Na⁺,K⁺-ATPase Activity and Subunit Protein Expression: Ontogeny and Effects of Exogenous and Endogenous Steroids on the Cerebral Cortex and Renal Cortex of Sheep

Reproductive Sciences
18(4) 359-373
© The Author(s) 2011
Reprints and permission:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1933719110385137
http://rs.sagepub.com

Chang-Ryul Kim, MD^{1,2}, Grazyna B. Sadowska, DVM¹,
Stephanie A. Newton, MS¹, Maricruz Merino, MD¹,
Katherine H. Petersson, PhD¹, James F. Padbury, MD¹, and
Barbara S. Stonestreet, MD¹

Abstract

We examined the effects of development, exogenous, and endogenous glucocorticoids on Na⁺,K⁺-ATPase activity and subunit protein expression in ovine cerebral cortices and renal cortices. Ewes at 60%, 80%, and 90% gestation, newborns, and adults received 4 dexamethasone or placebo injections. Cerebral cortex Na⁺,K⁺-ATPase activity was higher ($P < .05$) in placebo-treated newborns than fetuses of placebo-treated ewes and adults, α_1 -expression was higher at 90% gestation than the other ages; α_2 -expression was higher in newborns than fetuses; α_3 -expression was higher in newborns than 60% gestation; β_1 -expression was higher in newborns than the other ages, and β_2 -expression higher at 60% than 80% and 90% gestation, and in adults. Renal cortex Na⁺,K⁺-ATPase activity was higher in placebo-treated adults and newborns than fetuses. Cerebral cortex Na⁺,K⁺-ATPase activity was higher in dexamethasone- than placebo-treated adults, and α_1 -expression higher in fetuses of dexamethasone- than placebo-treated ewes at 60% and 80% gestation. Renal cortex Na⁺,K⁺-ATPase activity and α_1 -expression were higher in fetuses of dexamethasone- than placebo-treated ewes at each gestational age, and β_1 -expression was higher in fetuses of dexamethasone- than placebo-treated ewes at 90% gestation and in dexamethasone- than placebo-treated adults. Cerebral cortex Na⁺,K⁺-ATPase activity, α_1 -expression, β_1 -expression, and renal cortex α_1 -expression correlated directly with increases in fetal cortisol. In conclusion, Na⁺,K⁺-ATPase activity and subunit expression exhibit specific developmental patterns in brain and kidney; exogenous glucocorticoids regulate activity and subunit expression in brain and kidney at some ages; endogenous increases in fetal cortisol regulate cerebral Na⁺,K⁺-ATPase, but exogenous glucocorticoids have a greater effect on renal than cerebral Na⁺,K⁺-ATPase.

Keywords

brain, kidney, glucocorticoids, Na⁺, K⁺-ATPase

Introduction

Sodium-potassium-adenosinetriphosphatase (Na⁺,K⁺-ATPase) is one of the principal regulators of intracellular ion homeostasis. Its primary role is to maintain high intracellular K⁺ and low intracellular Na⁺ concentrations. This exchange generates electrochemical gradients that control the ionic cellular environment critical for water and electrolyte homeostasis, cell volume regulation, and generation of action potentials.¹ This enzyme is also critical for growth, differentiation, and survival of cells, central nervous system development, neuronal guidance,² and renal tubular ion transport.³ It is expressed abundantly in the brain, skeletal muscle, cardiac muscle, and kidney.

Na⁺,K⁺-ATPase comprises α - and β -subunit protein isoforms that are essential to normal enzymatic function.⁴ The α polypeptide subunit of Na⁺,K⁺-ATPase serves as the

¹ Department of Pediatrics, Women and Infants Hospital of Rhode Island, The Warren Alpert Medical School of Brown University, Providence, RI, USA

² Department of Pediatrics, Hanyang University College of Medicine, Seoul, Korea

Corresponding Author:

Barbara S. Stonestreet, Department of Pediatrics, Women and Infants Hospital of Rhode Island, The Warren Alpert Medical School of Brown University, 101 Dudley Street, Providence, RI 02905, USA
Email: bstonestreet@wihri.org

catalytic insertion site for ATP hydrolysis. The β -subunit is a smaller polypeptide that plays a role in ensuring the proper orientation of the α -subunits in the cell membrane. The β -subunits are also enzymatically active, as they are responsible for the K^+ -dependent reactions of the P-type ATPases, including Na^+,K^+ -ATPase.⁵

Multiple isoforms for both the α - (α_1 , α_2 , α_3 , and α_4) and β - (β_1 , β_2 , and β_3) subunits have been identified based on their amino acid composition, molecular weight, and sensitivity to ions, alkylating agents, and cardiac glycosides such as ouabain.^{6,7} The major isoforms expressed in most epithelial cells are the α_1 - and β_1 -isoforms.⁸ Of the 3 α -subunits identified in brain,⁹ in situ hybridization techniques demonstrated α_1 -subunit expression in glial cells and neurons, α_2 in glial cells, and α_3 in neurons.⁹⁻¹¹ Three β -subunits have been identified in brain; the β_1 -subunits are found in neurons, β_2 predominantly in glial cells,¹¹ and β_3 in oligodendrocytes.¹² The kidney contains predominantly α_1 - and β_1 -subunits.^{13,14}

The tissue-specific expression of Na^+,K^+ -ATPase subunits is coupled to the physiological demands of the particular tissue.² The existence of different isoforms most likely serves 2 primary physiological functions: they permit differential expression with slightly modified characteristics, and/or they allow different hormones or second messenger systems to act on the transcription of different genes in specific cell types.¹⁵

Na^+,K^+ -ATPase is activated and deactivated by hormonal, nutritional, and electrical influences. Glucocorticoids are one of the most potent drugs used in perinatal medicine and have been shown to facilitate the transition from fetal to neonatal life by their beneficial effects on multiple organ systems.^{16,17} The effects of glucocorticoids during development are species-, tissue-, and age-specific.¹⁸⁻²¹ Glucocorticoids can result in precocious changes in developmentally regulated proteins in a variety of different tissues.²²

Antenatal glucocorticoid therapy has been widely used to reduce the incidence of respiratory distress syndrome and intraventricular hemorrhage in premature infants.¹⁹ Postnatal dexamethasone is used to attenuate lung damage associated with mechanical ventilation in premature infants. However, recent findings suggest that postnatal glucocorticoid use may be associated with adverse neurodevelopmental outcomes.²³ In addition, adrenocorticotrophic hormone (ACTH) and glucocorticoids are often used in newborn and young infants to treat infantile spasms.

We have shown that maternal glucocorticoid treatment reduces blood-brain barrier permeability and regional brain water contents in fetal sheep^{20,24,25} and that postnatal treatment of newborn lambs with relatively high doses (0.5 mg/kg) of dexamethasone increases Na^+,K^+ -ATPase activity and α_1 -subunit expression in the cerebral cortex but not the renal cortex.²⁶ Hence, antenatal glucocorticoids affect the fetal brain, and postnatal treatment with glucocorticoids upregulates Na^+,K^+ -ATPase activity in the cerebral cortex.

Evidence also suggests that glucocorticoids affect fetal and neonatal water and electrolyte homeostasis.²⁷ In the fetal kidney, glucocorticoids alter renal gene expression and accelerate renal functional maturation.^{16,17} Antenatal glucocorticoid

treatment also accelerates the maturation of renal function in premature human infants potentially by inducing tubular Na^+,K^+ -ATPase activity.²⁸ A study by Petershack et al supported this concept, by demonstrating that intraperitoneal cortisol infusions increased Na^+,K^+ -ATPase activity in the kidneys of late gestation fetuses,²⁹ suggesting that this is one of the mechanisms by which glucocorticoids accelerate renal maturation in premature infants.^{28,30}

Doses of glucocorticoids similar to those used in the clinical setting could have important effects on Na^+,K^+ -ATPase both before and after birth. Moreover, these effects could differ depending on the organ system and stage of development examined. Although Na^+,K^+ -ATPase is an important regulator of cellular function, limited information is available concerning the effects of development, as well as the influence of glucocorticoid treatment and the effects of changes in endogenous fetal cortisol concentrations on Na^+,K^+ -ATPase activity and protein subunit expression in the same mammalian species during development. The sheep fetus has been widely used to study the development of both the brain and kidney.^{16-18,29,31,32}

Given the above considerations, we tested the hypotheses that development, as well as exogenous and endogenous glucocorticoids, increases Na^+,K^+ -ATPase activity and its isoform expression in cerebral cortex and renal cortex of ovine fetuses, lambs, and adult sheep.

Methods

This study was conducted after approval by the Institutional Animal Care and Use Committees of Brown University and Women and Infants Hospital of Rhode Island according to the National Institutes of Health Guidelines for use of experimental animals.

Animal Preparation

Surgery was performed on 81 pregnant mixed-breed ewes at 60% of gestation ($n = 34$), 80% of gestation ($n = 20$), and 90% of gestation ($n = 27$), newborn lambs ($n = 20$), and adult sheep ($n = 7$). The plasma and tissue samples in the current study were obtained as part of our previously published studies to examine the effects of glucocorticoid pretreatment on blood-brain barrier permeability and tissue water contents.^{20,21,25,32} As previously described in detail, surgery was performed under 1% to 2% halothane anesthesia in the pregnant ewes and adult sheep.^{20,25,32} Fetal catheters for the blood-brain barrier permeability studies were placed into the brachial vein and thoracic aorta. Catheters were placed in the femoral veins and arteries in the adult sheep. In the 2-day-old lambs, venous and arterial catheters were placed in the superior vena cava and thoracic aorta under 0.5% to 1% isoflurane anesthesia.²¹

Experimental Protocol and Methodology

After 2 to 6 days of recovery from surgery for the ewes and 24 hours for the adult sheep, the subjects were randomly

assigned to receive either four 6 mg doses of dexamethasone (Fujisawa, Deerfield, Illinois; concentration = 4 mg/mL, 1.5 mL was given to each ewe or adult sheep) or placebo (0.9% sodium chloride [NaCl]) as intramuscular injections every 12 hours over the course of 48 hours. Twenty-four hours after recovery from surgery, the newborn lambs were assigned to receive either four 0.01 mg/kg per dose of dexamethasone or placebo (0.9% NaCl).²¹ The plasma and tissue samples were obtained 66 hours after the initial injection of dexamethasone or placebo and 18 hours after the final injections were given to the ewes, lambs, and adult sheep. For the purpose of this report, the fetuses of the placebo-treated ewes, placebo-treated newborn lambs and adult sheep hereafter are designated as the control fetuses, newborn lambs, and adult sheep.

Total cortisol plasma concentrations were measured in duplicate using Clinical Assays GammaCoat Cortisol¹²⁵ I-radioimmunoassay (DiaSorin, Stillwater, Minnesota). The GammaCoat antiserum exhibits 100% cross-reactivity with cortisol. The observed coefficient of variation for inter- and intra-assay precision was 10.1% and 7.9%, respectively.

At the end of the study, each ewe was given intravenous pentobarbital (15-20 mg/kg) to achieve a surgical plane of anesthesia. A hysterotomy was performed, and the fetus was withdrawn from the uterus.^{20,25} The ewe was sacrificed with an overdose of pentobarbital (100-200 mg/kg). After carefully removing all of the meninges, the frontal cerebral cortex was sectioned. The renal cortex was separated from the renal medulla. Tissue samples were not obtained from the renal medulla for analysis. The frontal cerebral cortex and renal cortex were immediately frozen in liquid nitrogen and stored at -80°C until analysis. For the purpose of this study, the frontal cerebral cortex hereafter is designated as the cerebral cortex.

Preparation of Crude Membrane Fractions

Cerebral cortical and renal cortical crude total membranes were separated from soluble proteins by homogenization and differential centrifugation,³³ using an homogenization buffer (5% sorbitol, 5 mmol/L histidine brought to pH 7.5 with 5 mmol/L imidazole, 0.5 mmol/L Na_2EDTA , and proteolytic enzyme inhibitors: 10 μg of aprotinin, 1 μg of leupeptin, and 25 μg of 4-aminoethyl-benzensulfonyl fluoride per ml of extraction buffer). Protein concentrations of the homogenates were determined with a bicinchoninic acid protein assay (BCA, Pierce, Rockford, Illinois).

Na^+, K^+ -ATPase Activity

The membrane preparations were assayed for ouabain-sensitive Na^+, K^+ -ATPase activity according to the method of Lo et al,³⁴ as modified by Schmitt and McDonough³⁵ by incubation of the membranes in 0.1% sodium deoxycholate, immediately before the ATPase assay to permeabilize the membrane vesicles. Briefly, a 0.1 mL aliquot of membrane preparation (5-10 mg protein/mL) was added to 0.8 mL homogenization buffer (5% sorbitol, 0.005 mol/L histidine, 0.003 mol/L imidazole, and

0.0005 mol/L Na_2EDTA). To this was added 0.1 mL of 1% deoxycholate for vesicle permeabilization. After the membrane preparation, 0.8 mL of assay medium (0.004 mol/L MgCl_2 , 0.001 mol/L Na_2EDTA , 0.04 mol/L Tris- HCL, 0.004 mol/L NaN_3 , 0.142 mol/L NaCl, 0.025 mol/L KCl), with and without 0.003 mol/L ouabain, was equilibrated for 5 minutes at 37°C . To this mixture, 0.1 mL of 0.03 mol/L ATP was added. The reaction was initiated by the addition of 0.1 mL membrane mixture. After 15 minutes, the reaction was stopped by the addition of 0.2 mL cold 30% trichloroacetic acid (TCA). The reaction tubes were placed on ice for 30 minutes. After centrifugation, the supernatant was collected and the liberated inorganic phosphate (P_i) assayed colorimetrically by the method of Fiske and Subbarow.³⁶ Briefly, 1.5 mL of ammonium molybdate solution (0.001 mol/L $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}/0.5\text{N H}_2\text{SO}_4$) was added to 0.5 mL of test supernatant or standard. To this, 0.25 mL of reducing agent (0.56 mol/L SO_3 plus 0.0042 mol/L 1-amino-2-naphthol-4-sulfonic acid) was added. Samples were incubated at room temperature for exactly 15 minutes and absorbance read at 660 nm (Perkin Elmer UV/VIS Spectrophotometer, Lambda 2 Newton Centre, Massachusetts). Na^+, K^+ -ATPase activity was calculated as the difference between total activity and ouabain-insensitive activity. Results were expressed as micromoles inorganic phosphate (P_i) per milligram homogenate protein per hour.³⁴ Each sample was analyzed twice, in triplicate. The final value represented the average of 6 values.

Western Immunoblot Detection and Quantification of α_1 -, α_2 -, α_3 -, β_1 -, and β_2 -Subunit Isoform Expression

The membrane preparations from the cerebral cortex and renal cortex described above were used for the Western immunoblots. The expression of α_1 -, α_2 -, α_3 -, β_1 -, and β_2 -subunits was measured with subunit-specific antisera. The α_1 - and β_1 -subunit isoform expression were examined in both the cerebral cortex and renal cortex of ovine fetuses, lambs, and adult sheep because these subunits are ubiquitous in both brain and kidney.^{26,37} The α_2 -, α_3 -, and β_2 -isoforms were examined only in the cerebral cortex because they are expressed predominantly in brain.³⁷ Samples from all age groups exposed to dexamethasone or placebo along with aliquots of the tissue extracts obtained from a single adult sheep brain or kidney were placed on each immunoblot.

We initially selected the adult sheep kidney to use as an internal control standard because we had sufficient tissue available to use tissue aliquots from a single adult kidney in all studies and the adult kidney expresses the α_1 - and β_1 -subunits.^{26,37} At first, we examined the cerebral cortical α_1 - and β_1 -subunit isoforms; we then expanded our study to include analyses of the additional subunits (α_2 , α_3 , and β_2) in the cerebral cortex. We used adult kidney as the standard for both the brain and kidney α_1 - and β_1 -subunit samples, as these subunits are present in both tissues, and so that one standard could be used for both tissues. Adult brain tissue was used as the internal control standard for the α_2 -, β_2 -, and α_3 -subunits as these subunits are expressed primarily in brain. For the purpose of this report, the adult brain or kidney internal control

samples hereafter are referred to as the internal control samples. Although the β_3 -subunit is expressed in rat and mouse oligodendrocytes, consistent with previous work in sheep, we were not able to identify this subunit in ovine cerebral cortex.¹²

As previously described,^{26,37} the internal control samples served as a control for quality of loading and transfer of the samples, for normalization of the cerebral cortical and renal cortical densitometric values, and to permit accurate comparisons among the different immunoblots.^{26,37} The experimental autoradiographic densitometry values were expressed as a ratio of the internal control, facilitating normalized comparisons among the groups.

Each immunoblot included samples from the different treatment groups along with 3 internal control samples. The internal control samples were included in 3 lanes, as the first, middle, and last samples on each immunoblot. The values for the experimental samples were accepted as valid only if the coefficient of variation for the internal control samples was less than 20% on each immunoblot. Molecular weight standards (Bio-Rad Laboratories, Hercules, California) were included in each immunoblot. Uniformity in inter-lane loading was also established using the Coomassie blue stain (Sigma, St Louis, Missouri) for the polyacrylamide gels, and uniformity of transfer to the polyvinylidene fluoride membranes (polyvinylidene difluoride [PVDF], 0.2 micron, Bio-Rad Laboratories) confirmed with the Ponceau S stain (Sigma).³⁸

Brain and kidney samples were extracted and protein concentration was determined as described above. Solubilized preparations were then fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis for the α_1 -, α_2 -, α_3 -, β_1 -, and β_2 -subunit isoforms and transferred onto PVDF membranes using a semi-dry technique. The membranes were blocked with 5% nonfat milk for 1 hour at 37°C and washed in Tris-buffered saline with Tween (TBST) 5 times for 5 minutes per wash. Immunoblots were then incubated in primary antibody in TBST.

The α_1 - and β_1 -subunits were probed with a mouse monoclonal anti-sheep Na^+, K^+ -ATPase subunit antibody (Affinity Bioreagents, Golden, Colorado) at a dilution of 1:5000 and 1:1000, respectively, for 1 hour at room temperature. The α_2 - and β_2 -subunits were probed with rabbit polyclonal anti-rat Na^+, K^+ -ATPase α_2 - and β_2 -isoform antibodies (Upstate Chemicon, Baltimore, Maryland) at a dilution of 1:5000 at 4°C over night. The α_3 -isoform was probed with a mouse monoclonal anti-sheep Na^+, K^+ -ATPase at a dilution of 1:5000 at 4°C over night. The immunoblots were washed in TBST 5 times for 5 minutes per wash and incubated for 1 hour at room temperature with donkey anti-mouse horseradish peroxidase-conjugated secondary antibody (Affinity Bioreagents, Golden, Colorado) at a dilution of 1:5000 and 1:1000 for the α_1 - and β_1 -subunits, respectively. The α_2 - and β_2 -subunits were incubated for 1 hour at room temperature with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Alpha Diagnostic, San Antonio, Texas) at a dilution of 1:10000 and the α_3 -subunit was incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Zymed, South San

Francisco, California) at a dilution of 1:10000. The immunoblots were again washed in TBST 5 times for 5 minutes per wash. Binding of the secondary antibody was detected with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia Biotech, Inc, Piscataway, New Jersey) before exposure to Hyperfilm ECL (Amersham Pharmacia Biotech, Inc).

Densitometric Analysis

Band intensities were analyzed with a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, Maryland). All experimental samples were normalized to the average of the 3 internal control values on each immunoblot. The final values represented an average of the densitometry values obtained from the different immunoblots as a ratio of the average internal control sample. This method correlates well with values that were normalized as ratios to β -actin.²⁶

Statistical Analysis

Two-way analysis of variance (ANOVA) was used to compare values among the different age groups pretreated with dexamethasone and placebo. The factors were age group (fetuses at 60%, 80%, and 90% of gestation, newborn, and adult), treatment (placebo or dexamethasone), and organ (brain or kidney). To further describe and enhance the statistically significant treatment by organ and/or age group interactions, separate ANOVAs were performed for each organ among the different age groups pretreated with dexamethasone and placebo. The ANOVA was then repeated with male and female gender of the fetus and newborn included as an additional factor to rule out the possibility that the gender of the fetuses and newborns influenced the results. If a significant difference was found by ANOVA, the Fisher least significant difference test was used to detect specific differences among the age groups and between the dexamethasone- and placebo-pretreated groups. The Na^+, K^+ -ATPase activity and subunit isoform values in the cerebral cortex and renal cortex were compared to the plasma cortisol concentrations using the least squares regression analysis. The Na^+, K^+ -ATPase activity and subunit isoform values were also compared with gestational age, because plasma cortisol concentrations increase during gestation.^{20,39} In this analysis, we only included the control fetuses because we have previously shown that increases in gestation and endogenous cortisol concentrations were associated with vascular brain maturation during normal fetal development.^{20,40,41} Multiple regression partial correlational analyses were used to compare the relative strength of the associations between the changes in Na^+, K^+ -ATPase activity and subunit isoform values in the cerebral cortex and renal cortex, and the increases in gestation and plasma cortisol concentrations during fetal development. Differences were considered statistically significant at $P < .05$. Values are expressed as mean $\pm$ SD.

Table 1. Study Groups, Gestational Age or Postnatal Age, Pre-Study Treatment, and Numbers of Sheep^a

Subjects	Gestational or Postnatal Age	Pre-Study Treatment	
		Placebo (n)	Dexamethasone (n)
Fetus at 60% gestation	86-88 days	16	18
Fetus at 80% gestation	120-124 days	9	11
Fetus at 90% gestation	133-135 days	14	13
Newborn lambs	3-5 days	11	9
Adult sheep	3 years	3	4

^a Full-term gestation is 150 days in sheep.

Results

Table 1 summarizes the study subjects, sampling age, and treatment group numbers. The plasma cortisol concentrations were higher in the control newborn lambs (141.3 ± 49.2 nmol/L, $P < .001$) than in the fetuses at 60% (18.7 ± 50.8 nmol/L), 80% (20.8 ± 50.8 nmol/L), and 90% ($53.4.1 \pm 49.2$ nmol/L) of gestation, and in the adult sheep (4.9 ± 3.3 nmol/L).^{20,21,25} As expected,³⁹ the plasma cortisol concentrations showed a direct correlation with gestational age (data not shown, $r = .68$, $n = 32$, $P < .0001$). Dexamethasone pretreatment did not alter the age-related changes in plasma cortisol concentrations. Although plasma cortisol values tended to be lower in the fetuses of the glucocorticoid treated ewes, and treated lambs and adult sheep, particularly in the fetuses at 80% and 90% of gestation, and in the adult sheep, the suppression in the treated groups did not reach statistical significance because of the variances in the cortisol values. None of the dexamethasone-treated animals developed premature labor.

Effects of Development on Na^+ , K^+ -ATPase Activity in the cerebral cortex and renal cortex

Na^+ , K^+ -ATPase activity ($\mu\text{moles Pi/mg protein/h}$; Figure 1) was significantly higher in the cerebral cortex than renal cortex (ANOVA, main effect for brain vs kidney, $F = 279.4$, $P < .0001$). The Na^+ , K^+ -ATPase activity in the cerebral cortex was higher in the control lambs than in the fetuses at all ages, and the adult sheep (Figure 1A, ANOVA, main effect for age in cerebral cortex, $F = 175.9$, $P < .0001$). In contrast, Na^+ , K^+ -ATPase activity in the renal cortex was higher in the control newborn and adult sheep than in the fetuses at all ages (Figure 1B, ANOVA, main effect for age in renal cortex, $F = 88.5$, $P < .0001$).

Na^+ , K^+ -ATPase activity were determined on both male and female fetuses and lambs. To establish potential contributions of male versus female gender to the changes in Na^+ , K^+ -ATPase, a separate ANOVA was performed. This analysis showed that the gender of the fetuses and lambs did not contribute to the changes in Na^+ , K^+ -ATPase activity during development (ANOVA, main effect for male vs female in cerebral cortex, $F = 0.26$, $P = .61$, and in the renal cortex, $F = 0.96$, $P = .33$).

Figure 1. Na^+ , K^+ -ATPase activity ($\mu\text{moles Pi}$ per mg protein per h) in cerebral cortex and renal cortex plotted for the fetuses of the ewes exposed to dexamethasone and for the control fetuses at 60%, 80%, and 90% of gestation, and dexamethasone pretreated or control newborn lambs and adult sheep. Closed bars represent fetuses of the dexamethasone-treated ewes and dexamethasone-pretreated newborn lambs and adult sheep, and open bars represent the control fetuses, and lambs and sheep. Number of cerebral cortical samples in fetuses: 60% gestation: dexamethasone, $n = 17$, control $n = 14$, 80% gestation: dexamethasone, $n = 11$, control $n = 9$, 90% gestation: dexamethasone, $n = 12$, control $n = 10$, newborn lambs: dexamethasone, $n = 9$, control $n = 11$, and adult sheep: dexamethasone, $n = 4$, control $n = 3$. Number of renal cortical samples in the fetuses: 60% gestation: dexamethasone, $n = 16$, control $n = 15$, 80% gestation: dexamethasone, $n = 8$, control $n = 9$, 90% gestation: dexamethasone, $n = 11$, control $n = 12$, newborn lambs: dexamethasone, $n = 9$, control $n = 11$, and adult sheep: dexamethasone, $n = 4$, control $n = 3$. Values mean $\pm$ SD. ^a $P < .05$ versus control fetuses at 60% of gestation; ^b $P < .05$ versus control fetuses at 80% of gestation; ^c $P < .05$ versus control fetuses at 90% of gestation; ^d $P < .05$ versus control group at the same age; ^e $P < .05$ versus control newborn lambs.

Effects of Exogenous Glucocorticoid Pretreatment on Na^+ , K^+ -ATPase Activity in the cerebral cortex and renal cortex

There was an overall effect of dexamethasone pretreatment in all the age groups considered together on Na^+ , K^+ -ATPase activity (Figure 1) in the cerebral cortex (ANOVA, main effect for dexamethasone vs placebo in cerebral cortex, $F = 7.3$, $P < .01$). Na^+ , K^+ -ATPase activity in the cerebral cortex was higher in the dexamethasone-treated than in

the control adult sheep. There was an overall effect of pretreatment with dexamethasone on Na^+, K^+ -ATPase activity in the renal cortex in all the groups considered together (ANOVA, main effect for dexamethasone vs placebo pretreatment in renal cortex, $F = 18.7$, $P < .0001$). Na^+, K^+ -ATPase activity in the renal cortex was higher in the fetuses of the dexamethasone-treated ewes than in the control fetuses at all ages.

Effects of Development on α_1 - and β_1 -Subunit Protein Isoform Expression in the cerebral cortex and renal cortex

Representative Western immunoblots illustrating the Na^+, K^+ -ATPase α_1 - and β_1 -subunit protein expression in the cerebral cortex and renal cortex of the fetuses of the dexamethasone-treated ewes and control fetuses, and the dexamethasone-pretreated and control lambs and adult sheep are shown in Figure 2A. The densitometry values were normalized as ratios to the internal control values (Figure 2B). Although the cerebral cortical α_1 -subunit expression appears similar to renal cortical values, and the cerebral cortical β_1 -subunit expression appears higher than that of the renal cortex, the subunit expressions are ratios and, hence, relative values. Therefore, the ratios of the subunits in the cerebral cortex and renal cortex were not compared statistically.

The Na^+, K^+ -ATPase α_1 -subunit expression in the cerebral cortex was higher in the control fetuses at 90% than at 60% and 80% of gestation, and than in the lambs and adult sheep (Figure 2B, ANOVA, main effect for age in cerebral cortex, $F = 25.8$, $P < .0001$). The α_1 -subunit expression in the cerebral cortex was lower in the control adult sheep compared with all other age groups.

The α_1 -subunit expression in the renal cortex was higher in the control adult sheep than in the control fetuses at all ages and lambs (Figure 2B, ANOVA, main effect for age in renal cortex, $F = 48.2$, $P < .0001$). The α_1 -subunit expression in the renal cortex was also higher in the control lambs and fetuses at 90% of gestation than in the fetuses at 60% and 80% of gestation. The β_1 -subunit expression in the renal cortex was higher in the control adult sheep than in the control fetuses at all ages, and than in control lambs, and also higher in the control fetuses at 60% than at 90% of gestation (Figure 2B, ANOVA, main effect for age in renal cortex, $F = 48.2$, $P < .0001$).

Effects of Exogenous Glucocorticoid Pretreatment on α_1 - and β_1 -Subunit Protein Isoform Expression in the cerebral cortex and renal cortex

There was an overall effect of dexamethasone pretreatment in all the age groups considered together on the α_1 -subunit expression in the cerebral cortex (ANOVA, main effect for dexamethasone vs placebo pretreatment in cerebral cortex, $F = 9.4$, $P < .01$). The α_1 -subunit expression was higher in the fetuses of the dexamethasone-treated ewes than the control

fetuses at 60% and 80% of gestation. Dexamethasone treatment did not affect the β_1 -subunit expression.

There was an overall effect of pretreatment with dexamethasone on the α_1 -subunit expression in the renal cortex (ANOVA, main effect for dexamethasone vs placebo pretreatment in renal cortex, $F = 10.38$, $P < .01$). The α_1 -subunit expression was higher in the fetuses of the dexamethasone-treated ewes than control fetuses at all ages and was lower in the dexamethasone-treated than control adult sheep.

There was an overall effect of pretreatment with dexamethasone on β_1 -subunit expression in the renal cortex (ANOVA, main effect for dexamethasone vs placebo pretreatment in renal cortex, $F = 23.1$, $P < .0001$). The β_1 -subunit expression was higher in the fetuses of the dexamethasone-treated ewes than in the control fetuses at 90% of gestation and in the dexamethasone-treated than control adult sheep.

Effects of Development on Na^+, K^+ -ATPase α_2 -, β_2 -, and α_3 -Subunit Isoform Expression in the cerebral cortex

Figure 3A summarizes representative Western immunoblots illustrating the Na^+, K^+ -ATPase α_2 -, β_2 - and α_3 -subunit isoform protein expression in the cerebral cortex of fetuses, lambs, and adult sheep pretreated with dexamethasone and placebo. The α_2 -subunit expression in the cerebral cortex was higher in the control lambs than in the fetuses at all ages (Figure 3B, ANOVA, main effect for age, $F = 15.2$, $P < .0001$). The α_2 -subunit expression was also higher in the control adult sheep and fetuses at 90% of gestation than the fetuses at 60% of gestation. The β_2 -subunit expression was higher in the control fetuses at 60% of gestation than at 80% and 90% of gestation and in the adults (Figure 3B, ANOVA, main effect for age, $F = 12.2$, $P < .0001$). The β_2 -subunit expression was also higher in the control lambs than the fetuses at 90% of gestation. The α_3 -subunit expression was higher in the control lambs than in the fetuses at 60% of gestation (Figure 3B, ANOVA, main effect for age, $F = 5.4$, $P < .01$).

Effects of Exogenous Glucocorticoid Pretreatment on Na^+, K^+ -ATPase α_2 -, β_2 -, and α_3 -Subunit Isoform Expression in the cerebral cortex

Dexamethasone treatment did not affect the α_2 -, β_2 - or α_3 -subunit expression.

Effects of Gestation and Endogenous Plasma Cortisol Concentrations on Na^+, K^+ -ATPase Activity and Subunit Protein Isoform Expression in the Control Fetuses

To examine the potential relationships between Na^+, K^+ -ATPase activity and subunit protein isoform expression and endogenous glucocorticoid concentrations during normal fetal development, Na^+, K^+ -ATPase activity, and subunit expression as a ratio of the internal control standard were compared to

Figure 2. Panel A shows representative Western immunoblots of the Na^+, K^+ -ATPase α_1 - and β_1 -subunit isoform protein expression in the cerebral cortex and renal cortex for the dexamethasone pretreatment (D) and control (C) groups at 60%, 80%, 90% of gestation, newborn lambs, and adult sheep. One representative sample is shown for each group. Panel B shows Na^+, K^+ -ATPase α_1 - and β_1 -subunit isoform protein expression in the cerebral cortex and renal cortex plotted as a ratio of the densitometry values to the internal control samples. Bar designations as of Figure 1. Number of cerebral cortical α_1 -subunit samples in the fetuses: 60% gestation: dexamethasone, $n = 6$, control $n = 6$, 80% gestation, dexamethasone: $n = 6$, control $n = 4$, 90% gestation, dexamethasone: $n = 6$, control $n = 6$, newborn lambs: dexamethasone, $n = 4$, control $n = 4$, and adult sheep: dexamethasone, $n = 4$, control $n = 3$. Number of cerebral cortical β_1 -subunit samples the fetuses: 60% gestation: dexamethasone, $n = 6$, control $n = 6$, 80% gestation: dexamethasone, $n = 6$, control $n = 4$, 90% gestation: dexamethasone, $n = 6$, control $n = 6$, newborn lambs: dexamethasone, $n = 4$, control $n = 4$, and adult sheep: dexamethasone, $n = 4$, control $n = 3$. Number of renal cortical α_1 -subunit samples in the fetuses: 60% gestation: dexamethasone, $n = 6$, control $n = 5$, 80% gestation: dexamethasone, $n = 6$, control $n = 6$, 90% gestation, dexamethasone, $n = 6$, control $n = 6$, newborn lambs: dexamethasone, $n = 6$, control $n = 6$, and adult sheep: dexamethasone, $n = 4$, control $n = 3$. Number of renal cortical β_1 -subunit samples in the fetuses: 60% gestation: dexamethasone, $n = 6$, control $n = 6$, 80% gestation: dexamethasone, $n = 6$, control $n = 6$, 90% gestation: dexamethasone, $n = 6$, control $n = 6$, newborn lambs: dexamethasone, $n = 5$, control $n = 6$, and adult sheep: dexamethasone, $n = 4$, control $n = 3$. Values mean $\pm$ SD. ^a $P < .05$ versus control for the same age group; ^b $P < 0.05$ versus control fetuses at 60% of gestation; ^c $P < .05$ versus control fetuses at 80% of gestation; ^d $P < .05$ versus control fetuses at 90% of gestation; ^e $P < .05$ versus control newborn lambs.

plasma cortisol concentrations in the control fetuses. The activity and subunit expression were also compared to gestational age, because the plasma cortisol concentrations increase with advancing gestation. Na^+, K^+ -ATPase activity demonstrated

direct relationships with increases in both gestation and endogenous plasma cortisol concentrations in the cerebral cortex, but not in the renal cortex (Figure 4A cerebral cortex: gestation: $r = .85$, $n = 33$, $P < .001$; plasma cortisol

Figure 3. Panel A shows representative Western immunoblots of the Na^+, K^+ -ATPase α_2 -, β_2 -, and α_3 -subunit isoform protein expression in the cerebral cortex of the dexamethasone (D) and control (C) pre-treatment groups at 60%, 80%, 90% of gestation, newborn lambs, and adult sheep. One representative sample is shown for each group. Panel B shows Na^+, K^+ -ATPase α_2 -, β_2 -, and α_3 -subunit isoform protein expression in the cerebral cortex plotted as a ratio of the densitometry values to the internal control samples for the fetuses, newborn lambs, and adult sheep. Bar designations as of Figure 1. Number of cerebral cortical α_2 -subunit samples in the fetuses: 60% gestation: dexamethasone, $n = 7$, control $n = 6$, 80% gestation: dexamethasone, $n = 4$, control $n = 3$, 90% gestation: dexamethasone, $n = 6$, control $n = 6$, newborn lambs: dexamethasone, $n = 5$, control $n = 6$, and adult sheep: dexamethasone, $n = 4$, control $n = 3$. Number of cerebral cortical α_3 -subunit samples in the fetuses: 60% gestation: dexamethasone, $n = 7$, control $n = 6$, 80% gestation: dexamethasone, $n = 4$, control $n = 3$, 90% gestation: dexamethasone, $n = 6$, control $n = 6$, newborn lambs: dexamethasone, $n = 6$, control $n = 6$, and adult sheep: dexamethasone, $n = 4$, control $n = 3$. Number of cerebral cortical β_2 -subunit samples in the fetuses: 60% gestation, dexamethasone, $n = 7$, control $n = 6$, 80% gestation: dexamethasone, $n = 3$, control $n = 3$, 90% gestation: dexamethasone, $n = 6$, control $n = 6$, newborn lambs: dexamethasone, $n = 6$, control $n = 6$, and adult sheep: dexamethasone, $n = 4$, control $n = 3$. Values mean $\pm$ SD. * $P < .05$ versus control fetuses at 60% of gestation; ** $P < .05$ versus control fetuses at 80% of gestation; *** $P < .05$ versus control fetuses at 90% of gestation.

concentrations: $r = .74$, $n = 27$, $P < .001$; Figure 4B renal cortex: gestation: $r = .11$, $P = .51$, $n = 36$, plasma cortisol concentrations: $r = .28$, $n = 31$, $P = .12$).

The α_1 -subunit expression demonstrated direct relationships with both increases in gestation and endogenous plasma cortisol concentrations in the cerebral cortex and renal cortex (Figure 5A cerebral cortex: gestation: $r = .74$, $n = 16$, $P < .001$, plasma cortisol concentrations: $r = .77$, $n = 13$, $P < .001$; Figure 5B renal cortex: gestation: $r = .63$, $n = 17$, $P < .001$, plasma cortisol concentrations: $r = .73$, $n = 16$, $P < .001$).

The β_1 -subunit isoform expression demonstrated direct relationships with both increases in gestation and endogenous plasma cortisol concentrations in the cerebral cortex. There was an inverse relationship between the β_1 -subunit expression and gestational age in the renal cortex, but no correlation between the β_1 -subunit and plasma cortisol concentrations (Figure 6A cerebral cortex: gestation: $r = .88$, $n = 16$, $P < .001$, plasma cortisol concentrations: $r = .92$, $n = 13$, $P < .001$; Figure 6B renal cortex: gestation: $r = -.51$, $n = 18$, $P < .05$, plasma cortisol concentrations: $r = -.28$, $n = 17$, $P = .27$).

The α_2 -subunit expression demonstrated a direct relationship with increases in gestation, but not with changes in endogenous plasma cortisol concentrations (data not shown; gestation: $r = .89$, $n = 12$, $P < .001$, plasma cortisol concentrations: $r = .46$, $n = 12$, $P = .13$). The β_2 -subunit expression demonstrated an inverse relationship with increases in gestation but not with changes in endogenous plasma cortisol concentrations (data not shown; gestation: $r = -.93$, $n = 11$, $P < .0001$, plasma cortisol concentrations: $r = -.49$, $n = 11$, $P = .13$). The α_3 -subunit expression did not demonstrate correlations with increases in gestation or changes in plasma cortisol concentrations (data not shown; gestation: $r = .24$, $n = 12$, $P = .45$, plasma cortisol concentrations: $r = .24$, $n = 12$, $P = .46$).

In Table 2, the multiple regression correlation coefficients "R" and the partial correlation coefficients are summarized for the changes in Na^+, K^+ -ATPase activity and subunit protein isoform expression versus gestational age and plasma cortisol concentrations in the cerebral cortex and renal cortex of the control fetuses. The multiple regression partial correlational analysis compared the relative strength of the association between the increases in gestation and plasma cortisol concentrations and the changes in activity and subunit expression. This analysis demonstrated relatively equal strengths of association between the increases in gestational age and plasma cortisol concentrations and increases in Na^+, K^+ -ATPase activity in the cerebral cortex. The analysis confirmed that the associations between the increases in α_2 - and β_1 -subunit expression, and the decreases in β_2 -subunit expression and increases in gestational age were greater than that of the changes in cortisol concentrations. In the renal cortex, the analysis confirmed the lack of association between changes in Na^+, K^+ -ATPase activity and increases in gestational age and plasma cortisol concentrations. This analysis demonstrated relatively equal strengths of association between the changes in gestational age and

Figure 4. Na⁺,K⁺-ATPase activity (μmoles P_i • mg protein⁻¹ • h⁻¹) in cerebral cortex (A) and renal cortex (B) plotted against gestational age in days and plasma cortisol concentration (nmol/L) in the control fetuses. Cerebral cortex: gestation $r = .85$, $n = 33$, $P < .000001$; plasma cortisol $r = .74$, $n = 27$, $P < .0001$. Renal cortex: gestation $r = .11$, $n = 36$, $P = .51$; plasma cortisol $r = .28$, $N = 31$, $P = .12$.

plasma cortisol concentrations and increases in α_1 -subunit expression.

Discussion

The purpose of our study was to examine the ontogeny as well as the effects of exogenous and endogenous glucocorticoids on Na⁺,K⁺-ATPase activity and its isoform expression in cerebral cortices and renal cortices of sheep during development. The major findings of our study were as follows: (1) Na⁺,K⁺-ATPase activity and each subunit exhibit specific differential developmental regulation unique to cerebral

cortex and renal cortex. (2) In the cerebral cortex, Na⁺,K⁺-ATPase activity appears to increase gradually during gestation with the highest values late in fetal development and in the newborn period, whereas the major increases in the renal cortex appear after birth. (3) In the cerebral cortex, the various individual Na⁺,K⁺-ATPase subunit isoforms do not necessarily exhibit the same ontogenic pattern as the changes in enzyme activity. (4) Exogenous glucocorticoids regulate Na⁺,K⁺-ATPase activity and subunit expression in brain and kidney at some but not all ages. (5) Exogenous glucocorticoids appear to have a relatively greater effect on Na⁺,K⁺-ATPase in the renal cortex than the cerebral cortex of the

Figure 5. Na^+, K^+ -ATPase α_1 -subunit isoform protein expression in the cerebral cortex and renal cortex plotted as a ratio of the densitometry values to the internal control samples in cerebral cortex (A) and renal cortex (B) plotted against gestational age in days and plasma cortisol concentration (nmol/L) in the control fetuses. Cerebral cortex: gestation $r = .74$, $n = 16$, $P < .002$, plasma cortisol $r = .77$, $n = 13$, $P < .002$. Renal cortex: gestation $r = .63$, $n = 17$, $P < .007$, plasma cortisol $r = .73$, $N = 16$, $P < .002$.

fetus. (6) Endogenous increases in cortisol appear to have a relatively greater regulatory affect on fetal cerebral cortical than renal cortical Na^+, K^+ -ATPase.

Consistent with our previous findings in fetal sheep and newborn lambs,^{26,37} Na^+, K^+ -ATPase activity was higher in the cerebral cortex than renal cortex during development. Na^+, K^+ -ATPase activity and the α_1 -, α_2 -, α_3 -, β_1 - and β_2 -subunits in the cerebral cortex were expressed early in fetal life and throughout ovine development. Na^+, K^+ -ATPase enzyme activity in

the cerebral cortex exhibited ontogenic increases during gestation and was highest in the near-term fetal sheep and in newborn lambs. The individual subunit isoforms did not necessarily follow the same developmental pattern of expression as the enzyme activity because the α_1 -isoform was highest in the fetal sheep at 90% of gestation, α_2 -isoform highest in newborn lambs and adult sheep, and α_3 -isoform highest in the newborn lambs. The β_1 -isoform was highest in newborn lambs and β_2 -isoform highest at 60% of gestation. Therefore, the

Figure 6. Na^+, K^+ -ATPase β_1 -subunit isoform protein expression in the cerebral cortex and renal cortex plotted as a ratio of the densitometry values to the internal control samples in cerebral cortex (A) and renal cortex (B) plotted against gestational age in days and plasma cortisol concentration (nmol/L) in the control fetuses. Cerebral cortex: gestation $r = .88$, $n = 16$, $P < .001$; plasma cortisol $r = .92$, $n = 13$, $P < .001$. Renal cortex: gestation $r = -.51$, $n = 18$, $P < .04$, plasma cortisol $r = -.28$, $n = 17$, $P = .27$.

mechanism by which Na^+, K^+ -ATPase activity increases during gestation most likely results from heterogeneous combinations of different isoforms that collectively contribute to the increases in enzymatic activity because the individual subunits in the cerebral cortex do not reflect patterns similar to those of the enzyme activity. These findings are consistent with previous work, demonstrating that increases in Na^+, K^+ -ATPase activity are not always reflected by increases in subunit messenger (mRNA) abundance.⁴²

The brain expresses 3 α - and 3 β -subunits in different cell types.⁹ The relative differences in the subunits expressed during ovine development could also reflect differences in the amount of glia and neurons at each age, as we measured enzyme activity and subunit expression on crude membrane fractions from samples of total frontal cerebral cortex rather than in specific cell types. We were not able to localize the protein subunits to specific cell types by immunostaining as we had limited frozen tissue samples available from our former

Table 2. Summary of Multiple Regression: Partial Correlational Analyses Comparing Na^+, K^+ -ATPase Activity and Subunit Isoform Protein Expression to Gestational Age and Plasma Cortisol Concentrations in the Control Fetuses

Region	R	P	Partial Correlation Gestational Age	P	Partial Correlation Cortisol Concentration	P
Cerebral cortex						
Activity	.88	<.001	.69	<.001	.42	<.05
α_1 -subunit	.76	<.010	.29	.31	.52	.06
α_2 -subunit	.91	<.001	.88	<.001	-.38	.25
α_3 -subunit	.26	.72	.12	.73	.10	.76
β_1 -subunit	.81	<.002	.71	<.005	.03	.91
β_2 -subunit	.95	<.001	-.93	<.001	.46	.18
Renal cortex						
Activity	.30	.28	-.08	.68	.26	.17
α_1 -subunit	.58	<.050	-.52	<.050	.56	<.05
β_1 -subunit	.41	.25	-.38	.13	.15	.56

studies.^{20,21,24,25} Nonetheless, the relative increase in the α_1 -subunit in the fetuses at 90% of gestation suggests that this isoform was upregulated in neurons and glia cells at this age, the increase in the α_2 -subunit in newborns and adults that this isoform was upregulated in glia at these ages, and the increase in the α_3 -subunit in newborns that this isoform was upregulated in neurons. Likewise, the increase in the β_1 -subunit in the adult sheep suggests relatively higher expression of this isoform in the neurons of the adult sheep, and the relative increase in the β_2 -subunit suggests that more of this isoform was expressed in glia at 60% of gestation. The ontogenic increases in the expression of these isoforms most likely do not represent increases in total neuronal or glial cellular numbers, as we have previously demonstrated decreases in total and neuronal cellular numbers, and no change in nonneuronal cellular numbers in fetal sheep between 70% and 90% of gestation.⁴³

In rodent brain, Na^+, K^+ -ATPase activity and the majority of the isoform subunits increase during gestation with continued increases after birth reaching adult levels by 20 to 30 days after birth.^{14,35} In contrast to the findings in rodents, Na^+, K^+ -ATPase activity in the cerebral cortex increased during gestation through the neonatal period but was lower in adult sheep. In general, the α_1 - and α_2 -isoforms followed a similar general pattern, but the other subunits varied in their maximal expression at different gestational and postnatal ages. The differences between our findings in sheep and those in rodents most likely result from differences in brain development among species.⁴⁴ A large proportion of brain development in the sheep occurs before birth and, similar to the human, the sheep exhibits 2 distinct brain growth phases.^{44,45} The first phase occurs between 40 and 80 days of gestation and represents neuronal multiplication and the second phase occurs between 95 and 130 days of gestation and represents neuroglia multiplication and myelination.⁴⁴⁻⁴⁶ In contrast, the majority of the rodent brain growth occurs after birth.⁴⁴

In contrast to the findings in the cerebral cortex, Na^+, K^+ -ATPase activity, α_1 - and β_1 -isoform expression in the renal cortex remained relatively low during gestation and increased after birth without further increases in the adult. The developmental pattern of Na^+, K^+ -ATPase in the renal cortex in sheep

also differed from those of rodents.^{14,47-49} In rats, the renal cortical enzyme activity increases from postnatal day 16 through 40, with the largest increase being between days 16 and 20 of age, when the corticosterone levels increase.⁴⁸ Consistent with the findings in rodents⁴⁸ and previous work in sheep,²⁹ the plasma cortisol levels were highest in the newborns, suggesting that the increases in renal cortical Na^+, K^+ -ATPase activity in sheep were related to the perinatal surge in cortisol. The differences between the sheep and rodents in renal Na^+, K^+ -ATPase maturation could also relate to differences in renal maturation between species. In sheep, there are abrupt renal functional maturational changes after birth,⁵⁰ but in rodents, the increases continue during postnatal maturation.⁴⁸ Our findings in sheep are also consistent with maturational changes observed in the guinea pig renal cortex, in which there was an upregulation of Na^+, K^+ -ATPase activity and subunit abundance immediately after birth.⁵¹

Exogenous glucocorticoids regulated ovine Na^+, K^+ -ATPase activity and subunit expression in brain and kidney at some but not all ages. Treatment with exogenous glucocorticoids increased Na^+, K^+ -ATPase activity in the cerebral cortex only in adult sheep. The reason that exogenous glucocorticoids increased Na^+, K^+ -ATPase activity in the adult sheep but not the other age groups cannot be ascertained by our studies. However, the adult sheep received a dose of 6 mg, whereas the newborn lambs received 0.01 mg/kg per dose. In our former work,²⁶ 0.01, 0.25, and 0.50 mg/kg per dose were administered directly to newborn lambs in the same regimen as in the current study, but only the 0.50 mg/kg dose was associated with a significant increase in cerebral cortical Na^+, K^+ -ATPase activity and α_1 -isoform expression. Consistent with our previous findings, the dose of 0.01 mg/kg did not result in an increase in Na^+, K^+ -ATPase activity in the lambs.

Although maternally administered dexamethasone readily crosses to fetus as placenta 11 β -hydroxysteroid dehydrogenase has a low affinity for synthetic glucocorticoids,⁵² penetration of dexamethasone into the brain could be limited by the presence of the multidrug-resistance gene 1-type P-glycoproteins (*MRD1*). *MRD1* forms a functional barrier to lipid-soluble drugs that potentially could limit the access of dexamethasone

and endogenous glucocorticoids to the brain.⁵³ Thus, it is possible that higher doses of dexamethasone could have had a greater effect on Na^+, K^+ -ATPase in the brain at the other ages. Nonetheless, we have previously shown that fetuses of ewes receiving the same dexamethasone regimen exhibited lower brain water contents at 60% of gestation²⁴ and less apoptosis at 70% of gestation.⁴³ In addition, exposure to multiple courses of dexamethasone actually reduced Na^+, K^+ -ATPase activity at 70% of gestation.³⁷ Hence, the maternal treatment with glucocorticoids may have varying affects on different aspects of brain development in the same species.

In the renal cortex, maternal dexamethasone treatment increased Na^+, K^+ -ATPase activity at 60%, 80%, and 90% of gestation, but not in newborn or adult sheep, and increased α_1 -isoform expression at 60%, 80%, and 90% of gestation and the β_1 -subunit only at 90% gestation. In contrast, dexamethasone treatment decreased the α_1 - and increased the β_1 -subunit in the adult sheep. These findings suggest that exogenous glucocorticoid exposure has a greater effect on the renal cortical than the cerebral cortical Na^+, K^+ -ATPase, and that the kidney could be more sensitive to exogenous glucocorticoids during fetal life than after birth in newborn²⁶ and adult sheep. The greater effect of exogenous glucocorticoids on Na^+, K^+ -ATPase activity and α_1 -isoform expression in the fetal than adult renal cortex could be because glucocorticoid and mineralocorticoid receptors are higher in the fetal than adult kidney.⁵⁴ The increases in Na^+, K^+ -ATPase activity in the renal cortex are confirmatory of previous work.^{29,55} Our findings further suggest that glucocorticoids administered to the mother accelerate the maturation of renal Na^+, K^+ -ATPase in fetuses both early and later in gestation. Consistent with our findings that dexamethasone increased Na^+, K^+ -ATPase in the fetus, but not adults, prior work has shown that immature tubular cells are more sensitive than mature tubular cells to the inductive effects of hormones.⁴⁷

The pituitary–adrenal cortical axis is well known to mature during fetal development and cortisol concentrations increase particularly in the latter part of gestation.³⁹ Endogenous glucocorticoids modulate the developmental processes of numerous tissues including the brain and kidney.^{20,22,29} We have previously shown that endogenous increases in cortisol are associated with decreases in blood–brain barrier permeability during normal fetal development, and increases in the expression of the tight junction protein, ZO-2, in the fetal cerebral cortex.^{20,41} Our current findings support the contention that increases in endogenous plasma cortisol are also associated with increases in Na^+, K^+ -ATPase activity, α_1 - and β_1 -subunit isoform expression in the cerebral cortex, whereas, in the renal cortex, increases in cortisol are only associated with increases in the α_1 -subunit, but not with enzymatic activity or the β_1 -subunit. These findings suggest that the endogenous increases in cortisol could be in part responsible for the ontogenic increases in Na^+, K^+ -ATPase in the cerebral cortex during development in the fetus.

Our findings that maternal treatment with exogenous glucocorticoids increases renal, but not cerebral Na^+, K^+ -ATPase,

and that endogenous increases in glucocorticoids appear to have a greater effect on cerebral than renal Na^+, K^+ -ATPase suggests differential responsiveness of these tissues to both endogenous and exogenous glucocorticoids. These findings are consistent with the view that during gestation increases in endogenous cortisol are associated with increases in Na^+, K^+ -ATPase in the cerebral cortex, and thus cerebral Na^+, K^+ -ATPase is not responsive to maternal treatment with exogenous glucocorticoids. Endogenous glucocorticoids bind to the mineralocorticoid receptor, but synthetic glucocorticoids bind predominantly glucocorticoid receptor.^{56,57} Consistent with our findings that endogenous cortisol might have a greater impact on Na^+, K^+ -ATPase in the cerebral cortex, previous work has shown that the affinity of cortisol is greater for the mineralocorticoid than glucocorticoid receptor in sheep brain.⁵⁸ In contrast, in the renal cortex, the endogenous increases in cortisol were not associated with increases in Na^+, K^+ -ATPase and therefore renal Na^+, K^+ -ATPase remains responsive to exogenous glucocorticoids. Therefore, the differential responsiveness of Na^+, K^+ -ATPase in brain and kidney to endogenous and exogenous glucocorticoids could possibly relate to developmental differences in the proportion of the glucocorticoid and mineralocorticoid receptors in these tissues.

We conclude that developmental increases in endogenous cortisol concentrations contribute to maturational increases in Na^+, K^+ -ATPase activity in cerebral cortex, but not in renal cortex during ovine gestation. Maternal treatment with dexamethasone increases Na^+, K^+ -ATPase activity in renal cortex but not in cerebral cortex. The effects of ontogeny, and endogenous and exogenous glucocorticoids effects on Na^+, K^+ -ATPase activity and its subunits during the development are age- and organ-specific in sheep.

Perspectives

Maternal glucocorticoid therapy is widely used to treat pregnant women in premature labor. The relatively low-dose treatment regimen that was given to the ewes in our study was similar to that used in the clinical setting. Our findings suggest that changes in endogenous cortisol concentration have a relatively greater effect on fetal Na^+, K^+ -ATPase maturation in brain, but exogenous glucocorticoid treatment has greater impact on Na^+, K^+ -ATPase in the kidney during fetal development. The relatively greater effects of exogenous glucocorticoid treatment on the kidney than brain could suggest that this treatment has potential consequences for body volume and/or electrolyte homeostasis, and potentially for later cardio-renal homeostasis. Our findings also emphasize that the effects of endogenous glucocorticoids and exogenous treatment with glucocorticoids may differ in an organ-specific manner during development.

Acknowledgments

We are grateful to Alicia M. McDonough, PhD, Physiology and Biophysics, University of Southern California, Los Angeles,

California, for her assistance and guidance in setting up the Na⁺,K⁺-ATPase activity and Western blot subunit assays in our laboratory as well as for her general expert guidance during the development of this project.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research and/or authorship of this article: NIH R01-HD-34618 and 1R01-HD-057100.

References

- Skou JC. The Na,K-pump. *Methods Enzymol.* 1988;156:1-25.
- Mobasheri A, Avila J, Cozar-Castellano I, et al. Na⁺, K⁺-ATPase isozyme diversity; comparative biochemistry and physiological implications of novel functional interactions. *Biosci Rep.* 2000;20(2):51-91.
- Jahnukainen T, Chen M, Berg U, et al. Antenatal glucocorticoids and renal function after birth. *Semin Neonatol.* 2001;6(4):351-355.
- Therien AG, Blostein R. Mechanisms of sodium pump regulation. *Am J Physiol Cell Physiol.* 2000;279(3):C541-C566.
- Chow DC, Forte JG. Functional significance of the beta-subunit for heterodimeric P-type ATPases. *J Exp Biol.* 1995;198(pt 1): 1-17.
- Blanco G, Mercer RW. Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *Am J Physiol.* 1998;275(5 pt 2):F633-F650.
- Lopez LB, Quintas LE, Noel F. Influence of development on Na(+)/K(+)-ATPase expression: isoform- and tissue-dependency. *Comp Biochem Physiol A Mol Integr Physiol.* 2002;131(2): 323-333.
- Horisberger JD, Lemas V, Kraehenbuhl JP, et al. Structure-function relationship of Na,K-ATPase. *Annu Rev Physiol.* 1991;53(6):565-584.
- Hieber V, Siegel GJ, Fink DJ, et al. Differential distribution of (Na, K)-ATPase alpha isoforms in the central nervous system. *Cell Mol Neurobiol.* 1991;11(2):253-262.
- Shyjan AW, Levenson R. Antisera specific for the alpha 1, alpha 2, alpha 3, and beta subunits of the Na,K-ATPase: differential expression of alpha and beta subunits in rat tissue membranes. *Biochemistry.* 1989;28(11):4531-4535.
- Watts AG, Sanchez-Watts G, Emanuel JR, et al. Cell-specific expression of mRNAs encoding Na⁺,K⁺-ATPase alpha- and beta-subunit isoforms within the rat central nervous system. *Proc Natl Acad Sci U S A.* 1991;88(16):7425-7429.
- Martin-Vasallo P, Wetzel RK, Garcia-Segura LM, et al. Oligodendrocytes in brain and optic nerve express the beta3 subunit isoform of Na,K-ATPase. *Glia.* 2000;31(3):206-218.
- Herrera VL, Cova T, Sassoon D, et al. Developmental cell-specific regulation of Na(+)-K(+)-ATPase alpha 1-, alpha 2-, and alpha 3-isoform gene expression. *Am J Physiol.* 1994;266(5 pt 1): C1301-C1312.
- Orlowski J, Lingrel JB. Tissue-specific and developmental regulation of rat Na,K-ATPase catalytic alpha isoform and beta subunit mRNAs. *J Biol Chem.* 1988;263(21):10436-10442.
- Jaisser F, Jaunin P, Geering K, et al. Modulation of the Na, K-pump function by beta subunit isoforms. *J Gen Physiol.* 1994;103(4):605-623.
- Berry LM, Polk DH, Ikegami M, et al. Preterm newborn lamb renal and cardiovascular responses after fetal or maternal antenatal betamethasone. *Am J Physiol.* 1997;272(6 pt 2): R1972-R1979.
- Stonestreet BS, Hansen NB, Lupton AR, et al. Glucocorticoid accelerates renal functional maturation in fetal lambs. *Early Hum Dev.* 1983;8(3-4):331-341.
- Jobe AH. Animal models of antenatal corticosteroids: clinical implications. *Clin Obstet Gynecol.* 2003;46(1):174-189.
- NIH Consensus Development Panel. Effect of corticosteroids for fetal maturation on perinatal outcomes. NIH consensus development panel on the effect of corticosteroids for fetal maturation on perinatal outcomes. *Jama.* 1995;273(5):413-418.
- Stonestreet BS, Sadowska GB, McKnight AJ, et al. Exogenous and endogenous corticosteroids modulate blood-brain barrier development in the ovine fetus. *Am J Physiol Regul Integr Comp Physiol.* 2000;279(2):R468-R477.
- Sysyn GD, Petersson KH, Patlak CS, et al. Effects of postnatal dexamethasone on blood-brain barrier permeability and brain water content in newborn lambs. *Am J Physiol Regul Integr Comp Physiol.* 2001;280(2):R547-R553.
- Ballard PL, Ballard RA. Scientific basis and therapeutic regimens for use of antenatal glucocorticoids. *Am J Obstet Gynecol.* 1995;173(1):254-262.
- Yeh TF, Lin YJ, Lin HC, et al. Outcomes at school age after postnatal dexamethasone therapy for lung disease of prematurity. *N Engl J Med.* 2004;350(13):1304-1313.
- Stonestreet BS, Elitt CM, Markowitz J, et al. Effects of antenatal corticosteroids on regional brain and non-neural tissue water content in the ovine fetus. *J Soc Gynecol Investig.* 2003;10(2):59-66.
- Stonestreet BS, Petersson KH, Sadowska GB, et al. Antenatal steroids decrease blood-brain barrier permeability in the ovine fetus. *Am J Physiol.* 1999;276(2 pt 2):R283-R289.
- Kim CR, Sadowska GB, Petersson KH, et al. Effects of postnatal steroids on Na⁺/K⁺-ATPase activity and alpha1- and beta1-subunit protein expression in the cerebral cortex and renal cortex of newborn lambs. *Reprod Fertil Dev.* 2006;18(4):413-423.
- Wintour EM, Alcorn D, McFarlane A, et al. Effect of maternal glucocorticoid treatment on fetal fluids in sheep at 0.4 gestation. *Am J Physiol.* 1994;266(4 pt 2):R1174-R1181.
- Omar SA, DeCristofaro JD, Agarwal BI, et al. Effects of prenatal steroids on water and sodium homeostasis in extremely low birth weight neonates. *Pediatrics.* 1999;104(3 pt 1):482-488.
- Petershack JA, Nagaraja SC, Guillery EN. Role of glucocorticoids in the maturation of renal cortical Na⁺-K⁺-ATPase during fetal life in sheep. *Am J Physiol.* 1999;276(6 pt 2):R1825-R1832.
- al-Dahan J, Stimmler L, Chantler C, et al. The effect of antenatal dexamethasone administration on glomerular filtration rate and renal sodium excretion in premature infants. *Pediatr Nephrol.* 1987;1(2):131-135.

31. Back SA, Riddle A, Hohimer AR. Role of instrumented fetal sheep preparations in defining the pathogenesis of human periventricular white-matter injury. *J Child Neurol*. 2006;21(7):582-589.
32. Stonestreet BS, Oen-Hsiao JM, Petersson KH, et al. Regulation of brain water during acute hyperosmolality in ovine fetuses, lambs, and adults. *J Appl Physiol*. 2003;94(4):1491-1500.
33. Jorgensen PL. Purification and characterization of (Na⁺ plus K⁺)-ATPase. 3. Purification from the outer medulla of mammalian kidney after selective removal of membrane components by sodium dodecylsulphate. *Biochim Biophys Acta*. 1974;356(1):36-52.
34. Lo SC, August TR, Liberman UA, et al. Dependence of renal (Na⁺ + K⁺)-adenosine triphosphatase activity on thyroid status. *J Biol Chem*. 1976;251(24):7826-7833.
35. Schmitt CA, McDonough AA. Developmental and thyroid hormone regulation of two molecular forms of Na⁺-K⁺-ATPase in brain. *J Biol Chem*. 1986;261(22):10439-10444.
36. Fiske C, Subbarow Y. The colorimetric determination of phosphorus. *J Biol Chem*. 1925;66(2):375-400.
37. Mehter NS, Sadowska GB, Malaeb SN, et al. Na⁺, K⁺-ATPase activity and subunit isoform protein abundance: effects of antenatal glucocorticoids in the frontal cerebral cortex and renal cortex of ovine fetuses. *Reprod Sci*. 2009;16(3):294-307. Epub 2008 Nov 2011.
38. Tseng YT, Yano N, Rojan A, et al. Ontogeny of phosphoinositide 3-kinase signaling in developing heart: effect of acute beta-adrenergic stimulation. *Am J Physiol Heart Circ Physiol*. 2005;289(5):H1834-H1842.
39. Wintour EM. Developmental aspects of the hypothalamic-pituitary-adrenal axis. *J Dev Physiol*. 1984;6(3):291-299.
40. Stonestreet BS, Patlak CS, Pettigrew KD, et al. Ontogeny of blood-brain barrier function in ovine fetuses, lambs, and adults. *Am J Physiol*. 1996;271(6 pt 2):R1594-R1601.
41. Duncan AR, Sadowska GB, Stonestreet BS. Ontogeny and the effects of exogenous and endogenous glucocorticoids on tight junction protein expression in ovine cerebral cortices. *Brain Res*. 2009;1303:15-25.
42. Devarajan P, Benz EJ, Jr. Translational regulation of Na-K-ATPase subunit mRNAs by glucocorticoids. *Am J Physiol Renal Physiol*. 2000;279(6):F1132-F1138.
43. Malaeb SN, Hovanesian V, Sarasin MD, et al. Effects of maternal antenatal glucocorticoid treatment on apoptosis in the ovine fetal cerebral cortex. *J Neurosci Res*. 2009;87(1):179-189.
44. Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Hum Dev*. 1979;3(1):79-83.
45. McIntosh GH, Baghurst KI, Potter BJ, et al. Foetal brain development in the sheep. *Neuropathol Appl Neurobiol*. 1979;5(2):103-114.
46. Barlow RM. The foetal sheep: morphogenesis of the nervous system and histochemical aspects of myelination. *J Comp Neurol*. 1969;135(3):249-262.
47. Aperia A, Larsson L, Zetterstrom R. Hormonal induction of Na-K-ATPase in developing proximal tubular cells. *Am J Physiol*. 1981;241(4):F356-F360.
48. Rane S, Aperia A. Ontogeny of Na-K-ATPase activity in thick ascending limb and of concentrating capacity. *Am J Physiol*. 1985;249(5 pt 2):F723-F728.
49. Celsi G, Nishi A, Akusjarvi G, et al. Abundance of Na(+)-K(+)-ATPase mRNA is regulated by glucocorticoid hormones in infant rat kidneys. *Am J Physiol*. 1991;260(2 pt 2):F192-F197.
50. Turner AJ, Brown RD, Carlstrom M, et al. Mechanisms of neonatal increase in glomerular filtration rate. *Am J Physiol Regul Integr Comp Physiol*. 2008;295(3):R916-R921.
51. Guillery EN, Huss DJ, McDonough AA, et al. Posttranscriptional upregulation of Na(+)-K(+)-ATPase activity in newborn guinea pig renal cortex. *Am J Physiol*. 1997;273(2 pt 2):F254-F263.
52. Matthews SG. Antenatal glucocorticoids and the developing brain: mechanisms of action. *Semin Neonatol*. 2001;6(4):309-317.
53. Schinkel AH, Wagenaar E, van Deemter L, et al. Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest*. 1995;96(4):1698-1705.
54. Hantzis V, Albiston A, Matsacos D, et al. Effect of early glucocorticoid treatment on MR and GR in late gestation ovine kidney. *Kidney International*. 2002;61(2):405-413.
55. Jobe AH, Polk DH, Ervin MG, et al. Preterm betamethasone treatment of fetal sheep: outcome after term delivery. *J Soc Gynecol Investig*. 1996;3(5):250-258.
56. Matthews SG. Antenatal glucocorticoids and programming of the developing CNS. *Pediatr Res*. 2000;47(3):291-300.
57. Rose JC, Kute TE, Winkler L. Glucocorticoid receptors in sheep brain tissues during development. *Am J Physiol*. 1985;249(4 pt 1):E345-E349.
58. Richards EM, Hua Y, Keller-Wood M. Pharmacology and physiology of ovine corticosteroid receptors. *Neuroendocrinology*. 2003;77(1):2-14.