, is likely to be an important component of the endocrine control of fluid balance and the normal preterm suppression of ACTH.

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