Expression of ENaC subunits, chloride channels, and aquaporins in ovine fetal lung: ontogeny of expression and effects of altered fetal cortisol concentrations

Nathan M. Jesse, Jarret McCartney, Xiaodi Feng, Elaine M. Richards, Charles E. Wood, and Maureen Keller-Wood

Departments of 1Pediatrics, 2Pharmacodynamics, and 3Pharmacodynamics, Colleges of Medicine and Pharmacy, University of Florida, Gainesville, Florida

Submitted 27 February 2009; accepted in final form 5 June 2009

The ovine fetal adrenal, like the human, gradually increases its mineralocorticoid receptor (MR), which has high affinity for cortisol, and by corticosteroids (7, 43). The sodium channel subunits is regulated both developmentally and by corticosteroids (7, 43).

In previous studies in our laboratory we demonstrated expression of the receptors for the adrenal corticosteroids, the mineralocorticoid receptor (MR), which has high affinity for cortisol, and the lower affinity glucocorticoid receptor (GR) in the late gestation ovine fetal lung. The fetal lung has low expression and activity of the cortisol inactivating enzyme 11β hydroxysteroid dehydrogenase activity (11β HSD2) (18, 50), allowing cortisol binding to both receptors in the fetal lung. MR expression is considerably greater in the distal lung of the fetus than in the newborn or adult (19), suggesting that MR may play a role in the secretion of cortisol in the lungs at the time of birth. In renal epithelia in adult animals, aldosterone action at MR induces ENaC channel activity by direct effects on ENaC mRNA transcription (31), indirect effects on transcription through the early intermediate gene sgk1 (8), and effects on ENaC localization in the apical membrane (44). The ovine fetal adrenal, like the human, gradually increases its production of cortisol in late gestation, followed by a dramatic

37 wk, but before the onset of labor, develop respiratory distress (17), indicating that both maturity and labor influence the clearance of lung fluid.

The secretion of lung liquid into the airways is thought to involve chloride secretion across the lumen epithelium by chloride channels, primarily the chloride channel 2 (CLCN-2) and the cystic fibrosis transmembrane conductance regulator (CFTR). At the time of birth, the reabsorption of sodium establishes an osmotic gradient for water reabsorption between cells or through aquaporins (AQP); in the ovine fetal lung AQP1 is expressed on vascular endothelium and AQP5 is expressed on alveolar type I cells (24). The reabsorption of sodium occurs through epithelial sodium channels (ENaC) expressed on the luminal surface of pulmonary epithelium and basal sodium-potassium active transport pumps (Na⁺,K⁺-ATPase) expressed on the basal surface (32). The epithelial sodium channel is composed of three subunits, α, β, and γ. Although deletions of β- and γ-subunits slow liquid reabsorption, these deletions do not cause respiratory distress; in contrast, deletion of the α-subunit leads to respiratory failure (14). Channels composed of the α-subunit alone produce nonselective cation channels, whereas channels composed of the β- and/or γ-subunits have little or no functional activity in the absence of the α-subunit, and optimal sodium conductance appears to require channels constructed of all three subunits (6, 16). It is not clear whether the ratio of these subunits is normally 2α:1β:1γ or 3α:3β:3γ (10, 11). The expression of the sodium channel subunits is regulated both developmentally and by corticosteroids (7, 43).

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THROUGHOUT MOST OF GESTATION the fetal lung acts as a secretory organ, producing lung liquid, which is essential for normal lung development and contributes to amniotic fluid volume. At the end of gestation reversal of this process from secretion to reabsorption is critical in the normal transition to extraterine life, and delayed clearance of fluid in the airways contributes to the pathophysiology of respiratory distress syndrome and transient tachypnea of the newborn (17). The incidence of transient tachypnea of the newborn has been shown to be inversely related to gestational age at delivery and is influenced by the mode of delivery with infants born via caesarean section at greater risk than those born vaginally. Approximately 6% of term neonates born by elective repeat caesarean section after term neonates born by elective repeat caesarean section after...
increase in cortisol production in the last few days before birth, and a further increase in cortisol production during active labor and vaginal delivery (36), suggesting that actions mediated by the adrenal steroids may also induce changes in sodium channels over the last several weeks of gestation in the ovine fetus.

In the present study, we have determined the ontogeny of the chloride channels CLCN2 and CFTR and the water channels AQP1 and AQP5 during late gestation in the ovine fetus and have quantitatively compared the expression of the genes for the ENaCα, ENaCβ, and ENaCγ subunits of the epithelial sodium channel. We have also determined the expression of both mature and immature forms of the ENaCα and -β subunit protein isoforms in lung membrane fractions as well as in whole cell homogenates. Both rodent and sheep models have previously been used to assess developmental changes in the various channels involved in fluid secretion or reabsorption. CFTR expression decreases and AQP expression increases in late gestation fetal sheep (4, 24). We have previously found distinct ontogenetic patterns of expression of ENaCα and Na\(^{+}\),K\(^{+}\)-ATPase\(_{1}\) subunit mRNAs and protein in the ovine fetal lung compared with the ovine fetal kidney or small intestine (18); these previous results indicate earlier increases in expression of these sodium transporters in lung than in kidney or small intestine. In contrast, in the fetal rat, expression of ENaC subunits and AQP1 in both lung and kidney occurs in the last days of fetal life or postnatally (21, 40, 41, 45, 46). In the present study, our determinations were made over the last 45% of gestation, spanning the developmental window from the cœniural phase through the alveolar phase and including samples collected from lambs during active labor and first days of postnatal life. We hypothesize that the changes in expression of genes critical for reabsorption, such as the sodium channel and AQPs, will be most pronounced in the fetuses of near-term and laboring ewes, and that expression of genes linked to secretory mechanisms, such as the chloride channels, will be lowest in late gestation. We also hypothesize that there is differential induction of the three subunits of ENaC during late gestation with greater expression of ENaCα mRNA at term than in the midgestation fetus, reflecting formation of active sodium channels near the time of birth. We further hypothesize that physiologic increases in cortisol, similar to those in the preterm fetus in late gestation but before the preparturient surge in cortisol, would significantly increase expression of ENaCα mRNA as it does in the postnatal kidney. Previous studies have suggested that high doses of cortisol or maternal or fetal antenatal glucocorticoid treatment induce AQP1 and AQP5 expression in ovine fetuses (24) and ENaC expression in rat fetuses (41); however, the effect of smaller increases in cortisol, potentially acting through MR, has not been studied in the late gestation ovine fetus.

**METHODS**

**Ontogenetic patterns of genes in fetal and neonatal lung.** To determine the ontogenetic pattern in expression of ENaC subunits, chloride channels and AQPs, time-dated pregnant ewes between 80 and 146 days gestation were killed with an overdose of pentobarbital and phenytoin (Euthol solution; Virbac AH, Fort Worth, TX) administered intravenously [80 days, n = 4–5; 96–100 days, n = 4; 120 days, n = 4; 130 days, n = 4; 143–146 days (near-term), n = 5]. None of these ewes showed any signs of labor. This range of fetal ages was chosen to include the period of development of the fetal lung from the end of the canalicular phase through the alveolar phase to the time of birth (1). Data regarding lung, small intestine, and renal medulla and cortex expression of MR, GR, 11β HSD1 and 2, Na\(^{+}\),K\(^{+}\)-ATPase\(_{1}\), ENaCα, and sgk1 from this set of animals have also been previously reported (18). Four additional ewes and their fetuses were also killed while in labor (142–148 days gestation) (labor, n = 5 fetuses), and newborn lambs (n = 5) were also killed in the first 48 h after delivery to determine the effects of labor and the associated increase in cortisol, and the changes in the immediate postpartum period after the high levels of cortisol have subsided and lung MR expression is decreased. In all cases portions of distal lung were rapidly dissected and frozen in liquid nitrogen and then stored at −80°C until use. Our protocol and all animal care and handling were approved by the Institutional Animal Care and Use Committee at the University of Florida.

RNA was extracted from the lung samples using Trizol reagent (GIBCO/BRL, Grand Island, NY) as previously described (18) and stored at −80°C. cDNA was subsequently synthesized using a high-capacity cDNA archive kit (Applied Biosystems; Foster City, CA) and stored at −20°C until use.

Gene expression was determined using quantitative real-time PCR using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA) and Taqman probes and primers. Probes and primers for ENaCα, ENaCβ, and ENaCγ were designed from ovine sequences (accession no: ENaCα, AF232715; ENaCβ, AF065146; ENaCγ, AF250862) and CLCN2 probe and primers were designed from the bovine sequence (accession no. AJ786345.1) using Primer Design software (Applied Biosystems). CFTR (4) and AQP (24) probes and primers have previously been described by others.

To quantitatively compare expression of ENaCα, ENaCβ, and ENaCγ mRNAs, we also included standard curves for ENaCα, -β, and -γ in the PCR plates in which expression in lung samples for each of these genes was determined. The standards used were a 100-bp sequence of ENaCα or ENaCγ, or a 110-bp sequence of ENaCβ. These sequences included the 67-bp ENaCα amplicon, the 71-bp ENaC γ amplicon, or the 75-bp ENaCβ amplicon, and produced standard curves that were linear and parallel over the range of standard and samples (10\(^{5}\) to 10\(^{6}\) molecules per reaction with efficiencies of 82.2 ± 1.5% for ENaCα, n = 4; 83.6 ± 3.5% for ENaCβ, n = 4; and 87.8 ± 6.2% for ENaCγ, n = 3).

**Ontogeny of ENaCα and -β subunit protein and its localization in the membrane fraction.** The appearance of functional sodium channels requires the localization of the mature forms of the ENaC subunits to the membrane of the alveolar cell. To determine the ontogenetic patterns in expression of the mature subunits for ENaCα and -β subunits, whole cell homogenates and membrane-enriched fractions were prepared from lung samples from the same fetuses and newborns as were used for determination of gene expression. Western blot analysis was performed as previously described (18), using specific antibodies against the α-subunit (used at 1:100, catalog no. 3464; AbCam, Cambridge, MA) and β- and γ-subunit (used at 1:1,000, catalog no. ENaCβ1-A; Alpha Diagnostics, San Antonio, TX) of ENaC, or against β-actin (catalog no. A-5441, used at 1:20,000; Sigma, St. Louis, MO). For blots of both ENaC subunit antibodies, specificity of the bands was confirmed in experiments in which the antibody was preabsorbed to the immunizing antigen. The blots were analyzed with a Chemi-Doc system and Quantity One software (Bio-Rad, Hercules, CA) and the ENaCα and -β density results were expressed as the optical density units normalized to the optical density of the β-actin band. In the case of ENaCα, specific bands were detected at ~68 kDa, as well as a higher molecular weight bands at ~100 kDa in whole cell and membrane preparations. Both of these bands were frequently visualized as doublets; both bands are dramatically reduced in intensity by preabsorption with immunizing peptide, indicating that they are specific. These bands may represent alternative glycosylations of the protein, as six different sites have been predicted (13). Our analysis of the immunoblots for ENaCα therefore included a volume
encompassing molecular weight of the doublet bands of ~100 and 68 kDa in each lane. For ENaCβ two bands of ~112 and 102 kDa were detected in both whole cell and membrane-enriched preparations. In transfected canine kidney cells it has been shown that all three ENaC subunits exist as both mature and immature protein forms in the membrane and in whole cell preparations (13). In the case of ENaCα, maturation results in a lower molecular weight form (65 vs. 95 kDa), whereas the mature form of the ENaCβ subunit is increased in weight (110 vs. 96 kDa). Thus we imaged and analyzed these two sets of bands with the assumption that the 112 kDa band of ENaCβ and the 68 kDa band(s) of ENaCα correspond to the mature forms of each subunit, whereas the 102 kDa band of ENaCβ and the 100 kDa bands of ENaCα correspond to the immature forms of each subunit.

Effects of altered fetal cortisol concentrations. To determine the effect of plasma cortisol on expression of ENaCα and Na⁺,K⁺-ATPase subunits in the fetal lung, samples from distal lung were also collected and analyzed from twin pregnancies in which one twin was treated with cortisol to produce circulating concentrations within the range observed in late gestation, but before the time of the final surge in subunits in the fetal lung, samples from distal lung were also collected.

In mRNA was calculated as 2⁻ΔΔCt, where ΔΔCt is the difference between the mean Ct for each gene and the mean Ct for β-actin from the same sample. Changes in gene expression over fetal and postnatal age were analyzed by one-way ANOVA using the Dunnett’s test, as appropriate. For graphical purposes, the fold change between ages were compared by Newman-Keuls Studentized range test.

On gestation, the level of ENaCβ protein as detected by immunoblot was lower in late gestation lung samples than in 80 days samples (Fig. 3, bottom). Both 102 and 112 kDa proteins in lung whole cell homogenates tended to decrease with advancing age; however, in lung membrane only the 102 kDa form, representing the immature form, significantly decreased with age. Despite the overall decrease in ENaCβ subunit proteins with maturation, there was significantly greater density of the mature form of ENaCβ compared with the immature (102 kDa) form in the near-term and newborn whole cell and membrane extracts. The ratio of the density of the mature 112-kDa band to the immature 102-kDa band was significantly increased in the near-term lungs in both membrane and whole cell homogenates (9.85 ± 15.13 at 145 days and 0.52 ± 0.21 at 80 days in whole cell homogenates; 58.85 ± 103.85 at 145 days and 0.68 ± 0.27 at 80 days in membrane). We have previously reported that the mature form of ENaCβ protein is increased in both whole cell and lung membrane extracts in late gestation, and decreases after birth (18). The weaker immature band at ~100 kDa for ENaCα observed in the whole cell homogenates did not significantly change with gestational age, and there was no appreciable immature ENaCα in membrane at any age after 80 days (Fig. 4, top). The ratio of the mature 68-kDa band to the immature 100-kDa band also did not significantly change with age.

Effects of cortisol on ENaC subunit, Na⁺,K⁺-ATPaseα1, and AQ expression. As expected, cortisol implants increased plasma cortisol in the treated fetuses to concentrations significantly greater than those in the control fetuses (14.3 ± 2.4 nM in cortisol-treated twin compared with 2.7 ± 0.9 nM in the
control twin) but well below the expected value for fetuses on the day of birth [\(\sim 180 \text{nM} \); (39)]. In cortisol-treated fetuses, there was a significant increase in expression of mRNA for ENaC (Fig. 4) compared with their control twins, but there was no change in the expression of mRNA for ENaC or ENaC, nor was expression of Na\(^{+}\),K\(^{+}\)-ATPase\(_{1}\), AQP1, or AQP5 mRNA (AQP data not shown) increased in the cortisol-treated fetuses relative to their untreated twins. Expression of ENaC increased by 1.82 \(\pm\) 0.33-fold in the cortisol-treated twins relative to their control twins, whereas expression of ENaC and ENaC\(_{\gamma}\) were changed 1.14 \(\pm\) 0.29- and 0.76 \(\pm\) 0.21-fold, respectively, relative to controls (Fig. 4). Expression of AQP1 and AQP5 were changed 0.83 \(\pm\) 0.11- and 0.81 \(\pm\) 0.16-fold, respectively, in the cortisol-treated twins relative to controls.

**DISCUSSION**

The results extend our previous studies showing increased expression of ENaC\(_{1}\) and Na\(^{+}\),K\(^{+}\)-ATPase\(_{1}\) subunits near term (18), and also show increases in ENaC\(_{\beta}\) and -\(\gamma\) subunit expression in the distal lung of the ovine fetus beginning several weeks before full term. Our results indicate that induction of mRNA for ENaC\(_{1}\) and ENaC\(_{\gamma}\) peaks in the lamb at 18.82 \(\pm\) 0.33-fold in the cortisol-treated twins relative to their control twins, whereas expression of ENaC\(_{\beta}\) and ENaC\(_{\gamma}\) were changed 1.14 \(\pm\) 0.29- and 0.76 \(\pm\) 0.21-fold, respectively, relative to controls (Fig. 4). Expression of AQP1 and AQP5 were changed 0.83 \(\pm\) 0.11- and 0.81 \(\pm\) 0.16-fold, respectively, in the cortisol-treated twins relative to controls.

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The induction of ENaC\(_{1}\) by a relatively small increase in cortisol suggests that in the preterm fetus induction of ENaC\(_{1}\) may be mediated by cortisol activation of MR. This effect via MR may precede the more dramatic GR-mediated effects on ENaC\(_{1}\) gene expression that would be expected to occur with the high concentrations of cortisol produced in the last days of fetal life and during active labor. Studies in a variety of species, including the sheep (35, 37), indicate that the MR has higher affinity for cortisol than does the GR (\(K_{d}\) of ovine MR: 0.52 \(\pm\) 0.09 nM; \(K_{d}\) of ovine GR: 1.48 \(\pm\) 0.1 nM); saturation of MR occurs at \(\sim\)5 nM cortisol, whereas saturation of GR occurs at 20–25 nM cortisol. The doses of cortisol used increased cortisol to a concentration that should nearly saturate the MR assuming \(\sim\)25% free cortisol in the fetus (49). The predicted free cortisol concentrations of 3.5 nM in the cortisol-treated twins would produce \(\sim\)85% occupancy of MR and 50% occupancy of the GR (35). Our assay of unbound cytoplasmic receptors (i.e., binding capacity) using 3H-cortisol as the ligand are consistent with relatively high occupancy of MR in vivo in the preterm fetal lung compared with occupancy of MR in the preterm renal cortex, or compared with occupancy of GR in the preterm lung. There was only a 30% decrease in MR available (i.e., not bound to endogenous steroid) in the
The ovine and human lungs appear to differ from the rodent lung in the relative expression of the ENaC subunits in the maturing lung. Expression of ENaCα occurs very early in embryonic lung development in humans, and is measurable in ovine fetal lung at 80 days, but there is no detectable expression of ENaCα until 19 days of fetal life in rats (40, 41, 46). In rats the expression of ENaCα mRNA appears to be greater than that of ENaCβ or ENaCγ mRNAs; however, in fetal rats at 20-days gestation, translational efficiency of ENaCα appears to be low compared with that of ENaCβ or ENaCγ (41, 46). In the fetal sheep, although ENaCβ mRNA was more abundant at all gestational ages, ENaCβ protein expression decreases with fetal maturation, although the relative change in both mature and immature forms of ENaCβ protein are less dramatic in the membrane than that in the whole cell. This suggests that in the mature fetal sheep, ENaCα mRNA might be more efficiently translated than ENaCβ mRNA or that ENaCβ protein turnover might be relatively high. Consistent with a higher turnover of ENaCβ, in A6 kidney cells the half-life of ENaCβ decreases mucus clearance and surface fluid by 3 wk of life.
suggesting that the decrease in ENaC/H9252 protein level is critical for postnatal lung function. Our data also indicate differential regulation of the mature and immature forms of the subunits in the maturing sheep lung. It is thought that assembly of channels containing mature or immature forms of the three subunits can occur, but that efficient sodium conductance requires channels containing mature subunits (13). The ovine lung appears to efficiently process the ENaC/H9251 subunit at all ages. However, the “maturation” of the ENaC/H9252 protein, appears to be less efficient in the younger fetuses than those near term, suggesting that this may limit effective sodium conductance in the preterm fetus. Thus

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regulation of the contribution of ENaCβ subunit to the active channel may be primarily posttranslational, while the primary regulation of ENaCα in the fetus appears to be transcriptional. It is thought that other hormones, including epinephrine may play a role in posttranslational effects on channel activity (29). However, further studies are necessary to more fully characterize the mature and immature forms in the intracellular and membrane compartments of the maturing ovine lung, to assess the relative abundance of protein for the subunits, and to determine the factors important for transcriptional and posttranslational regulation of the subunits.

We have previously found that expression of the α-subunit of the Na⁺,K⁺-ATPase is highest at 130 days of gestation, although membrane protein is similarly elevated at 130 and 145 days. In that study we did not examine expression in fetal lungs during active labor. The ontogenetic pattern of ENaCα and Na⁺,K⁺-ATPaseα1 protein appear to be very similar, and both are increased in lung membranes at the time of imminent delivery, allowing for sodium flux across the alveolar epithelium. However, in the present study a small increase in cortisol did not significantly change the expression of Na⁺,K⁺-ATPaseα1, suggesting that MR regulation of Na⁺,K⁺-ATPase is less important than regulation by GR and other factors, such as oxygenation (2).

Our results also confirm the studies by others showing increased expression of AQP1 and -5 and decreased expression of chloride channel subunits CFTR and CLCN2 as gestation advances, consistent with transition of the pulmonary epithelia from a secretory to a resorptive surface. In the fetal lung CFTR are expressed in alveolar type II cells, although postnatally CFTR expression appears in the submucosal glands and proximal airway epithelium (30, 42). Recent studies have suggested that CFTR may associate with ENaC and reduce the open state of the sodium channel (reviewed in Ref. 3); the decrease in CFTR expression in the alveolar phase of lung development may therefore allow for increased sodium reabsorption. The increase in AQP1 and AQP5 at 120 days is consistent with the previous results of Liu et al. (24) who found that expression of AQP5 and -1 increase in fetal sheep lungs between 100 and 135 days of gestation. We also found that AQP1 mRNA
increases more dramatically in fetuses in labor. Our data are consistent with a dramatic increase in expression of AQPI and -5 and decrease in CFTR channels with transition from the canalicular phase to the alveolar phase, followed by further changes in AQPI and -5 closer to the time of birth. The changes in AQPI and -5 in late gestation would be expected to allow a coordination of water flux from the alveolar space across type I alveolar cells and into capillaries, given the localization of AQPSY in type I cells and AQPI in microvascular endothelium (24). The pattern of AQPI and AQPSY in late gestation differs markedly from that in rodent fetuses. In rats, AQPI protein and mRNA in the lung is first detected at E19 but remains low until after birth; AQPSY is detected postnatally. In sheep, infusion of relatively high doses of cortisol from day 120 to day 132 of gestation increases expression of mRNAs for AQPI and AQPSY expression in the fetal lung (24). In our study there was no increase in AQPI or -5 mRNAs in fetal lung after 6 days of infusion with a lower dose of cortisol. Taken together with the results of Liu et al. (24) the data suggest that the late-term increases in AQPI and -5 expression in the fetal lamb are likely to be mediated by the late gestation increases in cortisol acting primarily at GR rather than MR. Consistent with GR regulation of AQPI, dexamethasone induces AQPI mRNA in brain microvascular endothelial cells (22). Administration of betamethasone to dams induces AQPI mRNA in the lungs of fetal rat pup and administration of betamethasone to adult rats also induces mRNA for AQPI in lung (20). Thus, these results indicate that full AQPI expression requires the GR activation by the surge in cortisol that occurs in the last days of fetal life in the ovine fetus.

Perspectives and Significance

Taken together our results confirm that changes in expression of chloride, sodium, and water channels occur in a pattern in the ovine fetal lung that is consistent with the physiologic need to transition from net secretion to net reabsorption at the time of birth. The timing of the changes in gene expression vary among the various genes, with relatively early decreases in chloride channel expression and increases in aquaporin expression associated with development of alveoli in the fetal lung and more gradual increases in the mRNAs for the sodium channel subunits. This study suggests that small increases in cortisol can induce ENaCβ, but not ENaCβ or -γ, Na+,K+-ATPase α1, AQPI, or AQPSY. The stimulation of ENaCβ by relatively small increases in cortisol suggest that as in the kidney, MR may induce ENaCα expression and increased membrane localization of this subunit (8, 31, 44). However, much higher levels of cortisol produced late in gestation and in active labor are necessary for full induction of ENaCα expression in the lung and may mediate the increases in ENaCβ and ENaCγ expression. The ability to form high-conductance sodium channels at the time of birth also appears to involve subunit-specific changes in protein expression and in protein maturation. In the case of ENaCα, which is required for subunit activity, transcription rather than protein maturation appears to be the step leading the dramatic increase near term. Although ENaCβ transcription also appears to be induced near the time of birth, it appears that protein maturation and/or turnover may also determine ENaCβ contribution to the membrane channels. This pattern contrasts with that in the rat in which expression of the ENaC subunits occurs later in development, with expression of ENaCβ and -γ low until after delivery. In the rat, the timing of delivery is predictable, occurring in a narrow window of time in normal pregnancies and without fetal adrenal corticosteroid secretion. Induction of channel activity in the rat seems to be in partly dependent on increased oxygenation occurring in the first day after birth; dexamethasone only effects ENaCα protein synthesis and ENaC function in fetal rat lung distal epithelium grown at postnatal P02 (33, 34). In contrast, normal delivery in lambs occurs over a window of two or more days; normal-term delivery in humans is similarly associated with an increase in fetal secretion of cortisol. In both species there is thought to be gradual adrenal maturation and increasing cortisol secretion over the last weeks of life. Our data suggest that lower levels of cortisol, similar to those produced by stress in the preterm infant, begin induction of the critical subunit of the sodium channel ENαCα before any change in oxygenation, and may be important to assuring that the further labor-associated increases in cortisol secretion fully induce ENaCα expression, increasing the whole cell pool of this subunit. This mechanism would allow for high levels of channel formation at the time of the first breath and thereby reduce the incidence of respiratory distress in the term neonate.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-62080 (to M. Keller-Wood) and a fellowship grant from the Florida Affiliate of the American Heart Association (to N. M. Jesse).

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ONTOMETRY OF ENaC SUBUNITS IN THE OVINE FETAL LUNG