EFFECTS OF MINERALOCORTICOID AND GLUCOCORTICOID AGONISTS ON GENE AND PROTEIN EXPRESSION, STRUCTURAL MATURATION AND COMPLIANCE IN THE OVINE PRETERM FETAL LUNG

By

JARRET MCCARTNEY

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To my mom, whose perseverance and love for her family has always been an inspiration to me, also to my entire family, friends and especially Julie for their support and understanding

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Jarret McCartney

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In mammalian pregnancy, the in utero fetal cortisol surge that occurs shortly before parturition stimulates lung maturation and is thought to occur through glucocorticoid receptor (GR) dependent effects. Antenatal treatment of women threatening preterm labor with synthetic glucocorticoids has been shown to significantly decrease respiratory distress in preterm infants. We have found that the mineralocorticoid receptor (MR) is abundantly expressed in ovine fetal lungs, as are several genes known to be induced by MR action in the adult kidney, including the epithelial sodium channel alpha subunit (α-ENaC) and sodium potassium adenosine triphosphatase (Na,K ATPase -α1). However, the role of MR activation in fetal lung maturation is not clear. The purpose of this study was to determine if administration of an MR agonist would increase lung structural maturation, effect gene and protein expression, and lung compliance in ovine fetuses at 130d gestation.

Fetuses were infused with a solution containing the MR specific agonist aldosterone (0.2 mg/48h), the synthetic GR specific agonist betamethasone (0.25 mg or 0.75 mg 48h), or a combined 0.2mg aldosterone and betamethasone solution with both

0.25mg and 0.75mg betamethasone doses. In situ fetal lung compliance was then determined by measurement of intrapulmonary pressure produced by inflation of the lungs by 4-5 10ml stepwise volumes of air injected into the trachea.

While there was not an overall aldosterone effect on fetal lung peak pressure measurements, inflation pressure measurements showed a distinct effect of aldosterone to decrease intrapulmonary pressure at the initial inflation volume and again at the majority of subsequent volumes, indicating an apparent initial and transiently sustained increase in ovine fetal lung compliance. There were few significant effects of either dose of betamethasone alone and the lower concentration of betamethasone infusion decreased the effect of aldosterone to increase fetal lung compliance. Combined 0.75 mg dose of betamethasone with aldosterone only transiently improved lung compliance.

These results suggest an effect of aldosterone to increase lung compliance in the preterm ovine lung that involves a mechanism occurring through MR-specific factors. Quantitative real-time polymerase chain reaction (PCR) data suggest that GR agonists increase expression of α-ENaC and Na,K ATPase, while MR agonists do not. Western blot of α and β ENaC protein did not indicate a significant change in α-ENaC abundance in membrane. However, whole cell abundance and relative ratios of these two subunits are greatly affected by corticosteroid infusion suggesting a complex stoichiometric relationship that is perhaps affecting the efficacy of these channels. Tissue histology confirmed a significant increase in elastin and collagen abundance in GR agonist infused fetuses but not in aldosterone infused fetuses. Perhaps indicating that structural changes in ovine lung extra cellular matrix (ECM) proteins are contributing to the observed changes in lung compliance.

CHAPTER 1
INTRODUCTION

Fetal Lung Development

The human fetal lung begins to develop at the third week of gestation and development of the lung continues until early adulthood. The stages of fetal lung development are divided into five separate and distinct phases consisting of: Embryonic, Pseudoglandular, Canalicular, Saccular, and Alveolar stages (Joshi and Kotecha, 2007; Kotecha, 2000).

The human embryonic stage of development encompasses the first 7 weeks of fetal life and during this time the lung initially develops as an outgrowth from the ventral wall of the fetal forgut with concurrent development of the trachea, bronchi, and segmental bronchi (Kotecha, 2000). The human embryonic stage of development is further characterized as the beginning of fetal pulmonary vasculogenesis. Vascular proliferation occurs from an outgrowth located on the aortic arch, to form the early pulmonary arteries and veins accompanying the developing airways. It is during the human embryonic stage of lung development that the majority of congenital malformations manifest, due to improper pulmonary structural or vascular development (Joshi and Kotecha, 2007). The ovine fetus was the model of pregnancy utilized in this study and the comparable embryonic gestational age for these fetuses occurs from days 17-40 of development of an approximate 147 day period of gestation. During this time the trachea divides into the right and left bronchi and further branching into lobar and segmental bronchi occurs by day 25 of gestation (Bryden et al., 1973).

Weeks 7-17 of fetal life mark the period of the pseudoglandular stage of development and it is during this time that further branching of the airways and

vasculature occurs. The pseudoglandular stage is characterized as the fastest stage of airway branching and proliferation with approximately 70 % of the total airway present at the time of birth formed by this completion of this stage at 17 weeks gestational age. It is also during this stage that a marked increase in epithelial cell differentiation into primitive type II pneumocytes occurs, and the beginning of pulmonary cartilage and smooth muscle formation (Joshi and Kotecha, 2007). In the ovine fetus, the psuedoglandular stage of development occurs during days 40-80 of gestation and the epithelium consist of branching tubular system embedded loosely in mesenchyme tissue. The larger central tubules are surrounded by cartilage aggregates and smooth muscle sheaths (Alcorn et al., 1981).

The pseudoglandular stage of human fetal lung development is followed by the canalicular stage during weeks 17-27 of gestation. It is during this phase that surfactant production is first observed at 24 weeks gestational age. Further cellular differentiation of epithelial cells into type I and surfactant producing type II pneumocytes and the formation of a functional alveolar capillary barrier for gas exchange occurs at this time (Joshi and Kotecha, 2007; Kotecha, 2000). The importance of these changes in lung development are evident in preterm fetuses born during this stage in development, with fetal survival often observed in neonates as young as 26 weeks gestational age. However, these fetuses do suffer from surfactant insufficiency and a lack of lung structural maturation does present a predilection for fetal respiratory distress syndrome and development of transient tachypnea of the newborn (Kotecha, 2000). In the ovine fetus, the canalicular stage of development occurs during days 80-120 of gestation and is not subdivided into a following saccular stage of development. During this time the

lung parenchyma consist of a fine network of alveoli and the peripheral airways develop from the primitive tubular structures to greatly increase the potential airspace volume of the lungs. Proliferation of the pulmonary vasculature also increases during this time (Alcorn et al., 1981).

Although, while the human lung is producing small amounts of surfactant and is much more structurally mature in the canalicular stage than in earlier stages of development, it is hypothesized that fetal survival during the canalicular stage is influenced by the chronic fetal stressors that precipitate preterm birth and it is the effect of these stressors that act to increase the structural maturation and viability of the lung and contribute to the survival of these neonates (Jobe, 2010).

The next stage of human fetal lung development is the saccular stage during 28-36 weeks gestational age. During this stage there is significant enlargement of peripheral airways and thinning of the airway walls resulting in subsequent increases in surface area available for gas exchange. Further differentiation of type I cells into type II cells is observed with concomitant increases in surfactant production. Structural maturation of the lung continues to progress with a reduction of the distance in the air-blood interface at areas of gas exchange due to increased proximity of pulmonary capillaries and type I cells (Joshi and Kotecha, 2007; Kotecha, 2000).

The final stage of fetal lung development is the alveolar stage from 36 weeks gestational age until two years of age. This stage is characterized by formation of secondary septa from the alveoli located in terminal airways. The protruding secondary septa act to bifurcate the air space, creating two distinct and functional alveoli and this process of secondary septa formation and alveolar bifurcation continues until 2-3 years

of age. At the time of parturition, alveoli can be readily identified and estimates of alveoli number range from 20-50 million with subsequent increases in alveolar number to approx 300-800 million by 2-3 years of age. Afterwards, alveoli proliferation ceases while alveolar size and surface area continue to increase until early adulthood (Joshi and Kotecha, 2007; Kotecha, 2000). In the ovine fetus, the alveolar stage of development occurs from 120 days gestational age and continues after birth. In this stage of development the lung parenchyma consists of a fine network of alveoli and the large bronchi are lined with a developed epithelium. Blood vessels are surrounded in a thick fibromuscular coat. Alveolar wall thickness decreases and alveolar number increases by bifurcation by secondary septal crests (Alcorn et al., 1981).

It is important to note that ovine lungs at term are more advanced than human lungs in terms of relative alveoli formation (Alcorn et al., 1981). However the ovine fetal lung at term is more similar to term human lungs than other species such as rabbit (Kikkawa et al., 1968), mouse (Amy et al., 1977), and rat (Burri et al., 1974). Compared to human and ovine fetuses, all of these species have large saccular structures at birth and lack considerable mature alveoli. The ovine lung is therefore a more appropriate analog for human lung development than these other species with the notable exception of non human primates.

Collagen, Elastin Fibers and Surfactant

A brief description of lung biomechanics is essential to understand the factors that contribute to the functional efficacy of the fetal lung. Furthermore, the major elements that comprise lung intrapulmonary structures, namely the extra cellular matrix (ECM) proteins such as elastin fibers, collagen, proteoglycans, and

glycosaminoglycans, can have profound effects on lung function depending on their relative abundance and distribution (Faffe and Zin, 2009; Suki et al., 2005).

Collagen and elastin fibers are the major constituents of the lung ECM. Generation and organization of lung elastin begins in the psuedoglandular stage of fetal lung development at sites of lung airway branching and elastin synthesis increases throughout gestation until peaking during the alveolar stage of development. Elastin is intrinsically associated to the process of alveolarization, forming fibers at areas of stress and concentrated deposits at alveolar junctions. Small elastin fibers form delicate lattice structures connecting newly formed alveoli and act to define boundaries between these developing cells. Elastin fibers further ensure that newly formed septa are continuously interconnected and that the newly forming alveolar cells do not obstruct alveolar ducts. For these reasons, it is thought that normal alveolarization is dependent upon proper elastin fiber formation (Starcher, 2000).

While elastin fibers are an important component of the lung extracellular matrix they are also an essential element for the normal stretch and recoiling properties of the lung. Lung tissue is constantly under stress and distension by a positive transpulmonary pressure. The chest wall and diaphragm distend in order for the lung to remain inflated and to increase the transpulmonary pressure to facilitate air movement into the lung and conversely relax to decrease transpulmonary pressure to allow air movement out of the lung. This stress is distributed throughout the lung by the hydrostatic pressure in the pleural space surrounding the lung and is heterogeneously distributed due to the shape of the lung (Suki et al., 2005). This prestress is in turn distributed throughout the extra cellular matrix and, in the lung parenchyma, it acts to properly support the distension of

intrapulmonary structures. Under low stress conditions, the highly stretchable nature of elastin provides the majority of the lungs elastic properties. This elastic recoil can be attributed to the tight linear arrangement of elastin fibers, and their increased responsiveness to lower stresses than that of other stiffer components of the extra cellular matrix, such as collagen (Faffe and Zin, 2009).

In a study using a rat model of lung growth to determine the relationship of elastin, collagen and their effects on the mechanical properties of the lung, increases in elastin content were found to also increase lung recoil (Nardell and Brody, 1982). Although, other studies have shown that the orientation of ECM proteins, rather than absolute concentration, has a greater effect on lung mechanical properties (Tanaka and Ludwig, 1999), elastin is clearly an important mediator of the recoil properties of the lung.

Collagen is by far the most abundant and important ECM protein for lung structural integrity. While over 20 different collagen subtypes have been identified, the majority of lung parenchyma collagen is comprised of only type I and type III collagen. These collagens form in thick networks of fibers and fibrils in random or semiordered orientation, and the thickness of these fibers can vary from several hundred nanometers to over a micrometer. In the lung parenchyma, collagen fiber networks extend down central airways to alveolar ducts and are also present in the lung pleura (Suki et al., 2005). Collagen abundance increases throughout gestation in the same fashion as elastin also peaking during the alveolar stage of development (Faffe and Zin, 2009). Studies examining the changes of collagen content during development and in models of lung fibrosis have shown collagen to affect the biomechanical properties of the lung

(Suki et al., 2005). However, different organs with similar collagen content can have very different biomechanical properties. This highlights that both complex interactions between ECM proteins and abundance of these proteins has profound effects on lung function.

Another element affecting the biomechanical properties of lung function is pulmonary surfactant. Pulmonary surfactant is composed of a mixture of phospholipids and proteins secreted by type II alveolar cells and it functions to attenuate recoil pressures and to decrease the surface tension that arises by the difference in attractive forces between molecules that reside on the air-alveolus interface. On the surface of alveolus buds, this surface tension resists expansion of these buds and acts to contract the surface area. The phospholipids in surfactant resist this force and are capable of lowering the surface tension due to their insolubility with water and their ability to form lipid bilayers. Surfactant is essential to prevent collapse of alveolus buds following exhalation and its importance is evident in fetuses that suffer respiratory distress from inadequate surfactant production. There are four surfactant proteins comprised of surfactant related protein (SP) A, B, C, and D. SP-B and C are thought to enhance the absorption of lipids at the air-alveolus interface, while SP-A and D are thought to play critical roles in host defense from bacteria, fungal, and viral infection (Nkadi et al., 2009; Suki et al., 2005).

The Hypothalamic Pituitary Adrenal Axis

The hypothalamic pituitary adrenal (HPA) axis plays an essential role in fetal; development, maturation, and responses to stress by increasing the release of β-endorphin, noradrenaline and cortisol (Giannakoulopoulos et al., 1999). The actions of the HPA axis are principally controlled through the release of corticotrophin releasing

hormone (CRH) and arginine-vasopressin (AVP). These neuropeptides are synthesized in the paraventricular nucleus (PVN) of the hypothalamus and are released in the hypophyseal portal blood system through axons that project down the median eminence to the anterior pituitary (Swanson et al., 1983). CRH then binds to type I CRH receptors in the anterior pituitary and stimulates the release of the proopiomelanocortin (POMC) derivative adrenocorticotropic hormone (ACTH). AVP released into the hypophyseal portal system is also capable of acting at these receptors to facilitate the synthesis and release of ACTH (Mastorakos and Ilias, 2003). In humans, plasma ACTH then binds to receptors at the adrenal glands and acts to increase cholesterol transport into the cell and biosynthesis of cortisol, aldosterone and dehydroepiandrosterone (DHEA) (Conaglen et al., 1984). The secretion of CRH is controlled by normal circadian rhythm as well as a complex glucocorticoid negative feedback mechanism capable of decreasing HPA axis activity at higher limbic brain regions, hypothalamus and anterior pituitary (Kapoor et al., 2006, Mastorakos and Ilias, 2003).

Pregnancy and Development of the Fetal HPA Axis

In the human fetus, development of the glands of the HPA axis occurs early and rapidly in pregnancy with detectable generation of steroids between the eight to tenth week of pregnancy (Tegethoff et al., 2009). Species that give birth to less mature offspring such as rodents have a less developed HPA axis, but experience maturity after birth (Sapolsky and Meaney, 1986). The human fetal HPA axis differs from fully developed adult HPA axis in that the fetal HPA axis undergoes significant structural changes during gestation and is under further regulation by placental control (Tegethoff et al., 2009).

The human fetal adrenal is unique in that it is comprised of three distinct regions consisting of the fetal, definitive, and transitional zones. The fetal zone is detectable by the eighth week of primate gestation. It is the primary area of adrenal enlargement and is responsible for the synthesis of DHEA that is then utilized in the production of fetal estrogens, while the definitive and transitional zones produce mineralocorticoids and glucocorticoids respectively (Kapoor et al., 2006; Mastorakos and Ilias, 2003). The fetal hypothalamus exhibits detectable releases of neuropeptides by the 11.5 week of gestation, while the pituitary reaches maturity by the eighth week of gestation (Tegethoff et al., 2009).

Similarly to human fetal HPA axis development, the ovine fetus HPA axis develops early and rapidly in pregnancy. The production of CRH by the ovine fetal hypothalamus has been observed as early as day 63 of gestation and by day 90 in isolated PVN and median eminence neurons (Brieu et al., 1988). AVP in the ovine hypothalamus is detectable by gestational day 63 and as early as day 42 in isolated PVN and median eminence neurons with an increase in abundance observed progressively until gestational day 143. However, the ratio of AVP to CRH decreases as the fetus approaches term. Indicating that AVP is likely a more potent stimulator of ACTH than CRH in early gestation while CRH becomes the dominate stimulator of ACTH release in late gestation (Brieu et al., 1988). A functional hypophyseal portal system between the ovine hypothalamus and the pituitary is present by gestational day 45 and pituitary ACTH production is observed as early as gestational day 40 (Levidiotis et al., 1987). The ovine adrenal gland is recognizable by gestational day 28 (Wintour et al., 1975) and the weight of the adrenal gland increases exponentially in the last 15-20 days of

gestation in two phases of growth. The first phase of adrenal growth occurs by adrenal hypertrophy between 124-136d and the second phase occurs through both hypertrohic and hyperplasic growth from gestational day 130 until birth (Durand et al., 1980). The primary ovine glucocorticoid cortisol secreted by the fetal adrenal is also observed to increase in the final 25 days of gestation with highest concentrations observed during the last 48h before birth (Bassett and Thorburn, 1969; Elsner et al., 1980).

Regulation and Action of Cortisol

Cortisol is a potent glucocorticoid and exerts great influence on fetal organ development and maturation necessary for extrauterine life by binding with high affinity to either the glucocorticoid receptor (GR) or the mineralocorticoid receptor (MR). The activity of a corticosteroid such as cortisol occurs by first binding to either MR or GR located at target organs to form a corticosteroid receptor complex. The corticosteroid receptor complex then dimerizes and translocates to the nucleus where it binds to hormone response elements located on genomic deoxyribonucleic acid (DNA). The receptor complex then acts as a transcription factor to upregulate the transcription of messenger ribonucleic acid (mRNA) molecules that direct translation of specific proteins necessary for organ development and maturation (Bizzarro and Gross, 2004). Through this mechanism, cortisol regulates cardiovascular, neurobiological, and immunological development and exerts organ specific maturation of the fetal lungs, liver and digestive tract (Tegethoff et al., 2009).

Cortisol is elevated in times of stress, but is also increased during pregnancy (Mastorakos and Ilias, 2003). In instances of insufficient cortisol during pregnancy; fetal growth, uterine blood flow, placental morphology and fetal hypoxia may result (Jensen et al., 2005). Conversely, excessive cortisol during pregnancy has been shown in late

gestation ovine fetuses to produce increased fetal heart mass by increasing hyperplastic cardiomyocyte proliferation (Giraud et al., 2006). Thus, cortisol has profound effects on fetal development and correct regulation of cortisol is critical to insure proper fetal development and survival in the extrauterine environment.

The biological activity of endogenous cortisol is regulated greatly by11β-hydroxysteroid dehydrogenase (11βHSD-1) and 11βHSD-2. Cortisol is converted to biologically inactive cortisone in a unidirectional manner by the action of 11βHSD-2 while cortisone is converted to biologically active cortisol bidirectionally by 11βHSD-1. In both the adult animals and developing fetus, tissue specific concentrations of 11βHSD-1 and 11βHSD-2 function to regulate access to MR and GR. In the kidney, high amounts of 11βHSD-2 ensure that cortisol is inactive and that abundant MR receptors are available to bind with the MR specific agonist aldosterone (Ferrari and Krozowski, 2000). Organs such as the heart that are both GR and MR rich contain only a small relative fraction of the 11βHSD-2 mRNA that is present in the kidney, suggesting that cortisol plays a greater regulatory role in these tissues (Reini et al., 2006). The fetal lung also has abundant expression of GR, MR and 11βHSD-1, suggesting that endogenous cortisol would similarly occupy both receptors in this organ (Keller-Wood et al., 2009).

Fetal Cortisol Surge

At term, fetal ACTH plasma concentrations increase even in the presence of high circulating glucocorticoid concentrations and this increase is likely due to changes in glucocorticoid feedback sensitivity (Kapoor et al., 2006; Wood, 1988). This disruption in normal feedback regulation causes the fetal adrenal to precipitate a cortisol “surge” that precedes parturition in order to facilitate rapid maturation of the lungs, liver, kidneys and brain (Liggins, 1994). In the lung, this increase in plasma cortisol acts to increase

surfactant production, engender structural maturation, and mediate lung fluid metabolism through GR and possibly MR dependent mechanisms (Bizzarro and Gross, 2004; Jobe, 2010).

Fetal Respiratory Distress

Preterm infants are predisposed to increased risk of lung immaturity and development of respiratory distress (RD) that can manifest in numerous conditions that include: respiratory distress syndrome (RDS), transient tachypnea of the newborn (TTN), meconium aspiration syndrome (MAS), and bacterial infection. TTN is the most common cause of respiratory distress in term neonates and is implicated in 40% of instances of neonatal RD (Hermansen and Lorah, 2007). The condition is typically benign and occurs when there is an excess of fluid in the lung and presents as neonatal shallow rapid breathing.

RDS is the most common cause of respiratory distress in neonates born prematurely, affecting the majority of fetuses born before 28 weeks gestational age and 1/3 of fetuses born between 28-34 weeks gestational age. RDS is attributed to lung structural immaturity and insufficient surfactant production engendering, decreased compliance, increased alveolar surface tension, and areas of atelectasis in the fetal lung (Hermansen and Lorah, 2007). At areas of atelectasis, localized ischemia and ventilation perfusion mismatch occurs, resulting in vascular constriction at atelectatic areas and shunting of blood by pulmonary and extrapulmonary means. This shunting engenders the circulation of poorly oxygenated mixed venous blood and is evident by subsequent fetal hypoxia and cyanosis observed in fetuses with RDS (Verma, 1995). While surfactant insufficiency associated with RDS produces a pathological overall decrease in surface area available for gas exchange, preterm lung structural immaturity

further contributes to the RDS pathology and decreases in surface area available for gas exchange. RDS is a serious life threatening condition that requires immediate supportive therapy and is one of the most common causes of preterm infant mortality (Hermansen and Lorah, 2007; Verma, 1995).

RD can occur in term and post term infants by evacuation of meconium into the amniotic fluid and subsequent aspiration of this meconium stained amniotic fluid through fetal breathing movements resulting in MAS (Nkadi et al., 2009). MAS is a significant contributor to infant morbidity and mortality and the presence of meconium stained amniotic fluid is indicative of fetal distress. Meconium is a mixture of gastrointestinal secretions and aspiration of meconium obstructs airways, produces ventilation perfusion mismatches, inhibits pulmonary surfactant, and elevates proinflammatory cytokines (Donn and Dalton, 2009). While meconium is generally sterile, it does serve as an effective medium for bacterial growth in the extrauterine environment. Bacterial infection secondary to MAS or otherwise is another significant contributor to neonatal RD. Unlike other causes of RD, bacterial infection takes hours to days to develop in neonates and presents with elevated temperature in addition to the symptoms of respiratory distress (Hermansen and Lorah, 2007).

Synthetic Glucocorticoids and the Attenuation of Respiratory Distress

The original pioneering work of Dr. Graham Liggins, conducted in 1969, demonstrated that antenatal administration of synthetic glucocorticoids in ovine fetuses increased lung viability and decreased neonatal morbidity and mortality by accelerating the induction of surfactant production (Liggins, 1969). Glucocorticoid acceleration of fetal lung surfactant production was later confirmed in studies utilizing twin ovine fetuses in which one fetus served as a saline infused control while the other fetus received an

infusion of betamethasone (DeLemos et al., 1970). The results of the first control trial examining the role of synthetic glucocorticoids, for the attenuation of fetal RD in human subjects, were published in 1972. This study showed that the antenatal administration of the synthetic glucocorticoid betamethasone to pregnant women in danger of delivering prematurely decreased the incidence of fetal RD from 25.8 % in control subject to 9.0 % in subjects treated with synthetic glucocorticoids (Liggins and Howie, 1972). In the decades following these pivotal studies, the use of synthetic glucocorticoids and surfactant replacement therapy has become common practice by obstetricians and neonatologists for the prevention and treatment of fetal RD (Hermansen and Lorah, 2007).

Synthetic glucocorticoids such as dexamethasone differ from endogenous glucocorticoids in that they have approx 25 times higher affinity for GR than that of endogenous cortisol and very low affinity for MR, while cortisol retains affinity for both MR and GR (Rupprecht et al., 1993). Synthetic glucocorticoids and endogenous glucocorticoids further differ in their duration of biological activity, with betamethasone and dexamethasone having long acting characteristics between 36 to 54h duration and cortisol with relatively shot biological activity of 8 to 12h (Melby, 1977). Both endogenous and synthetic glucocorticoids are able to cross from the maternal circulation to the fetus via the placenta (Moss et al., 2003), but differ in that cortisol is actively metabolized by placental 11βHSD-2 into inactive cortisone, while synthetic glucocorticoids are not readily metabolized by 11βHSD-2 (Kapoor et al., 2006). Thus, synthetic glucocorticoids readily cross the placenta, are biological active and capable of exerting profound influence at GR rich fetal organs. While the importance of

endogenous and synthetic glucocorticoids to accelerate lung structural maturation and surfactant production clearly affects the biomechanical properties of the lung, the ability of glucocorticoids to accelerate lung fluid reabsorption shortly before birth also exerts profound influence on fetal lung function.

Fetal Lung Fluid Secretion

Throughout the majority of gestation, the fetal lung is a secretory organ producing lung liquid in a mechanism dependent upon the action of epithelial chloride channels secreting Cl^- ions against their electrochemical gradient by active transport with a passive secretion of Na^+ ions and interstitial fluid along this gradient into the intrapulmonary space (Olver and Strang, 1974). While mixing of lung liquid with amniotic fluid occurs during fetal breathing movements, the majority of lung liquid is contained inside the lungs by the closure of the vocal cords, larynx, and nasopharynx creating a positive intrapulmonary pressure (Fewell and Johnson, 1983). Maintenance of this positive pressure is necessary to prevent the collapse of developing lung structures and for normal lung development to occur. Hypoplastic lung growth occurs in instances of oligohydramnios, when amniotic fluid and lung liquid is depleted (Moessinger et al., 1986). Conversely, laryngeal atresia produces excessive lung liquid retention that results in an increased intrapulmonary pressure and hyperplastic lung growth (Wigglesworth et al., 1987). This effect was demonstrated using ovine fetuses in which one lung lobe was drained of lung liquid while the other lobe was ligated in order to increase lung liquid volume. The ligated lobe experienced hyperplastic growth while the drained lobe experienced hypoplastic growth (Moessinger et al., 1990). Thus demonstrating that proper balance of lung liquid throughout gestation is essential for normal lung development.

Fetal Lung Fluid Reabsorption

While lung liquid production and volume increases throughout the majority of gestation, shortly before birth, lung liquid production rapidly decreases with concomitant increases in lung liquid reabsorption and pulmonary perfusion to facilitate the transition of the fetal lung to effectively participate in gas exchange in the extrauterine environment (Bland, 1983; Kitterman et al., 1979; Pfister et al., 2001). The reabsorption of lung liquid is evident in studies involving newborn rabbits that have shown significant decreases in lung weight during the first two hours following parturition with further decreases throughout the first day of extrauterine life (Bland et al., 1980). In ovine fetuses, lung liquid production has also been shown to decrease in the days preceding term vaginal birth (Fewell and Johnson, 1983; Kitterman et al., 1979). However, rabbit fetuses born prematurely by cesarean birth before the onset of labor have excessive lung liquid, thus emphasizing the importance of the periparturient absorption of lung liquid and the beneficial effects of labor (Bland et al., 1979).

Epithelial Sodium Channel (ENaC), Sodium Potassium Adenosine Triphosphatase (Na, K ATPase), Serum Glucocorticoid Regulated Kinase (SGK) and Regulation by Glucocorticoids

The mechanism by which lung liquid is reabsorbed at birth is complex and its regulation is not fully understood, however it is known that glucocorticoids (synthetic or endogenous) effect fluid reabsorption by acting at GR in the fetal lung to increase transcription of the amiloride sensitive epithelial sodium channel (ENaC) (Itani et al., 2002; Venkatesh and Katzberg, 1997). In the lung, ENaC channels are abundantly present on type I and type II pneumocytes that compose the lung alveolar epithelium and function to actively transport Na^+ ions across the epithelium into the lung interstitial space. Transport of Na^+ ions through ENaC channels is facilitated by a favorable

electrochemical gradient generated by the activity of sodium potassium adenosine triphosphatase (Na,K-ATPase) pumps located on the basolateral surface of lung pneumocytes that pump intracellular Na^+ into the interstitial space at the expense of adenosine triphosphate (ATP) (Matalon, 1991). The active transport of Na^+ ion across the alveolar epithelium then precipitates a secondary active transport of an osmotic equivalent amount of Cl^- ions through cystic fibrosis transmembrane regulator (CFTR) and Cl^- channels located on the epithelial apical surface. Water then follows the osmotic gradient generated by the primary active transport of Na^+ ions and secondary active transport of osmoticly equivalent Cl^- ions across the apical surface and exits the epithelium into the interstitum passively through aquaporins and transcellular pathways (Eaton et al., 2009). The pathway and mechanism of fluid reabsorption in the lung epithelium is illustrated in figure 1-1.

The maintenance of the electrochemical gradient between the apical and basolateral membrane, and consequently the balance of fluid secretion and reabsorption, is dependent on the presence of tight junctions between type I and type II pneumocytes acting as a barrier to passive flux of ions and water. The importance of tight junction resistance to passive secretion of ions and fluid is evident in pathologies that increase pulmonary blood pressure or cause inflammation that affects the permeability of these junctions. The resultant increase in junction permeability increases passive secretion of fluid through these junctions and pulmonary edema occurs (Koval et al., 2010).

The presence of ENaC in the human lung has been detected as early as the embryonic stage of development on alveolar bud epithelium and is later dispersed

throughout the lung in late gestation (Smith et al., 2000). Studies utilizing cultured human fetal lung found glucocorticoids act to increase mRNA expression of α, β, and γ ENaC subunits by 2-3 fold and RNA polymerase II inhibitors attenuate this increase in ENaC mRNA expression (Venkatesh and Katzberg, 1997). While ENaC are three subunit domain protein channels, the alpha subunit is critical for adequate fluid clearance following parturition. Inactivation of the a-ENaC gene in mice has been shown to result in the development of RD and death within 40h following parturition from an inability to clear the lungs of fluid (Hummler et al., 1996). Down regulation of the β-ENaC subunit has also been shown to have deleterious consequences in mice. In β-ENaC knockdown mice, expression of β-ENaC was shown to be diminished by real-time polymerase chain reaction (PCR) and β-ENaC protein was not detectable by western blot. However, both the α and γ subunits of ENaC were shown to have increased expression in these animals. Measurements of lung liquid clearance showed a 32% decreased clearance ability than wild type mice despite the compensatory increase in α and γ ENaC subunit expression. These data imply that a complex stoichiometric relationship of the three subunits greatly affects the efficacy of ENaC channels (Randrianarison et al., 2008).

The activity of ENaC is further regulated by serum glucocorticoid regulated kinase (SGK) and the expression of SGK is principally regulated by MR and GR agonists. In vitro data utilizing bronchiolar epithelial and alveolar type II cells confirmed that exposure of these cells to corticosteroids increases expression of SGK mRNA (Itani et al., 2002). ENaC membrane proteins are subject to ubiquitylation by the ubiquitin protein ligases Nedd4-1 and Nedd4-2. Nedd4 attachment of ubiquitin polypeptides

inactivates ENaC protein by signaling intracellular pathways to internalize the protein from the plasma membrane and to then target ENaC for destruction. SGK increases ENaC activity and inhibits the activity of Nedd4 ubiquitin protein ligases by phosphorylating Nedd4 which attenuates the ability of Nedd4 to ubiquitinate ENaC (Snyder et al., 2002).

ENaC Activity in the Kidney and the Role of Aldosterone

Renal regulation of sodium reabsorption and excretion is critically important for the maintenance of blood pressure and extracellular volume homeostasis. Sodium reabsorption in the kidney is thought to occur by a mechanism similar to that of fetal lung liquid reabsorption and is likewise dependent upon the action of apical ENaC channels, basolateral Na,K-ATPase and SGK (Fuller and Young, 2005). The importance of ENaC in renal sodium regulation for proper volume homeostasis is evident in conditions of ENaC loss of function mutations (pseudohypoaldosteronism) that result in salt wasting or gain of function mutations (Liddle's syndrome) that cause excessive salt reabsorption and severe hypertension (Rossier and Stutts, 2009). While sodium is reabsorbed in multiple locations throughout the nephron, the principal area of the nephron that dictates the ultimate sodium concentration of urine is comprised of: the distal convoluted tubule, connecting tubule, and collecting duct. Together these structures are considered the aldosterone sensitive distal nephron (ASDN) and account for less than 10% of total filtered sodium reabsorption (Loffing et al., 2001). In the kidney, there is a preponderance of 11βHSD-2 that acts to attenuate the activity of glucocorticoids such as cortisol that bind with high affinity to MR and GR. By inactivating endogenous cortisol to cortisone, kidney sodium reabsorption is principally regulated by the MR agonist aldosterone binding to MR and subsequently acting at hormone

response elements to increase the transcription of ENaC, Na,K-ATPase, and SGK (Fuller and Young, 2005).

Prospective Study Rationale

While the importance of MR action in regulating sodium reabsorption and extracellular fluid homeostasis is evident in the kidney, MR may be equally important in mediating lung fluid reabsorption at birth (Fuller and Young, 2005). A study utilizing ex vivo whole lung preparations from near term guinea pigs found that low concentrations of the MR agonist aldosterone induced fetal lung liquid reabsorption. This effect was attenuated in the presence of the MR antagonist spironolactone, and spironolactone alone did not affect lung liquid production (Kindler et al., 1993). Aldosterone has been shown to mediate renal sodium reabsorption and extracellular volume homeostasis by increasing expression of ENaC, Na,K-ATPase, and SGK in an MR dependent mechanism (Loffing et al., 2001). When one considers the aldosterone mediated attenuation of lung fluid production, it is not unreasonable to hypothesize that stimulation of fetal lung MR, by MR agonists such as cortisol and/or aldosterone, could also induce a similar mechanism of sodium and fluid reabsorption in the fetal lung as that observed in the kidney.

The maturational benefits of the preparturient cortisol surge are evident in term fetuses delivered by vaginal birth that exhibit greater lung maturation, surfactant production and lowered risk of developing RD compared to fetuses delivered pre maturely and/or by cesarean section (Bizzarro and Gross, 2004; Bland et al., 1979). Endogenous cortisol possessing high affinity for both MR and GR would likely act at both receptors in target organs such as the fetal lung and exert a maturational influence that is both MR and GR mediated. However the general paradigm and clinical approach

of antenatal corticosteroid therapy involves the administration of synthetic glucocorticoids with specificity for only GR, under the assumption that the lung maturational influence of corticosteroids occurs through primarily GR mediated pathways (Gibbs, 2009; Itani et al., 2002).

A study of ontogenetic gene expression in ovine fetuses conducted by Keller-Wood et al, has shown by quantitative real-time PCR that expression of lung MR and Na,K-ATPase mRNA increases significantly at 130d gestation, followed by a significant increase in α-ENaC, 11βHSD-1, and SGK at 145d mRNA. Western blot analysis of membrane enriched lung protein extracts from these fetuses also showed an increase in α-ENaC and Na,K-ATPase protein at 130 days and an increase in whole cell protein SGK at 120d that is sustained for the remained of gestation (Keller-Wood et al., 2009). These data indicate both a marked transcriptional and translational increase in the cellular machinery necessary for fluid reabsorption that preceded parturition, with concomitant increases in MR and 11βHSD-1, implying that endogenous cortisol would retain its activity and exert influence at lung MR to regulate lung fluid homeostasis.

It is accepted that synthetic glucocorticoids have potent and long lasting biological activity, and nearly 40 years of empirical evidence supports the assertion that antenatal administration of synthetic glucocorticoids reduces fetal RD, stimulates lung maturation and increases preterm fetal viability (Gibbs, 2009; Helve et al., 2009; Itani et al., 2002; Jobe, 2010; Liggins and Howie, 1972, Liggins, 2000). However, data exist that indicates a possible MR mediated mechanism of fetal lung maturation that has not been adequately investigated (Kindler et al., 1993). Furthermore, it is possible that combined antenatal administration of MR and synthetic GR agonists will manifest a synergistic

benefit as a consequence of these two agonists acting as a biological mimetic of endogenous cortisol without the deleterious inactivation of antenatally administered cortisol by placental 11βHSD-2.

The objectives of this thesis were to determine if antenatal administration of MR, GR and combined MR/GR agonists to 130day ovine fetuses will increase the mRNA expression of genes and the abundance of proteins involved in the absorption of lung liquid, determine if MR agonists induce lung structural maturation, and ascertain if MR agonists improve lung compliance. In order to answer the specific aims of this thesis the following techniques were utilized; in vivo chronic catheterization of fetal sheep, in situ lung pressure measurements, tissue histology, radioimmunoassay (RIA), quantitative real-time PCR, and western blotting.

Specific Aims

Specific Aim 1: Using chronically catheterized 130d gestational age ovine fetuses, determine the effect of 48h antenatal administration of aldosterone, betamethasone, and aldosterone combined with betamethasone on ovine fetal lung compliance by in situ lung pressure measurements.

Specific Aim 2: To determine by quantitative real time PCR the effect of 48h corticosteroid infusion on mRNA expression of genes important in lung function, lung liquid reabsorption and lung structural maturation.

Specific Aim 3: To determine by Western blotting the effect of 48h corticosteroid infusion on abundance and location of α-ENaC and β-ENaC proteins using both membrane enriched and whole cell protein extracts of lung tissues.

Specific Aim 4: To use tissue histological staining methods to determine the effect of 48h corticosteroid infusion on elastin and collagen fiber abundance in lung parenchyma.

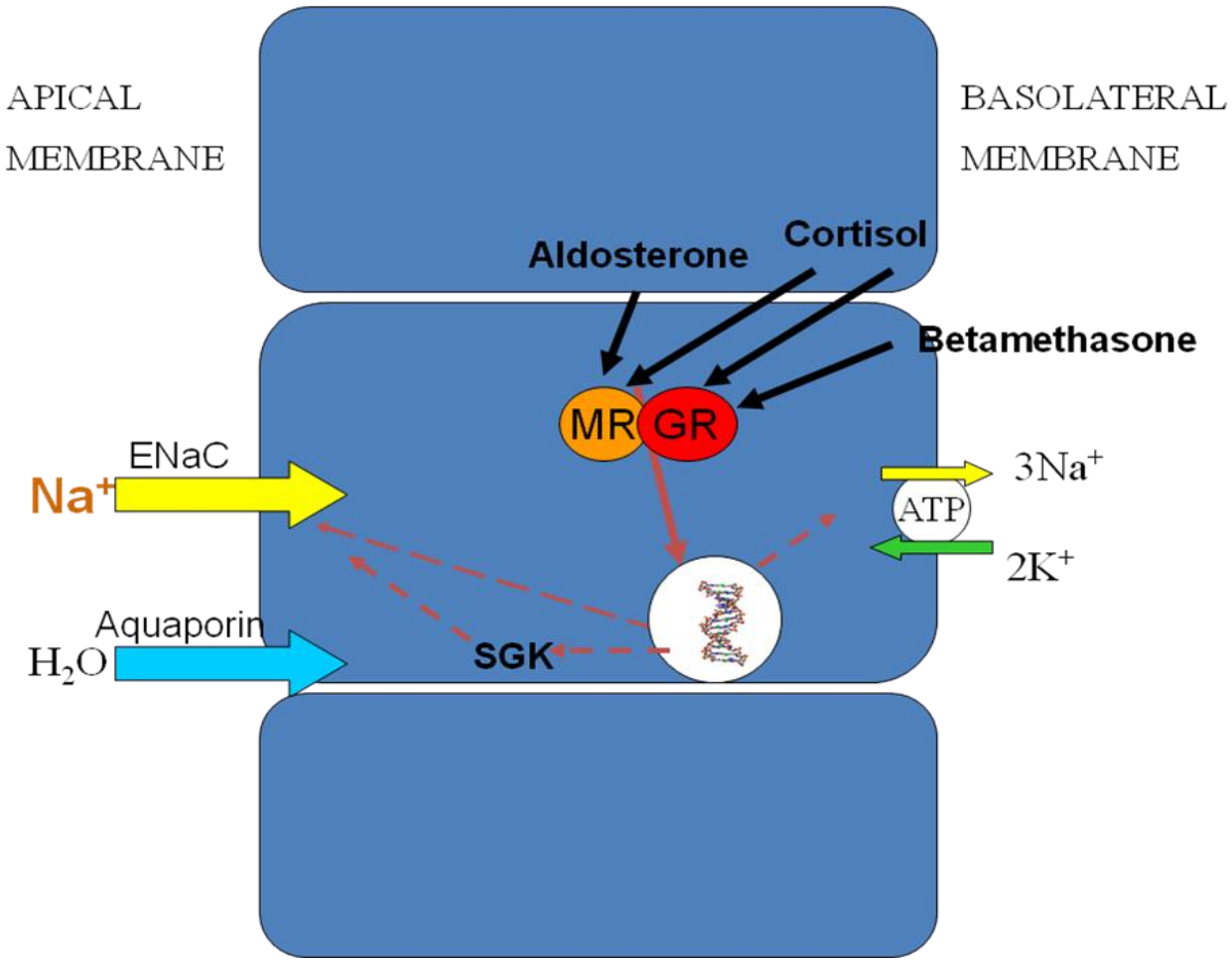

Figure 1-1. Proposed mechanism of lung fluid reabsorption across fetal lung epithelium

CHAPTER 2
EXPERIMENTAL METHODS

Surgical Procedures

As this study involved studying fetal lung development in utero, an analogous animal model of human fetal lungs was necessary. Ovine fetal lungs are more analogous to human fetal lungs than other animal species such as rabbit (Kikkawa et al., 1968), mouse (Amy et al., 1977), and rat (Burri et al., 1974). Compared to human and ovine fetuses, all of these species have large saccular structures at birth and lack considerable mature alveoli. The ovine lung is therefore a more appropriate analog for human lung development than these other species with the notable exception of non human primates. All animal use was approved by the University of Florida Institutional Animal Care and Use Committee and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Isoflurane (2-3%) in oxygen was used to anesthetize ewes before and during surgery. An incision was made along the ewe's abdomen to access the uterus, and a second incision was made in the uterus to access the fetus. The fetus's hind legs were then removed from inside the uterus and tibial artery and vein catheters were inserted. A catheter was then sutured to the leg for measurement of amniotic fluid and this catheter was placed inside the uterus along with the fetus at the end of the procedure. When twin fetuses were present the procedure was repeated in the twin. The uterus was then closed with umbrication of the myometrium with silk suture (Ethicon, Somerville NJ) and the fetal catheters were routed subcutaneously along the abdomen and out of an exit site in the ewes flank. After closure of the uterus and abdominal incision, catheters were placed in the maternal femoral artery and vein and exited along

with the fetal catheters through the maternal flank. The catheters were secured in a custom pouch to the ewe's flank. Upon completion of the surgical procedure all ewes were treated with Banamine (1 mg/kg IM; Fort Dodge Animal Health, Fort Dodge, IA) before recovery from anesthesia. The ewe was then returned to her individual pen and provided water, and salt blocks ad libitum. Ruminant pellet food was weighed to 2.5kg and placed in the pen with the ewe after the animal had completely recovered. Polyflex (500mg SC bid; Fort DodgeAnimal Health, Fort Dodge, IA) was administered for 3 days postoperatively. Banamine 1 mg/kg IM was again administered on the morning after surgery. Daily food intake was monitored on each postoperative day.

Experiment Procedures

Fetuses from 17 twin and 5 singleton pregnancies were catheterized at 122-124 days gestational age. After at least five days of recovery after surgery, (127-131 days gestation) fetal and maternal blood samples (approx 7 ml) were withdrawn for determination of blood gases, hematocrit, electrolytes, and plasma hormones (aldosterone and cortisol) concentrations. All blood samples were taken immediately after entering the room in which the ewes were housed in order to minimize the effect of handling on plasma adrenocorticotropic hormone (ACTH) and cortisol concentrations. A fetal vein catheter was subsequently used to deliver an infusion of steroid to the fetus over the following 48h. Infusions were delivered using a syringe pump at a rate of 1.45 ml/h. Fetuses in the five experimental groups were infused with a solution containing the mineralocorticoid receptor (MR) specific agonist aldosterone (0.2 mg/48h; n=5), the synthetic glucocorticoid receptor (GR) specific agonist betamethasone (0.25 mg or 0.75 mg 48h; n=4, n=4 respectively), or a combined 0.2mg aldosterone and betamethasone solution with both 0.25mg and 0.75mg betamethasone doses (n=4, n= 5 respectively).

The higher betamethasone dose consisted of a 0.25mg initial bolus of betamethasone followed by infusion of betamethasone 0.48 mg/48h (total dose 0.75 mg/48h; n=4). Control experiments were performed in twin pregnancies, one fetus received corticosteroids while the untreated fetuses served as controls (n=9 after exclusion of any hypoxic twins). The doses of steroids were chosen to simulate physiologic increases in corticosteroids based on relative efficacy of the agonists at MR and GR and the clearance of these agonists. Calculations of fetal GR occupancy after betamethasone infusion that considered fetal weight, betamethasone clearance rate, percent bound synthetic glucocorticoid and infusion rate, determined that 100% of GR would be expected to be activated by both 0.25mg and 0.75mg doses of betamethasone (Richards et al., 2003). Calculations of fetal MR occupancy found that approx 85% of MR receptors would be expected to be occupied by aldosterone alone, however it is likely that all MR receptors were occupied in these fetuses as endogenous cortisol will also occupy MR as well (Richards et al., 2003; Zipser et al., 1980).

After 48hours of infusion, fetal and maternal blood samples were collected (7ml) and analyzed for blood gases, hematocrit, electrolytes, and plasma hormones (aldosterone and cortisol) concentrations. Fetal blood pressure(s) and heart rate(s) were recorded over a 40 minute interval using LabView software (National Instruments, Austin, TX) and disposable pressure transducers (Transpac; Hospira, Lake Forest, IL). Amniotic fluid pressures were subtracted from fetal arterial pressures in order to normalize fetal arterial pressure. Following the blood pressure recording, fetal blood samples were collected again (7ml) and analyzed as before.

The ewe and fetus was killed with an overdose of pentobarbital (Euthasol solution, (Virbac AH, Fort Worth, TX). Immediately after euthanizing the ewe, the fetus(es) were removed from the uterus and the sex of the fetus was recorded as well as fetal weight. The fetus was then placed on a sterile pad and the chest was exposed to air. An incision was made in the upper fetal trachea and a 4mm endotracheal tube with an inflatable cuff was then inserted into the trachea and the cuff was inflated. The upper lobe of the right lung was then ligated and the endotracheal tube was connected via a three way stop cock to both a 60 ml syringe and to a pressure transducer (Transpac; Hospira, Lake Forest, IL). Pressure measurements from the transducer were recorded in real-time using LabView software (National Instruments, Austin, TX). With the chest wall open, lung compliance was then determined by measuring the airway pressure responses to injections of five 10ml boluses of air into the trachea for a total of 50ml of air infused into the lung. The lung was then allowed to equilibrate to room pressure after opening the three-way stopcock to air and then a second series of inflations were performed. After pressure data recording was complete and the endotracheal tube was removed, individual samples of lung tissue from the right lobe were collected and flash frozen in liquid nitrogen for messenger ribonucleic acid (mRNA) and protein extractions. These samples where then transferred to a -80 C freezer for long term storage. A sample of right lobe lung tissue was also collected and fixed in a 4% buffered paraformaldehyde solution for histology. The entire left lobe was collected, weighted to determine initial weight before baking in a 200 ˚C oven for 24h and then reweighed after baking to determine lung percent wet weight.

Experiment Analysis

Fetal blood gases and pH were measured with a blood gas/electrolyte analyzer (ABL77;Radiometer America, Westlake, OH). Maternal and fetal plasma electrolytes (sodium and potassium) were measured using an electrolyte analyzer (Roche 9180, Basel, Switzerland). For measurement of packed cell volume (PCV), blood was spun in microcapillary tubes for 3 minutes at 12,000 rpm (Damon Division, International Equipment, Needham Heights, MA). Plasma protein was determined using a refractometer. Fetal plasma cortisol (EA 65, Oxford Biomedical, Oxford, MI) and aldosterone (TKAL2, Siemens, Deerfield, IL) concentrations were measured using commercially available enzyme immunoassay and radio immunoassay kits respectively. Electrolyte, PCV, plasma protein, plasma cortisol, plasma aldosterone, and lung percent wet weight data were analyzed for differences among treatment groups by two-way analysis of variance (ANOVA) using Sigma Stat software (Systat Software Inc, Chicago, IL) The pulmonary airway pressure data was analyzed by determining peak pressures for each inflation volume and analyzing the relaxation curve following each volume peak pressure. The relaxation curve data was fit to a 3 parameter exponential decay regression curve: $y= y0 + ae-bx$, and the value of the parameter y0, representing the steady state pressure in the relaxation phase after each inflation was determined. Differences among treatment group for both peak pressure and y0 were analyzed by 2 and 3 way (ANOVA) corrected for repeated measures using Sigma Stat and SPSS software (SPSS Inc, Chicago, IL).

Western Blotting

Immunoblot detection with antibodies to epithelial sodium channel α-(ENaC), β-ENaC, and β-Actin proteins were performed on whole cell and membrane protein

fractions isolated from fetal lung samples. Tissue samples (0.5–1.0 g) were pulverized over dry ice, and placed in a cold Potter-Elvehjem tissue grinder with 2 volumes of ice-cold sucrose buffer (1 mM ethylenediaminetetraacetic acid disodium salt, 0.32 M sucrose, 1 mM (N -[2-hydroxyethyl]piperazine-N -[2-ethanesulfonic acid]), pH 7.4, 1: 100 protease inhibitor cocktail, Sigma P8340) and homogenized with three up and down strokes. The slurry was spun at 500g, at 4 ° C for 5 minutes. 300µl of the supernatant was mixed with 300µl of 2X lysis buffer (4% sodium dodecyl sulfate, 20% glycerol, 125 mM (Tris[hydroxymethyl]-aminomethane) pH 6.8, 5% 2-mercaptoethanol) and boiled for 5 minutes. The tubes were spun at 11,300 g for 5 minutes at room temperature. The supernatant was then aliquoted and frozen at –80°C for analysis of whole cell protein contents. The remaining supernatant from the 500g spin was spun at 5,000g for 5 minutes at 4°C. The supernatant was collected and spun at 45,000g for 20 minutes at 4°C. The pellet was reconstituted with an equal volume of 2X lysis buffer. The samples were placed in a warm water bath for 10 minutes, aliquoted and stored at –80°C for analysis of membrane protein contents.

Protein concentrations for whole cell and membrane extracts utilized in immunoblotting for α-ENaC, β-ENaC and β-actin were determined by protein assay using Biorad DC protein assay, (Bio-Rad, Hercules, Calif., USA) with bovine serum albumin (BSA) as the standard. The samples were diluted 1:100 to reduce interference from lysis buffer components and the standards were run with the same lysis buffer concentrations as the samples.

All samples were run on an 18 well 7.5% Tris-polyacrylamide gels at 150 V for approximately for 1 hour. Lanes containing molecular weight standards (Bio-Rad Dual

Color Standard) were also included on each gel. Sample sets required four gels and were run with a subset of samples from all treatment groups on each gel, and then transferred and developed at the same time. All gels were transferred at 100 V for 1 hour to 0.45- μm nitrocellulose membranes. All blots were blocked with a 10% non-fat dairy milk in Tris-buffered saline Tween-20 solution and exposed to appropriate primary antibodies for the α-subunit of ENaC (Abcam, Cambridge, Mass., USA; 3464; 1: 100 in blocking solution and 3% goat serum overnight), β subunit of ENaC (Alpha Diagnostics, San Antonio, TX, ENaCb21-A; 1:1,000) or the primary antibody for β-actin (Sigma, St. Louis,Mo., USA; A-5441; 1: 20,000 in blocking solution with 1% BSA for1 h). A goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma A0545; 1: 20,000) was used for α-ENaC and β-ENaC immunoblots. A rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody was used for β-actin immunoblots (Sigma, A9044; 1: 16,000). Immunoreactive proteins were visualized with electrochemiluminescence (ECL) detection reagents (GE Healthcare, Piscataway, N.J., USA) and developed with Kodak BioMax XAR film.

Western Blotting Analysis

The blots were analyzed with a Bio-Rad Chemi-Doc system and Quantity One software. The results of the densitometric analysis were then expressed as relative optical density units to β-actin or to total protein determined by Ponseau S staining (0.1% Ponseau S in 5% acetic acid solution) of the nitrocellulose membrane. The changes between treatment groups in abundance of the protein of interest were analyzed by two-way ANOVA of normalized optical density values.

Messenger Ribonucleic Acid (mRNA) Extraction and Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total ribonucleic acid (RNA) was extracted from lung tissue samples prepared over dry ice. Samples were homogenized in Trizol (Invitrogen, Carlsbad, CA) with an Ultra Turrax T25 tissue homogenizer (IKA Works, Inc., Wilmington, NC). Total RNA was then purified using Qiagen RNeasy Plus Mini Kit genomic deoxyribonucleic acid (gDNA) eliminator spin columns according to manufactures instructions. Correct usage of these kits has been demonstrated in our lab to yield high quality RNA. RNA was checked for genomic DNA contamination by real-time PCR using RNA extracted from lung tissues as a template in place of complementary deoxyribonucleic acid (cDNA) with probes and primers for GR (which produces a product within exon 2). No detectable GR amplification occurred after 40 cycles, while GR was detected in cDNA made from these RNA extracts at approx 30 cycles. Total RNA concentration was determined for each sample, as well as the (A260/A280) RNA/DNA to protein ratio using a Nanodrop spectrophotometer (Wilmington, DE) to assess the quantity and quality of RNA. RNA was then reverse transcribed into cDNA using a high capacity cDNA archive kit (Applied Biosystems, Carlsbad, CA) according to the manufactures instructions. cDNA was then aliquoted and dilutions were made for use in quantitative real-time PCR reactions. Gene expression was then measured using quantitative real-time PCR for each sample in triplicate for the following genes; beta actin (β-actin), serum glucocorticoid regulated kinase (SGK1), epithelial sodium channel (α-ENaC), sodium potassium adenosine triphosphatase (Na, K ATPase-α1), mineralocorticoid receptor (MR), glucocorticoid receptor (GR), aquaporin 5 (AQP5), aquaporin 1 (AQP1), surfactant related protein a (SP-A), and surfactant related protein b (SP-B).

Previously published sequences of ovine probes and primers were used for MR, SGK1, GR, α-ENaC (Keller-Wood et al., 2005), Na,K ATPase-α, β-Actin (Keller-Wood et al., 2009), AQP1 and AQP5 (Jesse et al., 2009). Primer Express 2.0 (Applied Biosystems, Carlsbad, CA) software was used to design probes and primers for SP-A and SP-B based on ovine sequences in the National Center for Biotechnology Information (NCBI) database. For SP-A probe and primer design, the ovis aries pulmonary surfactant-associated protein a sequence was used (accession number NM_001009728). The amplified sequence corresponds to (base pairs 62-136). For SP-B probe and primer design, the ovis aries pulmonary surfactant protein b sequence was used (accession number AF211857). The amplified sequence corresponds to (base pairs 445-514) and primer specificity was determined by BLAST analysis.

Quantitative Real Time PCR Analysis

All quantitative real-time PCR reactions were preformed in an ABI PRISM 7000 sequence Detection System (Applied Biosystems, Carlsbad, CA). Reactions were carried out with either 20 or 100ng of cDNA template; and all gene cycle threshold (Ct) values were normalized to β-actin Ct by calculating ΔCt. ΔCt was determined by calculating the difference between the mean sample Ct for the gene of interest and the mean Ct of β-Actin for the same gene. Template cDNA conc and reaction efficiency was checked for SP-A and SP-B using pooled lung cDNA from control animals. The changes in gene expression between treatment groups were analyzed by two-way ANOVA of the ΔCt values for the gene of interest.

Collagen Staining

To determine the localization and deposition of Collagen, fetal sheep lung tissues from the corticosteroid infusion study fetuses were collected and fixed in a 4% buffered

paraformaldehyde solution. The tissues were dehydrated with increasing concentrations of reagent alcohol followed by xylene, and embedded in paraffin wax. 5μm sections were cut by a Zeiss rotary microtome and placed onto tissue slides. Deparaffinization and rehydration was performed using standard methods. Sections from each fetal lung sample were stained with picrosirius red solution, 0.1% in saturated picric acid (Sigma, St. Louis,Mo., USA). Sections were hydrated and immersed in picrosirius red solution for one hour. The sections were then washed in two changes of acidified water (0.5% glacial acetic acid), dehydrated in three changes of 100% ethanol for three minutes each, cleared in two changes of xylene for ten minutes each, and mounted in permount.

Collagen Staining Analysis

All images were visualized using an Olympus DP71 microscope and Olympus software. Images of ten random fields of lung parenchyma were taken from each lung sample while avoiding blood vessels which stain heavily for collagen. The picrosirius red staining was then quantified using Image J software (NIH, Bethesda, MD). The percent area of collagen staining was calculated for each field and the average value of all ten measurements was determined. The changes in collagen content between treatment groups were analyzed by two-way ANOVA of the averaged percent area value for each sample.

Elastin Staining

To determine the localization and deposition of elastin, fetal sheep lung tissues from the corticosteroid infusion study fetuses were collected and fixed in a 4% buffered paraformaldehyde solution. The tissues were dehydrated with increasing concentrations of reagent alcohol followed by xylene, and embedded in paraffin wax. 5μm sections were cut by a Zeiss rotary microtome and placed onto tissue slides. Deparaffinization

and rehydration was performed using standard methods. Sections from each fetus were stained with Miller's solution (ScyTek Laboratories, Logan UT) which selectively stains elastin content. Sections were hydrated and immersed in 0.5% acidified potassium permanganate (Sigma, St. Louis,Mo., USA) for two minutes, rinsed in water, bleached in 1% Oxalic acid for 1 minute, washed in water, washed in 95% ethanol and then placed in Miller Solution for 1 hour. Samples were then washed in 95% ethanol and counterstained in 0.25% Tartrazine (Sigma, St. Louis,Mo., USA) in saturated picric acid solution for 1 minute. The sections were then washed in two changes of 100% ethanol, cleared in two changes of xylene, and mounted in permount.

Elastin Staining Analysis

All images were visualized using an Olympus DP71 microscope and Olympus software. Images of ten random fields of lung parenchyma were taken from each lung sample while avoiding blood vessels which stain heavily for elastin. Elastin staining was then quantified using Image J software (NIH, Bethesda, MD). The percent area of elastin staining was calculated for each field and the average value of all ten measurements was determined. The changes in elastin content between treatment groups were analyzed by two-way ANOVA of the mean percent area values for each sample.

CHAPTER 3
RESULTS

Fetal Lung Inflation Curves

Pressure curves indicative of the intrapulmonary dynamic compliance of mean peak pressure and standard error measurement at each inflation volume of corticosteroid infused fetuses and control fetuses are shown in figure 3-1. In the first inflation series, 0.2mg aldosterone treatment for 48h produced significantly lower initial mean pressure at 10ml than control fetus mean pressure at 10ml. This decreased mean pressure in aldosterone infused fetuses was again significant at 30, 40 and 50ml inflation volumes compared to control fetuses. This decreased pressure was not observed in fetuses infused for 48h with 0.25mg betamethasone (beta), 0.75mg betamethasone or 0.2mg aldosterone combined with 0.25mg betamethasone. Analysis of changes in mean pressure measurements after each volume of air infused into 0.2mg aldosterone infused fetuses showed that a significant increase in pressure occurred between 10ml and 20ml, but significant increases in pressure did not occur after 20ml. Analysis of changes in mean pressure after each successive increase in volume for control fetuses and fetuses infused with either 0.75mg beta or combined aldosterone with 0.25mg beta showed significant increases in pressure after each successive increase in volume. Fetuses infused with aldosterone and 0.75mg beta along with fetuses infused with 0.25mg beta had transient decreases in pressure at 50 and 40ml inflation volumes respectively. Treatment with combined aldosterone and 0.25mg beta did not result in the prevention of increases in pressure with increases in volume as was observed in fetuses infused with only aldosterone for 48h. In addition, aldosterone

infused fetuses also had significantly decreased mean pressures at 30, 40 and 50ml volumes when compared to fetuses infused with both aldosterone and 0.25mg beta.

In the second inflation series in figure 3-1, 0.2mg aldosterone infused fetuses had lower initial mean pressures at 10ml volume than control fetuses at 10ml. No statistical difference in mean pressure was observed during the remaining inflations between aldosterone infused fetuses and control fetuses. However, a dose dependent effect between infusion of combined 0.25mg beta with aldosterone and 0.75mg beta with aldosterone was observed at the 20ml inflation volume. At this volume, fetuses infused with aldosterone combined with 0.75mg beta had significantly lower pressures than that of fetuses infused with aldosterone combined with 0.25mg beta.

Inflation Curve Plateau Pressure Data

Pressure curves indicative of intrapulmonary static compliance of mean plateau relaxation pressures and standard error measurements after each inflation volume of corticosteroid infused fetuses and control group fetuses from both inflation series are shown in figure 3-2. In the first inflation series, 0.2mg aldosterone treatment for 48h produced significantly lower initial mean plateau relaxation pressure at 10ml as compared to control fetus mean plateau relaxation pressure at 10ml. This decreased mean plateau relaxation pressure in aldosterone infused fetuses was again significant at 30 and 40ml inflation volumes compared to control fetuses. Infusion of 0.75mg beta also produced a lower initial inflation relaxation plateau pressure compared to 10ml control fetuses. This decreased plateau relaxation pressure at 10ml volume was not observed in any other groups of corticosteroid infused fetuses Analysis of mean pressure measurements after each volume of air infused into 0.2mg aldosterone infused fetuses showed that a significant increase in pressure occurred between 10ml and

20ml, but there was no significant increase in pressure between 20 and 30ml volume. Fetuses infused with 0.25mg beta did not exhibit any significant increase in plateau relaxation pressure after the 20ml inflation volume. Analysis of changes in mean plateau relaxation pressure after each successive increase in volume for control fetuses and fetuses infused with either 0.75mg beta, combined aldosterone with 0.25mg beta or combined aldosterone with 0.75mg beta showed significant increases in pressure after each successive increase in volume.

In the second inflation series, no changes in initial plateau relaxation pressure occurred in any corticosteroid infused fetuses compared to control fetuses at 10ml. However, Analysis of mean plateau relaxation pressure measurements after each volume of air infused into 0.2mg aldosterone infused fetuses showed no significant increase in pressure after the initial 10ml volume. A significant decrease in plateau relaxation pressure occurred at both the 30 and 40 ml inflation volumes in aldosterone infused fetuses. Similarly, no increase in plateau relaxation pressure was observed in fetuses infused with 0.75mg beta and 0.25mg beta at 30 and 40ml inflation volumes respectively.

Expression of α-ENaC, Na, K ATPase and SGK mRNA

There were significant effects of betamethasone (beta) treatment on the expression of alpha epithelial sodium channel (α-ENaC) and sodium potassium adenosine triphosphatase (Na, K ATPase) messenger ribonucleic acid (mRNA) in lung. Both doses of betamethasone increased expression of α-ENaC and Na, K ATPase mRNA with and without out aldosterone. There were no differences between the effects of 0.25 mg and 0.75 mg beta. Aldosterone infusion did not alter expression of either

gene. Expression of serum glucocorticoid regulated kinase (SGK) mRNA did not differ in any treatment group compared to control fetuses or aldosterone infused fetuses. Figure 3-3 depicts these data in fold changes relative to control fetuses, as described in the figure legend.

Expression α-ENaC Membrane and Whole Cell Protein

Previous studies performed by Keller-Wood et al have described the immature and mature α-ENaC protein detected in ovine fetuses at multiple time points during gestation. Mature and immature α-ENaC protein have masses of approx 68 kDa and 100 kDa respectively (Jesse et al., 2009).

In fetal lung whole cell protein extracts, there was a significant overall effect of betamethasone on the 68kDa form of α-ENaC protein, but no overall effect of betamethasone on the 100kDa form. There was no effect of aldosterone on either form of α-ENaC protein. Fetuses infused with 0.25mg beta had significantly increased expression of both 100 kDa and 68 kDa forms of α-ENaC protein relative to fetuses infused with 0.75mg beta. There was a tendency for this effect to also occur in fetuses that received aldosterone with betamethasone, but these data were not statistically significant.

Analysis of expression of mature 68 kDa α-ENaC in fetal lung membrane enriched protein extracts showed no significant difference in protein expression among corticosteroid treated fetuses relative to control fetuses or aldosterone infused fetuses, but a tendency of 0.25mg beta with and without aldosterone to increase protein expression was observed. However this tendency was not statistically significant. No

detectable amount of 100 kDa immature α-ENaC protein was present in membrane enriched samples.

Analysis of the ratio of the mature 68KDa to the immature 100kDa form in whole cell showed an overall effect of betamethasone; 0.75mg beta tended to increase the ratio, but this effect was only significant when the fetuses were also infused with aldosterone. Analysis of membrane/whole cell ratio of the mature 68 kDa form of α-ENaC protein showed an overall significant increase in this ratio in 0.75mg beta fetuses relative to control and 0.25mg beta fetuses. There was also a tendency for this effect to persist in fetuses infused with aldosterone and beta, but this difference was not statistically significant. These data are contained in Figure 3-4 and significance is expressed as described in the figure legend.

Expression β-EnaC Membrane and Whole Cell Protein

Previous studies performed by Keller -Wood et al have described the immature and mature β-ENaC protein detected in ovine fetuses at multiple time points during gestation. Mature and immature β-ENaC protein have masses of approx 112 kDa and 102 kDa respectively (Jesse et al., 2009).

There were significant effects of betamethasone alone and significant aldosterone –betamethasone interactions on expression of the mature 112 kDa form of β-ENaC in fetal lung membranes and in the whole cell. Expression of mature 112 kDa β-ENaC protein in lung whole cell extracts showed an effect of 0.25mg beta to significantly increase protein expression in these fetuses relative to control and 0.75mg beta infused fetuses. However, aldosterone prevented this effect of 0.25 mg beta infusion. Infusion of aldosterone alone increased expression of the 112kDa form of β-ENaC protein in whole

cells as compared to control fetuses, or fetuses infused with 0.75beta with aldosterone. Additionally, fetuses receiving 0.25mg beta with aldosterone had similarly increased protein expression to that of aldosterone alone, and greater expression than fetuses receiving 0.75mg beta with aldosterone.

Analysis of expression of 112 kDa β-ENaC in fetal lung membrane enriched protein extracts showed that 0.25mg beta significantly increased protein expression relative to control, 0.75mg beta, and 0.25mg beta with aldosterone infused fetuses. In contrast, infusion of 0.75mg beta with aldosterone decreased expression of 112 kDa form of β-ENaC as compared to infusion of aldosterone alone.

There was also an overall effect of betamethasone and an interaction between betamethasone and aldosterone in the expression of the immature 102 kDa β-form of ENaC protein in whole cell extracts. 0.25mg beta infused fetuses had increased protein expression relative to control and 0.75mg beta fetuses. Fetuses receiving aldosterone with betamethasone had no increase in expression. No significant difference in 112/102 kDa β-ENaC expression ratio was found in any corticosteroid infused group. Likewise no significant difference in the expression ratio of 112 kDa β-ENaC membrane/whole cell ratio was found among corticosteroid infused fetuses. These data are contained in Figure 3-5 and significance is expressed as described in the figure legend.

Expression of Mineralocorticoid Receptor and Glucocorticoid Receptor mRNA

Expression of mineralocorticoid receptor (MR) mRNA in fetuses that received 0.25mg beta was significantly decreased relative to control fetuses. Expression of MR mRNA did not differ among fetuses receiving aldosterone or aldosterone with either dose of betamethasone. Expression of glucocorticoid receptor (GR) mRNA was

decreased in fetuses that received 0.75mg beta with aldosterone relative to fetuses that only received 0.75mg beta, and no significant changes in GR mRNA expression occurred among either betamethasone dose and control fetuses. Figure 3-3 expresses these data in fold changes relative to control fetuses, as described in the figure legend.

Expression of Aquaporin (AQP)-1 and AQP-5 mRNA

No significant changes in expression of aquaporin (AQP)-1 or AQP-5 mRNA occurred among fetuses infused with corticosteroids relative to control fetuses. Corticosteroid infused fetuses expression of lung AQP1 and AQP-5 mRNA did not significantly differ relative to aldosterone infused fetuses. Figure 3-3 depicts these data as fold changes relative to control fetuses, as described in the figure legend.

Expression of Surfactant Related Protein (SP)-A and SP-B mRNA

No Significant change in SP-A or SP-B mRNA expression occurred among fetuses infused with corticosteroids relative to control fetuses or aldosterone infused fetuses. Corticosteroid infused fetuses expression of lung SP-A and SP-B mRNA did not significantly differ relative to aldosterone infused fetuses. Figure 3-3 depicts these data as fold changes relative to control fetuses, as described in the figure legend.

Plasma Cortisol and Aldosterone

Ovine fetal plasma aldosterone and cortisol concentrations at 0h and 48h after beginning corticosteroid infusion are represented in Figure 3-6. Fetuses infused with aldosterone, both without and with either dose of betamethasone, have significantly elevated plasma aldosterone concentrations at 48h post beginning of infusion. No difference in plasma aldosterone concentration was present in any group before beginning the corticosteroid infusion. Fetuses at 0h did not have significant differences

in plasma cortisol concentration and no difference in plasma cortisol was detected in any corticosteroid infused group or in control fetuses at 48h post beginning of Infusion.

Histology

Table 3-1 contains percent left lung lobe wet weight and percent total area of collagen and elastin staining in ovine fetal lung sections. No significant change in left lung lobe percent wet weight was found between corticosteroid infused and control fetuses. The percent total area of collagen staining in lung sections from fetuses infused with 0.75mg beta was significantly greater than that in control fetuses or fetuses infused with 0.25mg beta. The percent total area of elastin staining in lung sections from fetuses infused with 0.75mg beta was significantly greater than that in control fetuses. Fetuses infused with 0.75mg beta with aldosterone also had significantly greater elastin percent area than aldosterone fetuses. Figures 3-7 and 3-8 are representative examples of typical elastin and collagen staining in ovine fetal lung parenchyma.

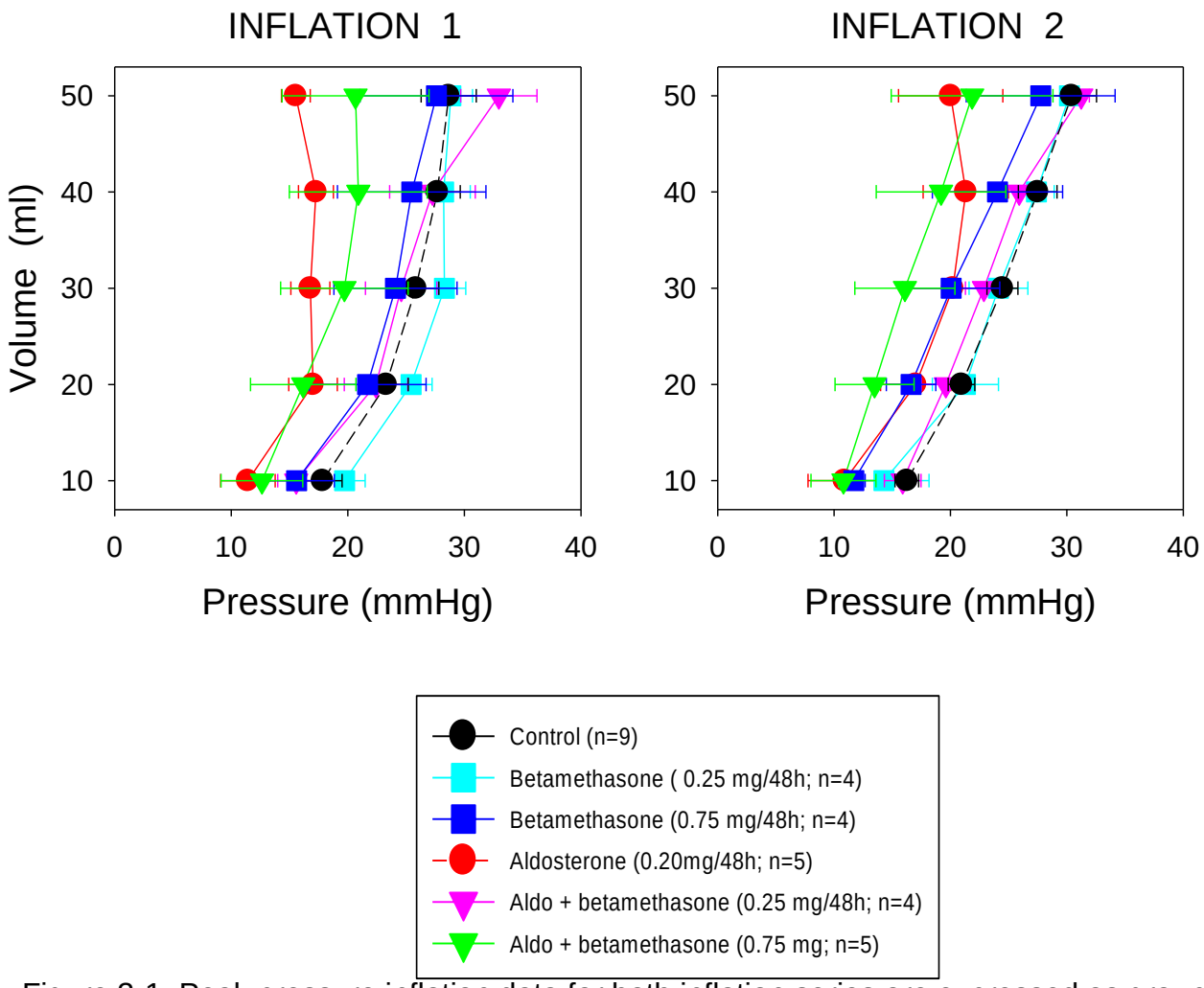

Figure 3-1. Peak pressure inflation data for both inflation series are expressed as group means and standard errors for each treatment group over all 5 inflation volumes

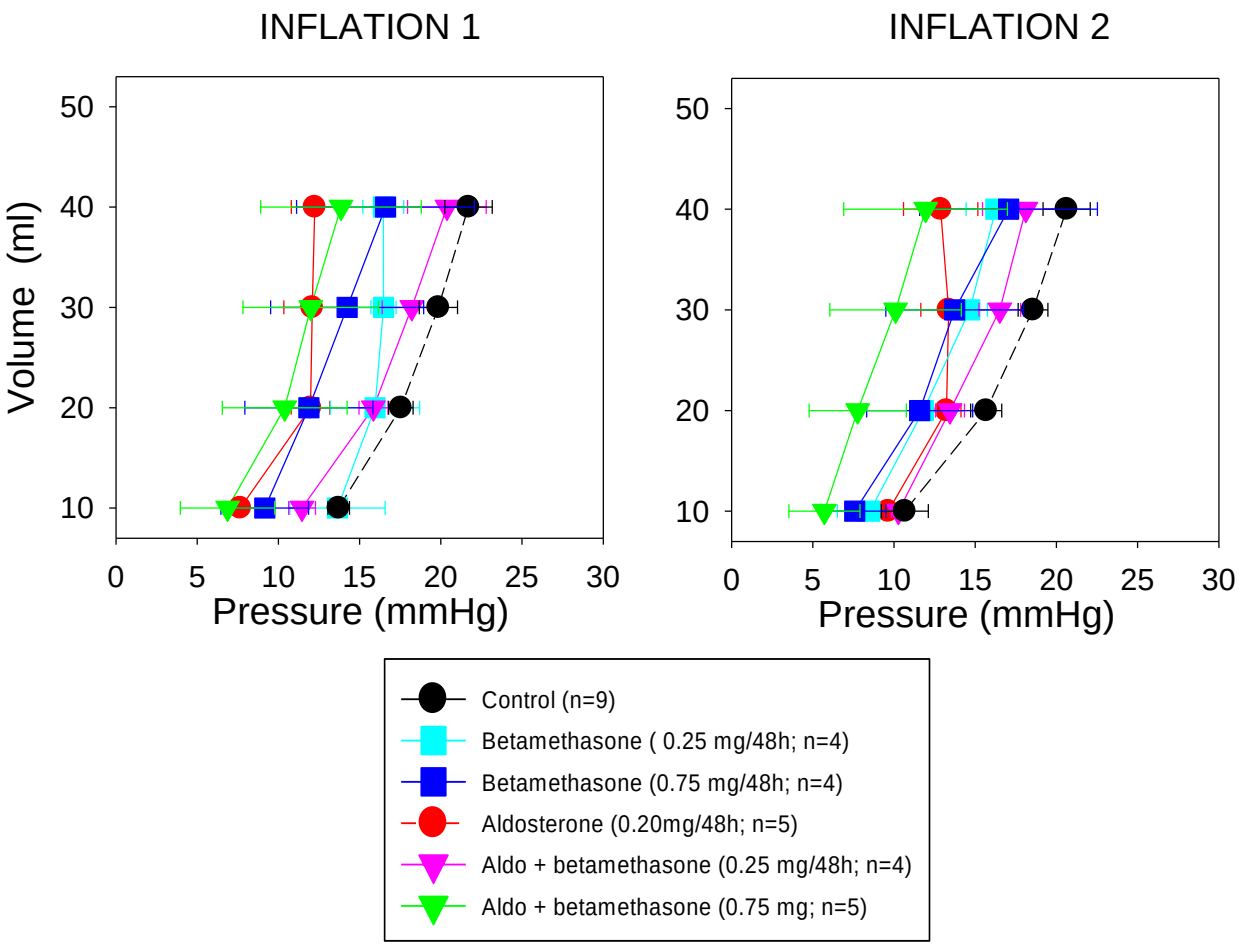

Figure 3-2. Plateau pressure measurements for in situ lung compliance data as determined by steady state pressure between inflations.

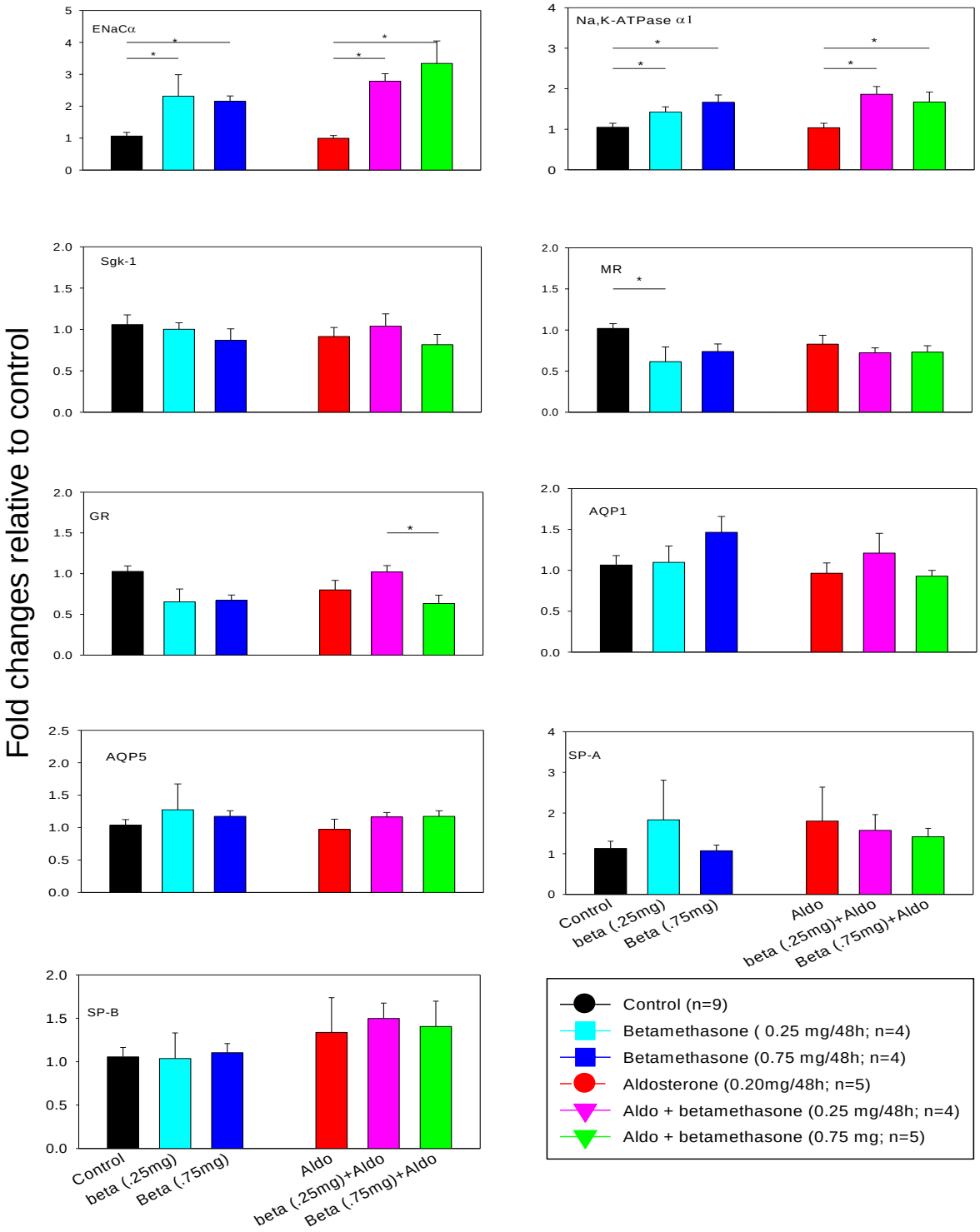

Figure 3-3. Quantitative real- time PCR data of genes involved in lung fluid homeostasis. Data are depicted as mRNA fold changes relative to control using the expression 2^ΔΔCt and expressed as a mean fold change ± SEM. Asterix over horizontal bars indicate significant differences ($p<0.05$) Vertical bars from left to right, 1: Control, 2: 0.25mg Betamethasone (beta), 3: 0.75mg Betamethasone, 4: 0.2mg Aldosterone, 5: 0.2mg Aldosterone+ 0.25mg Betamethasone, 6: 0.2mg Aldosterone + 0.75mg Betamethasone fetuses.

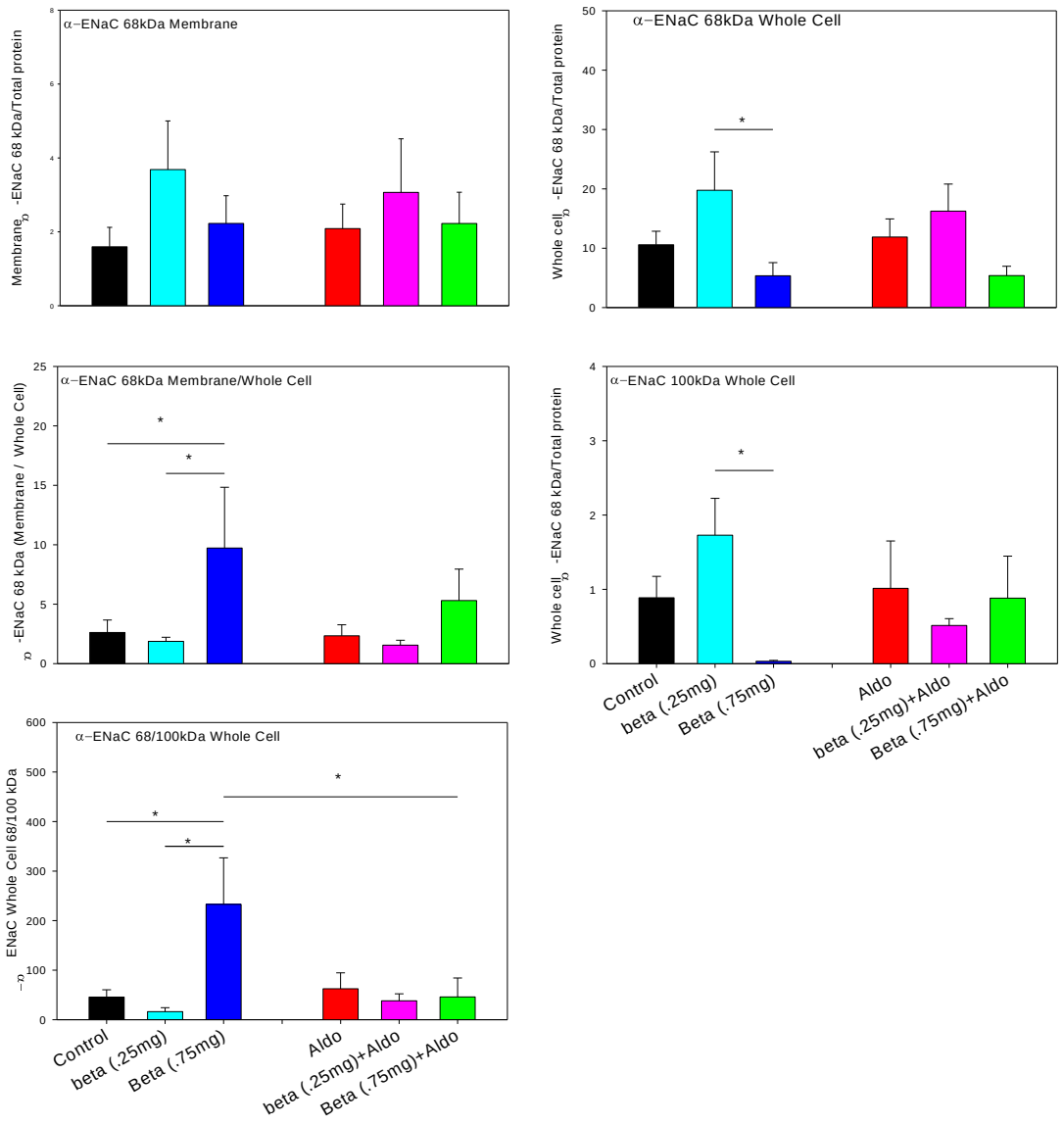

Figure 3-4. Western blots of epithelial sodium channel alpha (ENaC)-α protein in whole cell and membrane enriched extracts from fetal lungs. Data are expressed as group mean ± SEM. Significant differences are denoted by lines. Bars below each end of a line are significantly different from each other. ($p<0.05$)

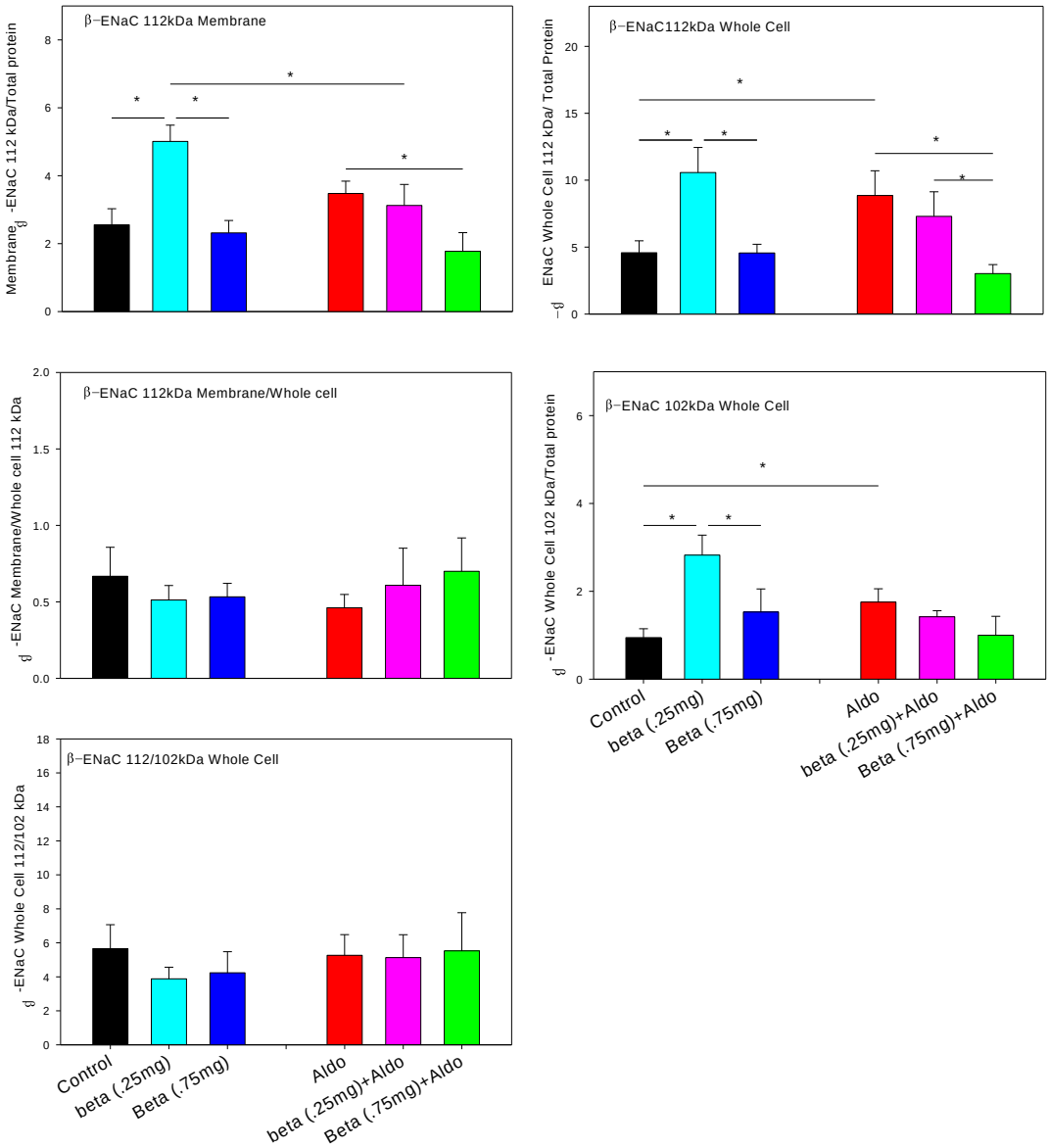

Figure 3-5. Western blots of β-ENaC protein in whole cell and membrane enriched extracts from fetal lungs. Data are expressed as group mean ± SEM. Significant differences are denoted by lines. Bars below each end of a line are significantly different from each other. ($p<0.05$)

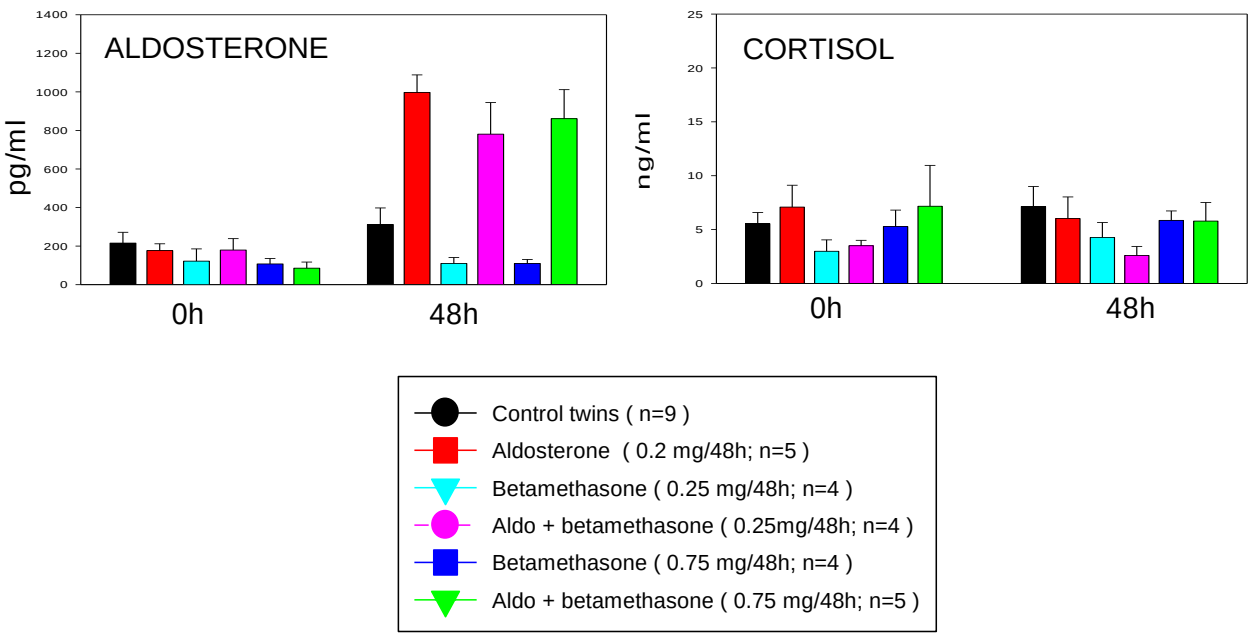

Figure 3-6. Fetal plasma aldosterone and cortisol concentrations at 0h pre infusion and 48h post start of corticosteroid infusion. 0h and 48h plasma aldosterone and cortisol concentrations of control fetuses that did not receive infusion of steroid are also included.

Table 3-1. Percent lung weight determined after baking the lungs. Percent collagen and elastin in corticosteroid treated fetuses and control animals were determined by fraction of total area stained for collagen and elastin in fetal lung sections.

	Control	Aldo	Beta (0.25 mg)	Aldo + Beta (0.25 mg)	Beta (0.75 mg)	Aldo+ Beta (0.75 mg)
% Wet Weight	92.63 ± 0.18	93.83 ± 1.47	93.13 ± 0.50	93.30 ± 0.46	92.63 ± 1.01	93.08 ± 0.70
% Collagen	7.948 ± 0.81	6.92 ± 2.21	6.37 ± 0.32	7.41 ± 0.90	11.37 ± 1.36 *^	10.59 ± 1.03
% Elastin	1.32 ± 0.15	1.43 ± 0.09	1.56 ± 0.32	1.90 ± 0.19	2.16 ± 0.19 *	2.46 ± 0.26 #

Data are expressed as means ± SEM, (*) denotes significance relative to control, (#) denotes significance relative to Aldo, and(^) denotes significance relative to 0.25mg Beta fetuses,($p<0.05$)

Table 3-2. Primers and Probes used in real-time PCR assays.

Gene	Forward Primer Nucleotide Sequence (5'-3')	Reverse Primer Nucleotide Sequence (5'-3')
AQP-1	CCATCGTCGCCACTGTCAT	GAGGCCAAGCGAGTTGTCA
AQP-5	CGCCGCAATCCTCTATGG	CGCAGTCGTGTTGTTGTTGAG
a-ENaC	CCCGGGTGATGGTGCATGAA	TCACGCCCGGCCGCAG
GR	ACTGCCCCAAGTGAAAACAGA	GCCCAGTTTCTCCTGCTTAATTAC
MR	ATTTCACTGAGTACCTGTTGATTATCATC	GGGAAACTTAATATGATTGCACTAAATAAA
Na,K ATPase a1	GGTGTTGCCCTGAGGATGTATC	CCGGACTTCGTCATACACGAA
SGK-1	GACTTTGGACTCTGCAAGGAGAA	CGGGCGTGCCACAGAA
b-actin	TTCCTTCCTGGGCATGGA	GACGTCACACTTCATGATGGAATT
SP-A	TGACCCTTATGCTCCTCTGGAT	CAGGGCTTCCAAGACAAACTTC
SP-B	TCCCTGCCTGGAGAATGG	CTGCCTGAGTGGTCACAAACA

Gene	Probe Nucleotide Sequence (5'-3')
AQP-1	TCTCGGGCATCACCTCCTCTCTGC
AQP-5	CAATGCCCGAAGCAATCTGGCTG
a-ENaC	TAAAGCCAGCATCATCCATAAAGGCG
GR	AAAGAAGATTTTATCGAACTCTGCACCCCTGG
MR	CTTTTCCAAGATTAATTTGGCCTCTATTCAAAGCA
Na, K ATPase a1	TTCTGTGCCTTCCCCTACTCGCTTCTCATT
SGK-1	TTGAACACAATGGCACGACGTCCAC
b-actin	TCCTGCGGCATTCACGAAACTACCTT
SP-A	TTCTGGCCTCGAGTGCGACACAAA
SP-B	TGCCACAAGTCTCTGAGTGCCAGCT

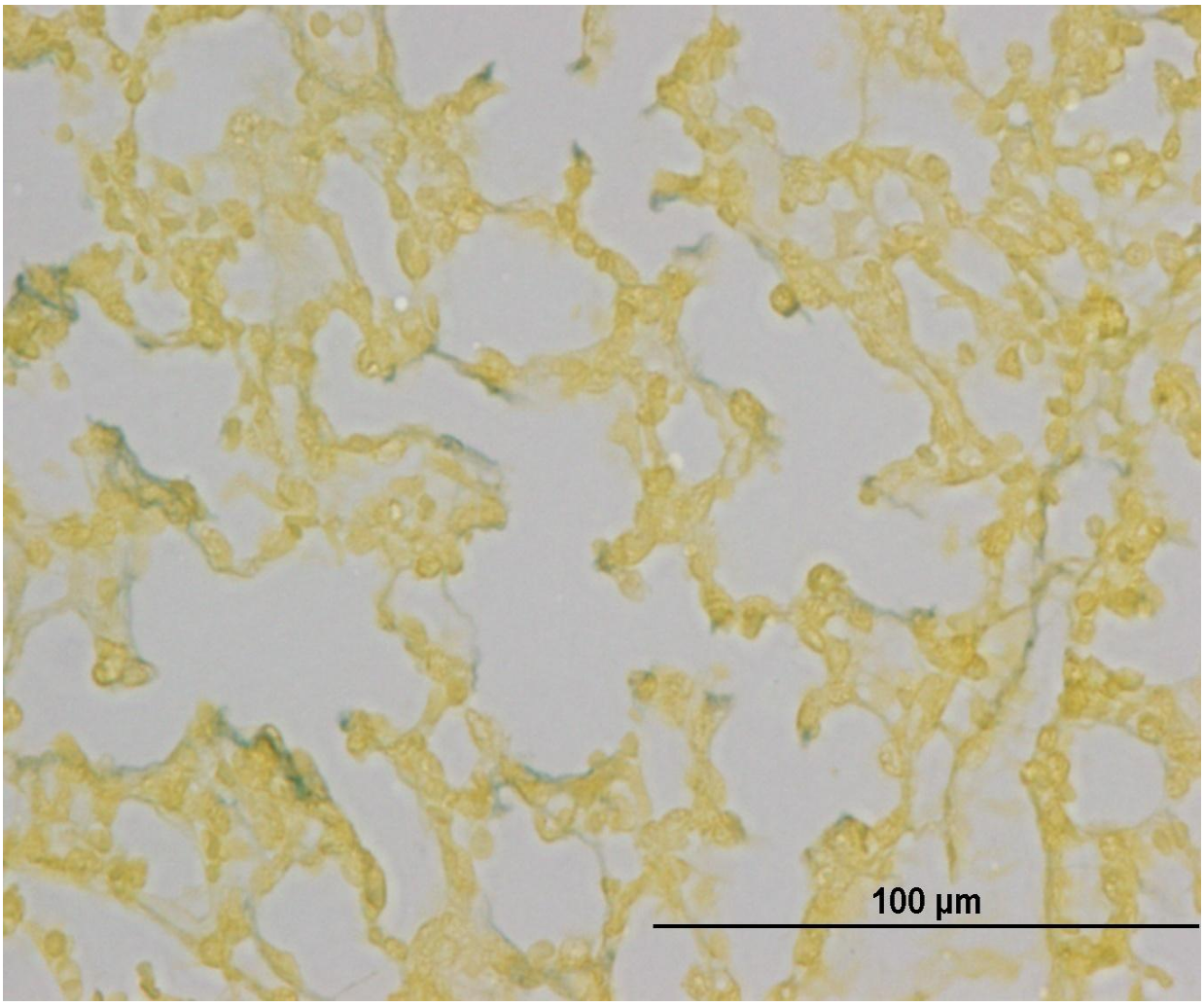

Figure 3-7.Typical Miller's elastin staining of ovine fetal lung parenchyma. Elastin fibers are clearly visible as black fibers over yellow secondary tartrazine stained tissue.

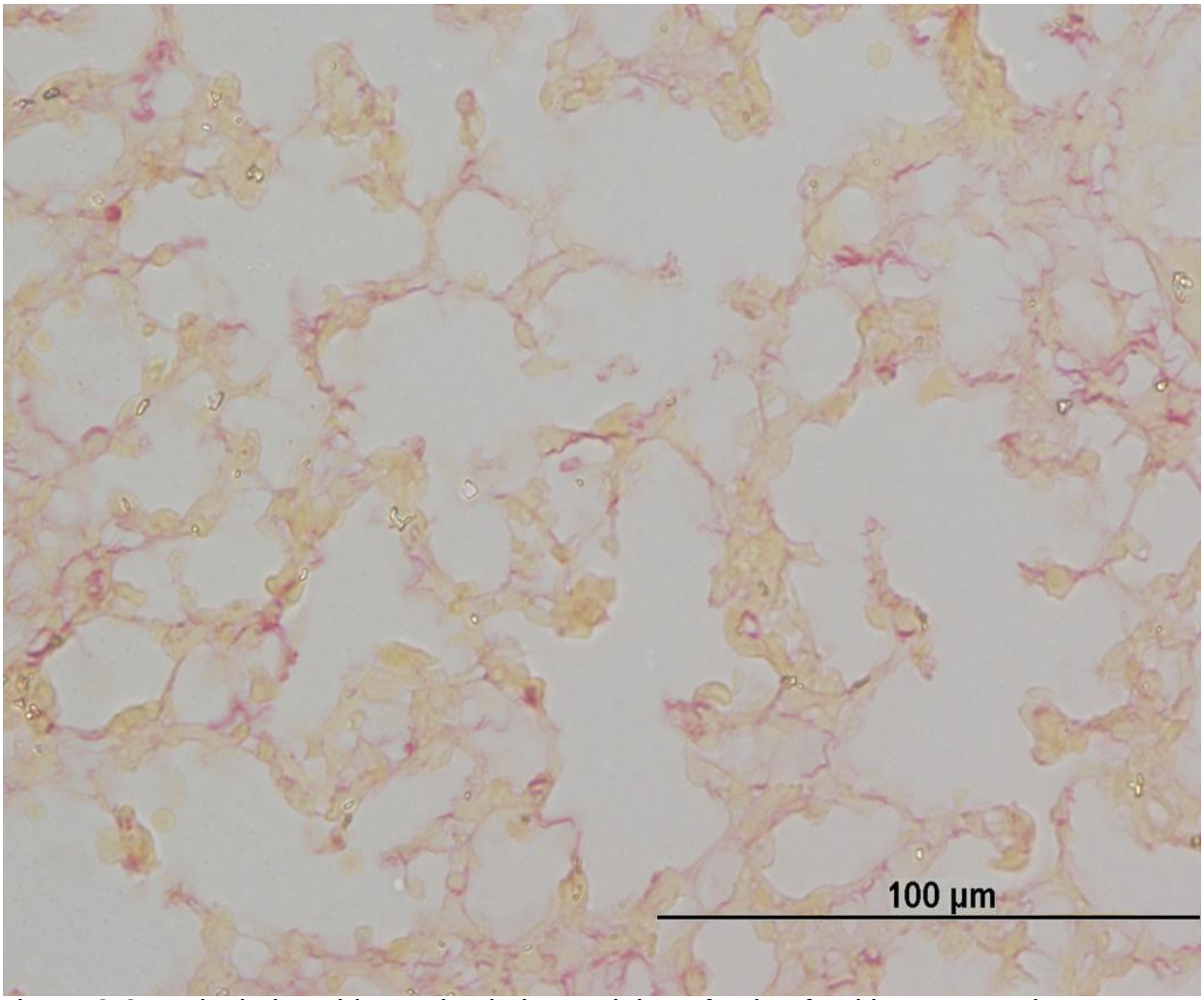

Figure 3-8.Typical picrosirius red solution staining of ovine fetal lung parenchyma. Collagen fibers stain red over yellow background tissue.

CHAPTER 4
DISCUSSION

Effects of Corticosteroid Infusion on Lung Compliance

While there was not an overall aldosterone effect on fetal lung in situ peak pressure measurements, the results of first and second inflation pressure measurements showed a distinct effect of aldosterone to decrease intrapulmonary pressure at the initial inflation volume and also at the subsequent higher volumes as compared to control fetuses, indicating an apparent initial and transiently sustained increase in ovine fetal lung dynamic compliance as determined by the intrapulmonary volume/pressure ratio. Initial intrapulmonary volumes are most analogous to the first breaths of fetal extrauterine life and these data indicate that aldosterone infusion had beneficial effects on fetal lung function during a time that would correlate to the initial breaths of life. This increased dynamic compliance is again apparent in the first inflation series as aldosterone infused fetuses did not have significant increases in intrapulmonary pressure after inflation to 20ml, while control fetal lungs had significant increases in pressure with each stepwise increase in intrapulmonary volume.

The aldosterone effect to increase fetal lung dynamic compliance was not entirely expected, however it was surprising that neither dose of betamethasone (beta) had significant effects on ovine lung dynamic compliance and betamethasone infused fetal intrapulmonary pressures differed little from control animal pressure measurements. These results were unexpected because antenatal administration of synthetic glucocorticoids has been demonstrated to effectively enhance preterm fetal lung function (Liggins and Howie, 1972). Both doses of glucocorticoid receptor (GR) agonist were expected to occupy all GR receptors and aldosterone infusion would occupy

approx 85% of fetal mineralocorticoid receptor (MR). However, it is important to note that the doses of GR agonists used in this study were physiological and not representative of clinical doses. Rather than attempt to reproduce clinical observations and make direct clinical correlations, this study was designed to provide insight into possible mechanisms of action of MR and/or GR agonists in response to a physiological increase in these agonist and to determine what effect combined administration of MR/GR agonist have on fetal lung maturation. These data do indicate a plausible mechanism of MR agonist inducible lung maturation that is ameliorating the intrapulmonary pressure in aldosterone infused animals while GR agonist did not induce this mechanism.

Interestingly, the beneficial effect of aldosterone infusion to increase fetal lung dynamic compliance was ablated in fetuses that received either dose of betamethasone combined with aldosterone. Only fetuses that received 0.75mg beta with aldosterone showed any significant difference from control fetuses during the first inflation, but this effect did not occur until the 50ml inflation volume. The initial decreased pressure and insignificant changes in pressure with stepwise increases in volume observed in aldosterone infused fetuses did not occur in other corticosteroid infused fetuses. As GR agonists are known to increase surfactant production (DeLemos et al., 1970) and induce potential mediators of lung fluid reabsorption (Venkatesh and Katzberg, 1997), it was expected that an increase in compliance would occur in these fetuses relative to control animals. Why these data do not indicate a change in compliance and why betamethasone combined with aldosterone ablates the aldosterone effect is not clear. There could possibly be an unknown inhibitory effect of betamethasone on aldosterone

or combined infusion of both betamethasone and aldosterone may possibly result in formation of heterodimer receptor GR/MR complexes that are ineffective at HRE (Liu et al., 1995), further investigation is clearly needed. However, the significance of these results directly correlates to the apparent increased viability of these aldosterone infused fetuses and their ability to survive in the extrauterine environment.

Determination of plateau relaxation pressures yielded similar results in the first inflation series to those of peak pressure measurements, with aldosterone again mediating lower intrapulmonary pressure at initial inflation volume and again at the majority of subsequent volumes compared to control fetuses. Indicating an apparent initial and transiently sustained increase in ovine fetal lung static compliance compared to control fetuses. Other than aldosterone infused fetuses, only fetuses infused with 0.75mg beta and aldosterone also had lower initial pressures than control fetuses. Again infusion of 0.25mg beta with aldosterone attenuated the positive effects of aldosterone infusion.

Plateau pressure data from the second inflation series did not show any difference in initial pressure measurements in any corticosteroid infused fetuses. However, aldosterone infused fetuses did not have significant increases in pressure after each stepwise increase in pressure. Surprisingly, in fetuses that received aldosterone significant decreases in pressure were observed at the 30 and 40ml inflation volumes. Indicating the possibility that perhaps a pressure threshold at some point before 30ml was exceeded, allowing the highly compliant nature of these lungs to afford repeated increases in volume without concomitant increases in pressure. While these decreased pressures are indicative of increased static compliance, the specific cause of this

increased compliance is not clear. There could potentially be remodeling of collagen and elastin fiber abundance and/or organization that could result in profound changes in the biomechanical properties of the lung (Tanaka and Ludwig, 1999). However lung tissue histological staining did not indicate significant changes in abundance of collagen or elastin in aldosterone treated fetuses when compared to control fetuses. It is possible that the arrangement of these fibers was affected by aldosterone infusion and a more sophisticated analysis of elastin and collagen stained fetal lung sections by scanning electron microscopy could potentially provide useful data.

Expression of α-ENaC, Na, K ATPase, and SGK-1 mRNA

As transient tachypnea of the newborn (TTN) is attributed to excessively wet lungs and is a major contributor to neonatal respiratory distress, it is reasonable to hypothesize that increased lung liquid reabsorption before birth would potentially ameliorate the risk of developing TTN. GR agonists are known to increase alpha epithelial sodium channel α-(ENaC) messenger ribonucleic acid (mRNA) expression (Itani et al., 2002; Venkatesh and Katzberg, 1997), and it was expected that infusion of MR or GR agonists would increase expression of α-ENaC mRNA in all fetuses that received corticosteroid infusion. With this in mind, and the known function of ENaC activity in the aldosterone sensitive distal nephron (ASDN) to increase sodium reabsorption and volume (Loffing et al., 2001), lung ENaC could potentially contribute to increased lung fluid reabsorption in a similar mechanism through induction by MR and GR agonists. Of specific interest was the potential of aldosterone to greatly upregulate ENaC activity, as was observed in ex vivo fetal hamster lung data in which aldosterone increased lung liquid reabsorption (Kindler et al., 1993).

However quantitative real-time polymerase chain reaction (PCR) data of α-ENaC, sodium potassium adenosine triphosphatase (Na, K ATPase) and serum glucocorticoid regulated kinase (SGK) mRNA extracts from corticosteroid infused fetal lungs did not indicate an effect of aldosterone infusion to increase expression of these genes, and no changes in SGK mRNA expression occurred among any fetal group. As expected, both doses of betamethasone increased expression of α-ENaC and Na, K ATPase compared to control animals (Itani et al., 2002; Venkatesh and Katzberg, 1997). This increase in α-ENaC and Na, K ATPase mRNA expression was also observed in fetuses receiving either dose of betamethasone with aldosterone compared to fetuses that received only aldosterone. As the in situ lung pressure data did not indicate that betamethasone infused fetuses had significant increases in pulmonary compliance, the increase in expression of these genes did not substantially affect the biomechanical properties of the lung. Also, as percent wet weight did not differ among corticosteroid infused fetuses and control fetuses it is unlikely that there was an appreciable change in lung fluid homeostasis. However, due to the large percentage of water weight in these lungs, small changes in percent wet weight may not have been able to be distinguished through our detection method.

Expression of MR and GR mRNA

The mRNA expression of both MR and GR did not differ greatly among corticosteroid infused fetuses. However there were significant decreases in MR mRNA expression in 0.25mg beta fetuses, the significance of which is unclear as synthetic glucocorticoids have very little affinity for MR receptors (Rupprecht et al., 1993). It would not be expected for betamethasone to directly affect MR expression, but betamethasone occupation of GR receptors could potentially afford increased binding of

endogenous cortisol to MR receptors and this decrease in MR mRNA expression could then be attributed to MR mRNA expression negative feedback regulation in response to increased MR activity. Also, transcriptional regulation of MR expression by increased GR activation is a potential explanation for the observed decrease in MR expression as well as complex MR/GR heterodimer formation downregulating MR expression (Liu et al., 1995).

A dissimilar expression pattern is observed for GR mRNA expression in which 0.25mg beta with aldosterone has significantly greater GR expression then 0.75mg beta with aldosterone infused fetuses. However these data can also be attributed to increased occupancy of GR receptors and negative feedback regulation of GR mRNA expression. While these data are only significant among fetuses that received betamethasone with aldosterone, there appears to be a specific dose dependent effect of betamethasone decreasing GR mRNA expression that is in agreement with the plausible explanation of increased negative feedback.

Expression of AQP-1, AQP-5, SP-a, and SP-b mRNA

There was a tendency for 0.75mg beta to increase transcription of aquaporin (AQP)-1 mRNA expression but these data did not reach statistical significance and AQP-5 expression did not differ among corticosteroid infused fetuses. AQP 1 and 5 are thought to provide the principal route for osmotic water transport of intrapulmonary fluid into the pulmonary capillaries with AQP-5 associated with type I cells located on the apical membrane of distal airways and contribute to intrapulmonary lung fluid reabsorption, while AQP-1 is more closely associated with microvascular endothelia and absorbing institutial fluid into the vascular compartment (Verkman, 2007). Increased lung AQP-1 and/or AQP-5 mRNA expression would possibly upregulate passive

reabsorption of lung liquid through either route, but corticosteroid infusion did not affect expression of these genes.

It was expected that mRNA expression of surfactant related proteins (SP) A and B would both be increased in fetuses that received betamethasone (Liggins, 1969) and potentially be increased in aldosterone infused fetuses, but surprisingly mRNA expression of both SP-A and SP-B did not differ from control fetuses in any corticosteroid infused groups. There was a tendency for 0.25mg beta to increase expression of SP-A, but variability among these fetuses was too great for these data to be significant. However, SP-A protein is generally associated with increased host defense from bacterial, fungal, and viral infection and increases in SP-A would not affect the biomechanical properties of the lung to the same extent as increased mRNA expression of SP-B(Nkadi et al., 2009). There did appear to be a general increase in mRNA expression of SP-B in all fetuses that received aldosterone with or without betamethasone. However the variability among these fetuses was again too great for significance to be obtained. As SP-B is more closely associated with decreasing alveolar surface tension and increasing efficacy of the air-alveolus interface (Nkadi et al., 2009) increased mRNA expression of SP-B could potentially contribute to the observed increased compliance of aldosterone infused fetuses.

The variability in SP-A and SP-B mRNA expression can possibly be attributed to the gestational age at which these fetuses were studied, and that initial surfactant production first occurs in the later canalicular stage of lung development in human fetuses (17-27 weeks) and the equivalent ovine stage of lung development occurs at (80-120d) gestation (Alcorn et al., 1981; Kotecha, 2000). When the gestational age of

these fetuses is considered, significant variability in the expression of surfactant related protein mRNA would be expected as surgery was performed on these fetuses between (122-124d) gestation and tissue collection occurred 7 days after surgery at a time in which upregulation of these genes is still only initially beginning. Therefore, there may be inherent variability in specific timing of SP-A and B mRNA expression upregulation from fetus to fetus, and this variability may prevent the actual effect of aldosterone from obtaining statistical significance.

α-ENaC Whole Cell and Membrane Protein

For lung apical alveolar epithelial ENaC channels to effectively participate in sodium reabsorption, insertion of ENaC protein into the alveolar apical membrane is critical. Western blot data of fetal lung whole cell and membrane enriched protein extracts confirmed that corticosteroid infusion or MR, GR or combined MR/GR agonists distinctly affect the abundance and potentially the stoichiometric relationship of α and β ENaC subunits. The most prominent increase in abundance of the vitally important mature α subunit occurred after administration of 0.25mg beta, with significant and nearly significant increases in whole cell and membrane protein extracts respectively. While numerous data have shown betamethasone to increase ENaC transcription (Itani et al., 2002; Venkatesh and Katzberg, 1997), it was surprising to not see a similar effect in fetuses that received 0.75mg beta. However, when the ratio of whole cell to membrane mature α-ENaC protein was determined, a substantial increase in the relative ratio of membrane to whole cell α-ENaC was found in fetuses that received 0.75mg beta. These data indicate that there is perhaps increased insertion of intracellular mature α-ENaC into the alveolar membrane in fetuses receiving 0.75mg beta, which could potentially stimulate increased sodium reabsorption and passive lung

fluid reabsorption. This effect of betamethasone appears to be dose dependent, and while greater abundance of mature α-ENaC is observed in 0.25mg beta infused fetal whole cell protein extracts, this dose may be insufficient to increase insertion of these channels into alveolar membrane.

These data also indicate that fetuses receiving 0.25mg beta have significantly increased immature 100kDa α-ENaC protein, while 0.75mg beta infused fetuses have a significant reduction in immature α-ENaC protein. This suggests that there may be an increase in the necessary post translational modification of these channels (Hughey et al., 2003) and increased production of more efficient mature α-ENaC channels in response to the 0.75mg dose of betamethasone but that 0.25mg beta dose is not sufficient to cause increased post translational modification. Aldosterone attenuated the effects of both doses of betamethsone infusion but similar patterns of protein expression were produced in whole cell mature α-ENaC protein extracts as well as a similar ratio of mature α-ENaC protein in membrane and whole cell fractions. These data suggest that aldosterone is reducing but not completely inhibiting the dose dependent action of betamethasone to increase α-ENaC abundance and membrane insertion. However, as in situ intrapulmonary pressure has demonstrated aldosterone infused fetuses to have the most significant increases in compliance, these betamethasone modifications of α-ENaC protein expression do not appear to have a significant impact on lung biomechanical properties.

β-ENaC Whole Cell and Membrane Protein

Similar changes in β-ENaC protein expression were also observed in corticosteroid infused fetuses. Again 0.25mg beta most significantly affected β-ENaC expression and greatly increased abundance of the β subunit in both membrane

enriched and whole cell protein extracts as well as Immature 102 kDa β-ENaC protein. The significance of this is not entirely clear as highly selective sodium channels are thought to contain β subunits (Jain et al., 2001) but expression of β-ENaC protein in ovine fetuses is also known to decrease greatly before parturition when rapid reabsorption of lung liquid occurs (Jesse et al., 2009). When considering the in situ pressure data in 0.25mg beta fetuses, these data do not suggest that there is a correlation between increased β-ENaC protein expression and changes in fetal lung compliance. Aldosterone again attenuated the increase in β-ENaC when combined with betamethasone and as fetuses infused with only aldosterone had the greatest increase in lung compliance, increases in β-ENaC protein expression likely do not affect lung biomechanical properties. This observation is in agreement with knockout studies indicating that the β-ENaC subunit is not critical for the transition into the extrauterine environment at birth (Randrianarison et al., 2008), while similar studies have shown that knockout of α-ENaC results in death shortly after birth (Hummler et al., 1996). These data considered along with the lack of agreement on exact ENaC subunit stoichiometry (Eskandari et al., 1999; Firsov et al., 1998) prevents any definitive conclusion to be drawn as changes in subunit abundance may also effect the formation and activity of nonselective, and highly selective channels (Jain et al., 2001) in a manner that is not easily predicted. As the role of ENaC to increase fluid reabsorption is thought to occur shortly before birth, it may not be feasible to increase lung liquid reabsorption at the stage of gestation these fetuses were studied. Lung liquid secretion is an important and vital component of normal lung structural development, and there are likely multiple feedback mechanisms to insure that this process continues until shortly before birth.

Also, the dose of MR and GR agonist utilized may not be sufficient to adequately affect ENaC protein abundance or subunit relationship in a manner that would significantly increase lung fluid reabsorption. Therefore, it is not entirely surprising that these changes in ENaC protein did not apparently affect fetal lung dynamic or static compliance.

Histology and Lung Percent Wet Weight

GR and MR agonists were expected to potentially effect expression of mediators of lung fluid reabsorption, and increased expression of these proteins could potentially have resulted in decreased lung percent wet weight. However, no changes in percent wet weight occurred in these fetuses, suggesting that corticosteroid infusion did not affect lung liquid reabsorption. As fluid reabsorption did not appear to be affect by these corticosteroids, infusion of MR and/or GR agonists could also potentially change expression of collagen and elastin proteins, resulting in significant changes in lung biomechanical properties. Betamethasone has been shown to increase expression of both collagen and elastin in lung (Beck et al., 1981), therefore it was not surprising that 0.75mg beta infusion significantly increased both lung percent collagen and elastin in these fetuses. However, it was interesting that increases in collagen were ablated when 0.75mg beta was infused along with aldosterone and that infusion of aldosterone alone did not affect collagen expression. Aldosterone did not attenuate the increase in elastin expression in fetuses that received both 0.75mg beta and aldosterone, but increases in elastin were not observed in aldosterone infused fetuses.

Changes in percent lung collagen and elastin does not appear to explain the observed change in lung compliance of MR agonist infused fetuses, but as elastin was increased in fetuses that received aldosterone with 0.75mg beta, this increase in elastin

could potentially explain why these fetuses did not have the same increases in lung compliance as that of aldosterone infused fetuses in which percent elastin did not increase. As increases in elastin content have been shown by others to increase the recoil properties of rat lungs, the increase in elastin content of synthetic glucocorticoid infused fetuses in this study may explain the lack of increases in lung compliance observed when elastin content also increased (Nardell and Brody, 1982).

Significance and Future Directions

This study indicates that antenatal administration of aldosterone can potentially enhance the viability of ovine preterm fetal lungs and these data are significant as no similar study in ovine fetuses has been previously conducted. In contrary to our hypothesis, the affect of aldosterone infusion does not appear to occur through increases in lung liquid reabsorption. Rather, there is potentially a novel undiscovered mechanism of MR activation in the ovine lung facilitating the increased lung compliance observed in aldosterone infused fetuses. Analysis of mRNA and protein expression of potential mediators of lung fluid homeostasis from aldosterone infused fetuses did not indicate a change in expression in either mRNA or protein expression that would explain the decreases in intrapulmonary pressures, however lack of increases in percent extra cellular matrix (ECM) proteins in aldosterone infused fetuses may provide valuable insight into why these pressures are decreased in aldosterone infused fetuses and are not decreased in betamethasone infused fetuses.

As the exact cause of the increases in lung compliance observed in aldosterone infused fetuses remain unclear, there are many potential changes in the fetal lung that may be contributing to this effect. The increased compliance may potentially be the result of aldosterone causing lung hypertrophy relative to non aldosterone infused

fetuses. This would result in decreased intrapulmonary pressures at equivalent volumes of air infused into the lungs of aldosterone infused fetuses. Increased thinning of the alveolar epithelial membrane after infusion of aldosterone would result in increased lung expansion at equivalent volumes of air in these lungs and also result in decreased intrapulmonary pressures and the observed increased compliance. Changes in the relative abundance of type II epithelial cells could potentially increase overall surfactant production without increased mRNA expression of surfactant related protein detectable by quantitative real-time PCR. Increases in the amount of type II cells would increase overall pulmonary surfactant secretion and would afford greater expansion of the lung. Also, while western blot and quantitative real-time PCR analysis of ENaC did not indicate increased transcriptional or translational expression in aldosterone infused fetuses, increased insertion of ENaC into the alveolar epithelial apical membrane would afford greater reabsorption of sodium and intrapulmonary fluid and potentially result in increased compliance as well.

Further investigation by immunohistochemistry (IHC) of lung parenchyma to determine α-ENaC and MR abundance, localization and/or colocalization could potentially elucidate the mechanism of aldosterone action in these fetuses. As ENaC and MR abundance may potentially be increased in the alveolar apical membrane and nucleus of the alveolar epithelial cells respectively in a manner not detectable by western blot. Membrane enriched protein extracts analyzed by western blot contained both apical and basolateral membrane proteins along with other cellular contents. Thus, only general changes in membrane abundance, but not specific changes in abundance of apical membrane ENaC was visible by western blot. However, IHC of lung sections

would afford determination of ENaC abundance in lung epithelial apical membrane as well as MR abundance in epithelial cell nucleus. If increased insertion of ENaC into the apical membrane did occur after aldosterone infusion, this could potentially explain the observed increases in compliance in these fetuses. Also, increased MR localization into the nucleus of lung epithelial cells would imply that the mechanism of action responsible for the observed increased lung compliance is MR mediated.

As the use of synthetic glucocorticoids has become more commonplace over the last 30 years for the attenuation and prevention of respiratory distress in preterm infants, the possible deleterious consequences of their overuse are of particular concern. Recently, published finding have shown that in a cohort of women given multiple doses of synthetic glucocorticoids, multiple doses did not decrease infant morbidity and mortality more so than a single dose of synthetic glucocorticoids. These data show that multiple doses resulted in reduced birth weight, body length and head circumference at birth. Therefore the judicious use of a single course of antenatal synthetic glucocorticoids is recommended over the use of multiple courses (Murphy et al., 2008). However, while these steroids are intended to mimic the action of endogenous cortisol that increases before labor to rapidly mature fetal organs, synthetic glucocorticoids lack the same biological activity as cortisol and do not have appreciable biological activity at MR receptors. Antenatal administration of combined MR and GR agonists may more accurately mimic the action of endogenous cortisol and may provide additional benefits for fetal lung maturation. As this study demonstrated that a physiological infusion of MR agonist decreased intrapulmonary pressure in ovine fetal lungs, clinical administration of MR agonist may potentially provide benefits not provide

by GR agonists alone. Further research into the role of MR agonists and the mechanism by which these corticosteroids decrease fetal intrapulmonary pressure would provide valuable insight into their future therapeutic potential in clinical doses. Moreover as antenatal synthetic glucocorticoids are known to potentially cause deleterious side effects in fetuses that may not manifest until later in life, reduced dosages of glucocorticoids or alternative usage of antenatal mineralocorticoid agonists may avoid these side effects and provide improved fetal outcomes. Further research of mineralocorticoid agonist mechanism of action and therapeutic potential may provide important advances in the prevention and treatment of preterm infant respiratory distress and should be pursued.

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BIOGRAPHICAL SKETCH

Jarret McCartney was born in Morgantown, West Virginia in 1982, the youngest of three boys and the son of Stephanie McCartney King and Fred McCartney. He moved to Port Charlotte, Florida at the age of five where he attended public school and graduated from Port Charlotte High School in 2000. Afterwards, Jarret received an Associate of Arts degree from Edison State College in 2002 and proceeded to attend the University of South Florida. At the University of South Florida, Jarret received his Bachelor of Arts degree with a major in chemistry with emphasis for health professions in 2005.

Afterwards Jarret was employed in the laboratory of Dr. Maureen Keller-Wood as a veterinary technician. After developing an interest in fetal physiology, Jarret enrolled in the University of Florida, College of Medicine, Medical Sciences Master of Science graduate program in August 2008. His thesis work was completed with Dr. Maureen Keller-Wood in the department of pharmacodynamics and focused on the effects of mineralocorticoid and glucocorticoid agonists on fetal lung development. Jarret plans to live in Charlotte, North Carolina and pursue a career in scientific research.