MECHANISMS BY WHICH OVEREXPOSURE TO CORTISOL CAUSES FETAL HEART ENLARGEMENT IN LATE GESTATION

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To my mother, Janet, whose tireless work ethic and strong moral character are an inspiration to me. Also to my Father, George, who has always challenged me to think critically.

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MECHANISMS BY WHICH OVERVEXPOSURE TO CORTISOL CAUSES FETAL HEART ENLARGEMENT IN LATE GESTATION

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Elevated cortisol levels during late gestation can lead to fetal heart enlargement. However, the mechanism by which cortisol increases fetal heart size has not been fully identified. These experiments were designed to 1) investigate left ventricle (LV) expression of genes potentially responsible for increasing heart size, 2) determine the ontogenetic expression of those genes in the LV and right ventricle (RV), 3) elucidate whether the mineralocorticoid receptor (MR) or glucocorticoid receptor (GR) mediate the cortisol-induced cardiac enlargement, and determine if cardiac fibrosis accompanies the enlargement, and 4) investigate the role of cell proliferation in causing the cardiac enlargement. I found that mRNA expression of 11β-hydroxysteroid dehydrogenase 2 (11βHSD2), the insulin-like growth factor receptor type 1 (IGF1R), and the angiotensin type 1 receptor (AT1R) to angiotensin type 2 receptor (AT2R) mRNA ratio decreased in response to elevated cortisol in the LV. The decrease in IGF1R mRNA expression and the AT1R to AT2R ratio may have been an attempt by the heart to limit growth whereas the decrease in 11βHSD2 expression indicates cortisol is able to increase its action at MR and GR.

Ontogeny analysis revealed that MR and GR mRNA expression are high at all points and 11β-hydroxysteroid dehydrogenase 1 mRNA expression is significantly higher than 11βHSD2 at all times, implying cortisol action at MR and GR is important for heart growth throughout late gestation. I also found that angiotensin converting enzyme 1 mRNA dramatically increases in both ventricles in late gestation implicating angiotensin II production is important in maturing the heart for life after birth. This study also showed that a reduction in mRNA of growth promoting IGF family members towards term within both ventricles may contribute to the decrease in myocyte proliferation that occurs during the last third of gestation.

In vivo experiments demonstrated that cardiac specific blockade of MR negated cortisol-induced heart enlargement and that GR blockade lessened it, suggesting corticosteroid receptors mediate the enlargement. Picrosirius red staining of the hearts revealed that fetal heart enlargement in response to elevated cortisol is not accompanied by an increase in cardiac collagen deposition, but KI67 staining of the hearts revealed that enlargement may be due to an increase in myocyte proliferation.

CHAPTER 1
INTRODUCTION

Background and Significance

Proper fetal development is important for good health not only before birth, but also for after parturition and into adult life. One example of fetal development potentially affecting adult health is in the development of cardiovascular disease (CVD). For instance, we now know from studies conducted on adults who were conceived during the Dutch famine of 1944-1945 and born with low birth weights due to undernutrition that they carry higher risks for cardiovascular disease and diabetes in their adult years (Roseboom *et al.* 2001). There is also evidence in both animals (aghajafari *et al.* 2002, Newnham *et al.* 2001) and humans (Walfisch *et al* 2001, Banks *et al.* 1999, French *et al.* 1999) that overexposure of the fetus to corticosteroids can cause fetal growth restriction, potentially increasing the risk for development of disease later in life. Currently, CVD is one of the biggest health concerns facing the world, and it appears risk for this disease may be partially determined by fetal health.

Role of Cortisol in Pregnancy

Cortisol is a stress-response hormone, released from the adrenal cortex, which binds either mineralocorticoid receptors (MR) or glucocorticoid receptors (GR). Release of cortisol by the adrenal gland is controlled by the pituitary gland secretion of adrenocorticotropin (ACTH), which is controlled by the hypothalamus secretion of corticotropin releasing hormone (CRH). When cortisol levels increase beyond the set-point, the pituitary gland and hypothalamus sense the high cortisol levels and release of ACTH and CRH are inhibited (Figure 1-1).

Cortisol can be chronically elevated during chronic stress, but is also normally increased during pregnancy. In fact, maternal plasma levels of cortisol are elevated during human pregnancy (Carr *et al.* 1981), and doubled during late gestation in ewes (Bell *et al.* 1991, Keller-

Wood 1998). Cortisol plays an important role in fetal development including inducing maturation of the intestinal tract (Arsenault *et al.* 1985; Galand G *et al.* 1989) and inducing maturation of surfactant production in the lung (Ballard *et al.* 1996; Liggins *et al.* 1972). Highlighting the importance of glucocorticoids in fetal organ development is the fact that corticosteroids have been used to mature fetal lungs and prevent respiratory distress syndrome since 1972 (Liggins *et al.* 1972).

Importance of Maintaining Proper Cortisol Levels in Pregnancy

Whereas a single course of antenatal corticosteroids for fetal maturation in preterm birth instances appears to be safe (Crowley *et al.* 2002, NIH 2001), repeated treatments to women with recurring risk of preterm birth has become the norm (Quinlivan *et al.* 1998). Recently, it has been shown in animals (aghajafari *et al.* 2002, Newnham *et al.* 2001), and humans (Walfisch *et al.* 2001, Banks *et al.* 1999, French *et al.* 1999), that repeated antenatal corticosteroid treatments can cause fetal growth restriction, a symptom of pre-programming for cardiovascular disease or diabetes later in life (Law *et al.* 1996, Phillips *et al.* 1998). It has been suggested that this programming may be due to excess exposure of the fetus to glucocorticoids (Benediktsson *et al.* 1993, Clark *et al.* 1998, Roghair *et al.* 2005, Seckl *et al.* 1998, Seckl *et al.* 1997). Short-term glucocorticoid exposure to the fetus in the third trimester has been demonstrated to have adult-life programming effects in rats (Levitt *et al.* 1996). Amazingly, it has been demonstrated in rats that excessive corticosteroid exposure induced pre-programming of the fetus to adult cardiovascular disease can cause the same effects in the next generation, even when the next generation were not themselves exposed to excess corticosteroids during fetal life (Drake *et al.* 2005). Also, postnatal hypertension has been observed in sheep following glucocorticoid treatment in early or mid-gestation (Figueroa *et al.* 2005, Wintour *et al.* 2003).

Direct effects on the fetus have also been observed in response to improperly regulated glucocorticoid levels during pregnancy. It has been shown previously that consequences of a reduction of cortisol levels in late gestation ewes are a reduction in fetal growth, a reduction in maternal plasma volume and uteroplacental flow, an altered placental morphology, and a greater likelihood for fetuses to become hypoxic (Jensen *et al.* 2002, Jensen *et al.* 2002, Jensen *et al.* 2005). On the other hand, modest elevations in ovine maternal cortisol levels between ~120 and ~130 days gestation (term = ~145 days) have been shown to cause an increase in heart growth while reducing overall fetal growth (Jensen *et al.* 2005).

Corticosteroid Receptors and 11 Beta-Hydroxysteroid Dehydrogenases

MR and GR are the two main receptors cortisol acts on within the body. The selectivity of MR and GR for the endogenous steroid ligands differs among species. For instance, in the rat MR binds its primary glucocorticoid, corticosterone, and aldosterone with high affinity, but binds cortisol with slightly lower affinity (Sutano *et al.*1987). Conversely, in the hamster corticosterone and cortisol bind MR with high affinity while aldosterone binds with lower affinity (Sutano *et al.* 1987). Interestingly, dogs secrete both cortisol and corticosterone (Westphal *et al.* 1971, Keller-Wood *et al.* 1983) with MR having greater affinity for corticosterone than cortisol and aldosterone, which have similar affinity for MR (Reul *et al.* 1990). On the other hand, human MR has similar affinities for cortisol and aldosterone and corticosterone (Arriza *et al.* 1987). In sheep, corticosterone is not secreted in appreciable amounts while cortisol and aldosterone are the major corticosteroids of action (Westphal *et al.* 1971). Additionally, both the human and the ovine GR have greater affinity for cortisol than for aldosterone. However, whereas MR has been shown to be the higher affinity receptor for cortisol (K_d = 0.52 ± 0.09 nM) in ovine hippocampal cells, GR is the lower affinity (K_d = 1.48 ± 0.11 nM) but more abundant and higher capacity receptor (Richards *et al.* 2003). This study also

showed that aldosterone affinity for MR is similar to that of cortisol. However, unless local levels of 11 beta-hydroxysteroid dehydrogenase 2 (11βHSD2) are high (i.e. kidney), cortisol would be expected to occupy MR in vivo rather than aldosterone because of the higher relative concentration of cortisol in the plasma (<0.1 nM aldosterone as compared to 1-10 nM cortisol). Furthermore, in sheep it is estimated that approximately 20% of circulating is free and not bound to cortisol binding globulin. This means that in the sheep fetus, where average the average cortisol concentration is ~1.5 ng/ml, free cortisol concentrations would be ~0.8 nM. It also means that based on the study of Richards *et al.* (Richards *et al.* 2003), we would predict that these free concentrations would result in approximately 65% occupancy of MR and 35% occupancy of GR in the sheep fetus. Thus, at basal levels we would expect cortisol to exert more effects via MR than via GR activation.

Locally, the actions of cortisol are primarily dependent on the expression levels of 11βHSD1 and 2. 11βHSD2 is responsible for converting cortisol (active at MR and GR) into cortisone (inactive at MR and GR) (Figure 1-2). The counterpart of 11βHSD2 is 11βHSD1, which has a primary role that is opposite to 11βHSD2 by converting cortisone into active cortisol, although it has the ability do the same as 11βHSD2 also (Figure 2; Seckl *et al.* 2001). When large amounts of cortisol are converted into inactive cortisone by 11βHSD2, aldosterone is then free to bind MR receptors. Organs containing high amounts of epithelial tissue such as the kidney tend to highly express 11βHSD2 in order to prevent glucocorticoid action at MR, which is also highly expressed, and allow aldosterone binding (Young *et al.* 2007). This maintenance of proper 11βHSD2 levels in the kidney is critical as inactivation of this enzyme results in hypertension, potassium wasting, and sodium retention as a result of glucocorticoid activation of MR (Stewart *et al.* 1988, Edwards *et al.* 1989). On the other hand, while MR expression in the

hippocampus is similar to that found in the kidney, 11βHSD2 exhibits substantially less expression making the hippocampus primarily a site of glucocorticoid action at MR (Kim *et al.* 1995). Similarly, 11βHSD2 expression in the adult human heart is less than 1% of that found in the kidney (Lombes *et al.*1995), suggesting that the myocardium is more operationally similar to the hippocampus than it is to the kidney. MR, GR, and 11βHSD1 and 2 expression levels within the fetal heart have not been fully elucidated, but would be helpful in determining the role of cortisol in fetal heart growth and maturation.

Consequences of Elevated Cortisol on Fetal Heart Growth

As previously mentioned, modest elevations in ovine maternal cortisol levels between ~120 and ~130 days gestation have been demonstrated to cause an increase in heart growth (Jensen *et al.* 2005). Interestingly, in this study the enlargement was observed without a chronic rise in blood pressure, suggesting cortisol may be acting directly on receptors in the fetal heart to cause increased growth. Additionally, Giraud *et al.* have shown that cortisol infusion directly into the ovine fetal coronary artery increases heart mass due to an increase cell cycle activity, and this was without an increase fetal blood pressure also (Giraud *et al.* 2006). Furthermore, dexamethasone (GR agonist) administration into the maternal rat (48 μg/d from E17) resulted in increased myocyte proliferation and relative heart size in the fetal and newborn rat (Torres *et al.* 1997).

It has also been shown that acute infusion (~129 through 132 days gestation) of non-physiologically high amounts of cortisol (resulting in ~100 times higher plasma concentrations than controls) directly into the ovine fetus also leads to cardiac enlargement, but this was accompanied by a significant increase in mean arterial pressure (46.7 ± 1.5 vs. 59.7 ± 2.0) (Lumbers *et al.* 2005). Hearts from this study exhibited increased left ventricular cell volume

and higher expression of angiotensinogen mRNA in both ventricles, suggesting the increase in blood pressure was a major force in driving the increase in cardiac enlargement. Whether MR, GR, or both receptors within the heart are directly mediating the cardiac enlargement has not been studied. However, whereas the mechanism by which elevated cortisol levels cause enlargement of the fetal heart is not well understood, these studies suggest that the mechanism of enlargement may differ based on delivery method, amount, and duration.

Role of MR in the Heart

Whereas little has been done to elucidate expression of MR in fetal hearts, MR has long been known to be expressed in adult hearts, as Lombes et al. showed in rabbits (Lombes *et al.* 1992). MR appears to have a major role in hearts that have experienced ischemic injury or are experiencing heart failure. In rats MR blockade has been shown to improve modulate the inflammatory response improve vasomotor dysfunction and vascular oxidative stress after myocardial infarction (Fraccarollo *et al.* 2008 and Sartorio *et al.* 2007). In dogs with chronic heart failure, it was shown that eplerenone (MR antagonist) administration reduced LV filling pressure and end-diastolic wall stress and stiffness, and improved LV relaxation (Suzuki *et al.* 2002).

Promising studies have also recently been done in humans looking at the role of MR in heart failure. The Randomized Aldactone Evaluation Study (RALES) trial demonstrated that patients from various backgrounds and countries, who had severe heart failure, were dramatically helped upon daily administration (25mg/day) of spironolactone, an MR antagonist. Improvement in survival at 3 years was 30% while improvement in hospitalization was at 35%, causing the trial to be halted just over the halfway point of the projected time-course for lack of a need to continue the trial (Bertram *et al.* 1999). Similar benefits have been seen with eplerenone, a more specific MR antagonist, in the Eplerenone Post-Acute Myocardial Infarction Heart

Failure Efficacy and Survival Study (EPHESUS) (Bertram *et al.* 2001). Most think that the improvement seen in the patients of the RALES trial was due to blocking the effect of aldosterone (Bertram *et al.* 1999). This is reasonable because, although not much is known about aldosterone signaling (Fiebeler *et al.* 2003), aldosterone is known to promote harmful events in the heart such as endothelial dysfunction, water and sodium retention, and hypertrophy (Fraccarollo *et al.* 2004). It has been suggested, however, that many heart failure patients without elevated plasma aldosterone levels still receive the same benefits from MR blockade, indicating aldosterone may not be the only ligand (Young *et al.* 2007). Additionally, while it has long been known that MR and 11βHSD2 are co-expressed in hearts of many animals, including humans (Lombes *et al.* 1995), it has also been proposed that reductions in 11βHSD2 expression in adult human hearts can lead to cardiac damage via cortisol binding, and not necessarily aldosterone (glorioso *et al.* 2005, funder *et al.* 2005).

In cultures of neonatal myocytes, it is presumed that MR mediates aldosterone actions in directly stimulating myocyte surface area (Okoshi *et al.* 2004) and remodeling of the myocyte membrane (Kliche *et al.* 2006). Additional evidence of intracardiac action at MR is cortisol increases expression of atrial natriuretic peptide in cultured neonatal myocytes, and both cortisol and aldosterone potentiate the effect of phenylephrine on hypertrophy in these cultures (Lister *et al.* 2006).

Cardiac Collagen Deposition

Collagen plays a crucial role in the heart in maintaining ventricular function by regulating its shape and size (Baicu *et al.* 2003). Within the heart, collagen serves as connective tissue found between myocytes, nerves, and blood vessels. The two main types of collagen found within the heart are types I and III with I being the predominant type. Type I collagen is often associated with tissue that is more stiff and rigid than tissue containing predominantly type III

collagen (Pearlman *et al.* 1982). Interestingly, multiple studies have correlated increases in LV collagen concentrations and wall stiffness (Janicki *et al.* 1993, Jugdutt *et al.* 2005).

Elevations in stress from increased ventricular pressure or volume, or from injury, leads to remodeling of the ventricular wall until the wall stress in normalized resulting in near normal systolic and diastolic function. Once wall stress exceeds the compensatory ability of the ventricle, heart failure will eventually occur (Brower *et al.* 2006).

An increase in collagen concentration in hypertrophied hearts was first reported by Pearlman *et al.* upon examining postmortem hearts of patients with and without heart failure (Pearlman *et al.* 1982). Also, Pauschinger *et al.* reported an increase in the collagen I/III ratio in myocardium from patients suffering from dilated cardiomyopathy (Pauschinger *et al.* 1999). Whereas many studies have shown increases in collagen concentrations in response to chronic elevations in pressure and myocyte hypertrophy, it has also been shown that increases in collagen can occur without an increase in cardiac hypertrophy (Narayan *et al.* 1989), and that cardiac hypertrophy caused by increased pressures is not always accompanied by an increase in fibrosis (Gelpi *et al.* 1991, Douglas *et al.* 1991). This is also observed in the hearts of human athletes (MacFarlene *et al.* 1991, Nixon *et al.* 1991).

There is increasing evidence that the MR receptor may play a role in increasing wall stiffness following injury as blockade of MR can prevent collagen concentration increases and ventricular remodeling following injury. It has been shown in rats that MR antagonism provides additional benefit to angiotensin II type 1 receptor (AT1R) blockade in preventing increases in fibrosis in myocardial infracted hearts (Fraccarollo *et al.* 2004), possibly explaining the benefits seen during the RALES trial in humans. Similarly, Takeda *et al.* demonstrated in rats that spironalactone (MR antagonist) administration greatly improved collagen accumulation and

reduced apoptosis in infarcted hearts (Takeda *et al.* 2007). Furthermore, *Nagata et al.* observed that MR blockade attenuated LV hypertrophy and heart failure in rats with low-aldosterone hypertension, indicating glucocorticoid action at MR may have been mediating the harmful events within the heart (Nagata *et al.* 2006). Collagen concentrations within fetal hearts enlarged from excess glucocorticoid exposure has not been studied but it is not outside the realm of possibility that enlargement of the fetal heart is accompanied by an increase in fibrosis as is often seen in the adult.

Hyperplasia vs. Hypertrophy in the Fetal Heart

Cardiac growth occurs through proliferation of myocytes throughout most of gestation (Smolich *et al.* 1989). The ability of myocytes to proliferate ceases sometime within the perinatal period through entering a final round of DNA replication followed by a lack of cell division (Oparil *et al.* 1984). This results in binucleation, or terminal differentiation, of the myocytes (Barbera *et al.* 2000). The progression of the heart from proliferation to terminal differentiation is gradual, however, leaving two different populations of myocytes in latter gestation and early perinatal life. One group contains mononucleated myocytes which contain the ability to grow the heart through proliferation and increasing cell size while the other group contains binucleated myocytes which grow strictly through hypertophic means starting at the point of terminal differentiation. This idea has been confirmed in sheep (Burrell *et al.* 2003, Jonker *et al.* 2007) and humans (Adler *et al.* 1975, Garcia *et al.* 2002, Huttenbach *et al.* 2001) where it was shown that the heart experiences myocyte proliferation and terminal differentiation simultaneously throughout the last third of gestation. It is also known that myocyte volumes increase during the last third of gestation in fetal sheep (Burrell *et al.* 2003), indicating that an increase in cell size, along with an increase in cell number, contributes to cardiac growth at this time. Jonker *et al.* observed in fetal sheep that binucleation became the more frequent out of the

myocyte cycle at ~115 days gestation, at which time cardiac growth through increase in cell size became much more considerable than before that point (Jonker *et al.* 2007).

It is not fully understood whether heart enlargement in response to elevated cortisol is from an increase in cell size, cell number, or both. As mentioned previously, there is evidence to suggest cortisol increases myocyte cell cycle activity in both rats (Torres *et al.* 1997) and sheep (Giraud *et al.* 2006). However, Rudolph *et al.* observed a decrease in the LV DNA concentration in response to cortisone infusion for 72-80 hours into the left coronary artery of fetal sheep (125-133 days) (Rudolph *et al.* 1999). The decrease in DNA concentration caused by cortisol was interpreted as being a result of a decrease in replication, suggesting cortisol may inhibit myocyte proliferation. Additionally, evidence exists that extreme increases in cortisol can lead to an increase in myocyte volume in fetal sheep (Lumbers *et al.* 2005). While there is conflicting evidence as to the method of growth observed in the heart in response to elevated cortisol, the magnitude of cortisol increase along with whether the increase was accompanied by an increase in blood pressure may be important factors in determining the type of growth that occurs.

Role of the Renin-Angiotensin System in the Heart

Angiotensin II is a peptide hormone important in maintaining cardiovascular homeostasis. Angiotensin II is synthesized from the cleaving of angiotensinogen (Aogen) into angiotensin I and the further cleaving of angiotensin I into angiotensin II. Aogen is a 118 amino acid protein primarily made in and released from the liver, but is also made for local activity in other tissues. Renin, which is released from the juxtaglomerular apparatus of the kidney, then acts to cleave 4 amino acids from aogen resulting in the decapeptide angiotensin I. Angiotensin I is then converted to Angiotensin II, an octapeptide, by angiotensin converting enzyme I. Angiotensin II then acts on Angiotensin II type 1 and 2 receptors (AT1R and AT2R) throughout the body.

Additionally, angiotensin converting enzyme 2 (ACE2) converts angiotensin I into angiotensin 1-9 and angiotensin II into angiotensin 1-7 (Figure 1-3).

The renin-angiotensin system (RAS) has been implicated as playing a major role in cardiac hypertrophy and fibrosis. It has been discovered in mice that local over-production of angiotensin II within the heart, without involvement of the systemic RAS, acted locally to cause interstitial fibrosis within the heart and also accelerated the deterioration of hearts that were post myocardial infarction (Xu *et al.* 2007). Furthermore, there is well established evidence for benefits of administration of ACE inhibitors and AT1R antagonists following myocardial infarction in humans and these classes of drugs are both recommended as treatments for patients who have suffered a myocardial infarction (Mancia *et al.* 2007, Rosendorff *et al.* 2007).

The RAS has been implicated in playing a role in cortisol-induced heart enlargement in fetal sheep. Acute administration of high doses of cortisol directly into the sheep fetus in late gestation affects components of the RAS system and may contribute in cortisol-induced heart enlargement. A study by Lumbers *et al.* showed that aogen mRNA expression increased in the treated group as compared to the control group (Lumbers *et al.* 2005). A problem in that study is that the dose of cortisol caused premature labor and elevated fetal blood pressure. It is, however, possible that the increased cortisol levels are directly stimulating an increase in aogen. Interestingly, Sundgren *et al.* showed that AT2 stimulates hyperplasia, but not hypertrophy, in fetal cardiomyocytes (Sundgren *et al.* 2003). Also, infusion of angiotensin II into fetal sheep has also been shown to stimulate left ventricular growth (Segar *et al.* 2001). Furthermore, growth-retarded fetuses of nutrient-restricted ewes show protected heart growth but also a decrease in AT1R and AT2R protein expression in mid-gestation, indicating the angiotensin II receptors play

a distinct role under nutrient restricted conditions compared to normal cardiac development (Gilbert *et al.* 2005).

Schneider and Lorell have suggested that Angiotensin II action is actually reflective of the AT1R to AT2R receptor expression ratio (Schneider *et al.* 2001). Cardiac hypertrophy caused by angiotensin II in adults is thought to be mediated by the AT1R (Zhu *et al.* 2003), however, the role of the AT2R in the heart has been thought to inhibit the growth in response to cardiac hypertrophy (Carey *et al.* 2005, Booz *et al.* 2004). Most evidence points to angiotensin II acting at AT1R primarily to constrict blood vessels, whereas AT2R actions promote vasodilation in the coronary microcirculation, in small resistance arterioles, and larger vessels such as the aorta (Carey *et al.* 2005). It has also been observed in human heart failure that AT1R expression decreases whereas AT2R expression increases or stays the same (Suzuki *et al.* 2004). Disruption of AT2 receptors in mice does not result in any histologic changes within the heart (Hein *et al.* 1995) and myocytes do not express AT2 receptors at any age in rats (Shanmugam *et al.* 1996).

Most of these studies were performed in adult hearts. The roles of AT1R and AT2R within the heart are much more defined in adults than in fetal life, so the AT2R may or may not perform the same functions in the fetal heart. It is known that the AT2R tends to be more highly expressed in many fetal and neonatal tissues relative to adult levels. For instance, Cox and Rosenfeld demonstrated in sheep that fetal vascular smooth muscle (VSM) expressed only AT2R systemically and AT1R did not start to express systemically in the VSM until two weeks after birth (Cox *et al.*1999). This suggests it is possible that the AT2R performs different roles in prenatal and neonatal life than it does in adult life.

The primary job of ACE1 is to convert angiotensin I into angiotensin II, after angiotensinogen has been converted into angiotensin I by renin. Cardiac hypertrophy is known to

be augmented by over-expression of ACE1 in rat hearts (Tian *et al.* 2004). ACE1 over-expression in mouse hearts has also been shown to induce cardiac arrhythmia, enlargement of the atria, and sudden death (Xiao *et al.* 2004). ACE2, on the other hand, converts angiotensin I into angiotensin 1-9 and angiotensin II into angiotensin 1-7. ACE2 therefore limits the amount of angiotensin II that is produced and potentially plays a role in protecting the heart from negative consequences observed from increased aniotensin II action (Danilczyk *et al.* 2006).

Role of the Insulin-Like Growth Factors in the Heart

The insulin growth factors (IGFs) 1 and 2 can act in both an endocrine and paracrine manner (LeRoith *et al.* 1995) and have been shown to be potentially important in fetal heart development and maturation (Cecilia *et al.* 1996, Sundgren *et al.* 2003). Whereas most of the pro-growth actions of IGF1 and IGF2 occur through binding of the IGF-1R, IGF2 has the ability to also bind IGF-2R which is thought to act to eliminate excess IGF2 and be anti-mitogenic (Randhawa *et al.* 2005). Ontogeny studies suggest IGF-2 may be more important in early fetal heart development whereas IGF-1 appears to be more important to the fetal heart in late gestation and into adulthood (Cheung *et al.* 1996). Multiple studies have shown the importance of IGFs in regulating growth in the fetal heart. IGF-1R and IGF-2R protein has been shown to be increased in enlarged hearts of fetuses of undernourished ewes, implying IGFs may play a role in the process of cardiac growth during restricted circumstances (Dong *et al.* 2005). However, a decrease in IGF2 mRNA abundance in preterm (111-116 days gestation) ovine hearts was observed in response to umbilical cord occlusion, but no change was observed in near-term (132-138 days gestation) fetuses (Green *et al.* 2000). Also, Sundgren *et al.* have shown in cultured fetal cardiomyocytes that IGF-1 stimulates proliferation of the myocytes and that this is mediated by ERK and PI3K (Sundgren *et al.* 2003). Moreover, over-expression of IGF1 in transgenic

mice leads to a 50% increase heart weight and a 20-50% increase in total number of myocytes, with no increase in myocyte hypertophy (Reiss *et al.* 1996).

IGF1 has been shown to have cardiac specific benefits in injured hearts. For instance, IGF1 has been demonstrated to decrease myocyte apoptosis in rats suffering from ischemia-reperfusion injury (Buerke *et al.* 1995), and in mice following myocardial infarction (Li *et al.* 1999). In dogs with induced heart failure, IGF1 reduced myocyte apoptosis and increased contractile function of the heart (Lee *et al.* 1999). It is also known that in IGF1 levels are low in patients with heart failure and that there is a correlation between the severity of ventricular systolic dysfunction and IGF1 levels (Niebauer *et al.* 2001, Anker *et al.* 2001). It was also observed in the Framingham Heart Study that there is an inverse relationship between plasma IGF1 levels risk for congestive heart failure in elderly people who have not previously experienced a myocardial infarction (Vasan *et al.* 2003), whereas a positive correlation between blood pressure and circulating IGF1 levels has also been observed (Andronico *et al.* 1993, Valensise *et al.* 1996). It has also been shown that both IGF1 mRNA and protein increase in the heart with the development of hypertension (Donohue *et al.* 1994, Guron *et al.* 1996). Furthermore, IGF1 mRNA expression increases in myocardium of hearts exposed to pressure overload from aortic banding or renal hypertension (Hanson *et al.* 1993, Wahlander *et al.* 1992). Moreover, chronic infusion of IGF1 into rats has been shown to lead to cardiac hypertrophy and enhance hypertrophy of viable myocardium after infarction (Duerr *et al.* 1995). The observed increase in cardiac hypertrophy in this study, however, led to improved systolic and diastolic function without an increase in fibrosis. In humans, there is evidence from those with the disease acromegaly, in which growth hormone (the primary stimulator of IGF1 expression) secretion is increased, that natural over-production of IGF1 leads to an increase in LV wall thickness (Fazio

et al. 1993, Fazio *et al.* 1994), whereas humans with growth hormone deficiency exhibit reduced cardiac mass (Cittadini *et al.* 1994, Cuocolo *et al.* 1996, Amato *et al.* 1993) that is restored upon growth hormone replacement therapy (Amato *et al.* 1993, Fazio *et al.* 1997). These findings suggest a direct role for IGF1 in maintenance of adult cardiac morphology.

Whereas IGF1 is thought to be more important in extrauterine life, a ~40% reduction in birth weight has been observed in mice with an inactivated IGF2 gene suggesting IGF2 is important in fetal growth (Dechiara *et al.* 1990). The mice from that study were fertile and appeared normal aside from the reduced growth, but it has also been observed that IGF2 null mice exhibit delayed lung development at the end of gestation most likely due to decreasing the plasma corticosterone levels, indicating IGF2 plays an important role fetal lung development (silva *et al.* 2006) . Chronic hypoxia has been shown to increase IGF2 mRNA levels of term human placentas, implicating the importance of IGF2 in maintenance of nutrient exchange of the mother and fetus during pregnancy (Trollman *et al.* 2007). In addition, evidence suggests IGF2 expression is increased in the ovine cotyledon in response to intrauterine growth restriction at 55 days gestation (De Vrijer *et al.* 2006).

Cortisol is thought to be a potentially key regulator of IGF1 production within the developing fetus. In support of this, IGF1 in ovine fetal skeletal muscle appears to be regulated by plasma cortisol concentrations as IGF1 mRNA expression decreases at the same time as the prepartum rise in ovine plasma cortisol levels, and this decrease in IGF1 expression is abolished when fetuses were adrenalectomized while premature increase in circulating cortisol concentrations in the fetus leads to a premature decrease in IGF1 mRNA expression in fetal skeletal muscle (Li *et al.* 2002). Furthermore, the same thing was found to be true in the developing ovine liver (Li *et al.* 1996).

IGF binding proteins 1-6 function to prolong the half life of IGFs in plasma and act to regulate the biological actions of IGFs *vivo*. IGFBPs have the ability to modulate the actions of IGF through regulating transport, turnover, and tissue distribution (Jones *et al.* 1995). IGFBPs 4 and 6 seem to primarily inhibit IGF actions whereas IGFBPs 1, 2, 3, and 5 have been shown to both inhibit and potentiate IGF actions (Yin *et al.* 2004). Previous studies have shown IGFBP2 and IGFBP3 play roles in fetal development. It has been reported that over-expression of IGFBP2 (Hoeflich *et al.* 1999) and IGFBP3 (Modric *et al.* 2001) in mice leads to a ~10% decrease body weight. Maternal nutrient restriction leads to an increase in plasma IGFBP2 levels within the fetus between 90 and 135 days gestation (Ogersby *et al.* 2004), but did not alter fetal heart weights. In contrast, Green and coworkers found that umbilical cord occlusion for four days (107-108 d fetuses) led to no change in plasma IGFBP2 or IGFBP3, but did lead to an increase in RV mRNA expression of IGFBP2 (Green *et al.* 2000); there was no change in either body weight or heart weight with this 4 days of manipulation.

Summary

It has been shown in multiple studies that elevated fetal exposure to cortisol leads to cardiac enlargement; however, the exact methodology involved has yet to be elucidated. The objectives of this dissertation were to determine if the heart enlargement is mediated by corticosteroid receptors within the heart, determine whether the enlargement is due to increased myocyte proliferation or accompanied by fibrosis, and investigate which genes may be important in aiding/causing the enlargement. Maintaining proper cortisol levels during pregnancy appears to be important to proper heart development of the fetus and may have long lasting implications pertaining to maintenance of heart health and regulating risk for cardiovascular disease throughout life. This research addressed the mechanisms by which elevated cortisol increases fetal heart size. In order to answer the specific aims outlined in this dissertation, I used *in vivo*

chronic catheterization of fetal sheep, immunohistochemistry, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), quantitative real-time polymerase chain reaction, and western blot analysis.

Specific Aims

- Specific Aim 1: To look for changes in expression of genes potentially important in cortisol-induced enlargement of the ovine fetal heart.
    - The mRNA expression of MR, GR, 11βHSD1, and11βHSD2 along with the IGFs and IGF receptors and major components of the RAS was studied in LVs from fetal hearts enlarged from elevated maternal cortisol and control hearts.
    - Immunohistochemical analysis was performed to look for location of MR, GR, 11βHSD1, and11βHSD2 within the heart.
- Specific Aim 2: To establish the ontogeny of genes in Aim 1 within the ovine fetal LV and RV.
    - The mRNA expression of MR, GR, 11βHSD1, and11βHSD2 along with the IGFs and IGF receptors and major components of the RAS was studied at various gestational ages within the LV and RV.
- Specific Aim 3: To determine if the increase in fetal heart weight and wall thickness in response to increased maternal cortisol is mediated by cardiac corticosteroid receptors, MR and/or GR, and to determine if cardiac fibrosis accompanies the cardiac enlargement in response to cortisol.
    - Fetal heart mass and wall thicknesses from 4 groups were examined including one control group with no manipulation. The three other groups were exposed to elevated maternal cortisol but one group received cardiac blockade of MR, another one received cardiac blockade of GR, and the other group did not receive either.
    - Picrosirius red staining was utilized to quantify the amount of collagen deposition that had occurred in each heart.
- Specific Aim 4: To determine if the observed cardiac enlargement from elevated cortisol is due to an increase in cell proliferation.
    - Immunohistochemical analysis was performed to see if there was any difference in the number of myocytes expressing Ki67 between the four groups.
    - PCNA protein was quantified to check for differences in expression between the groups.

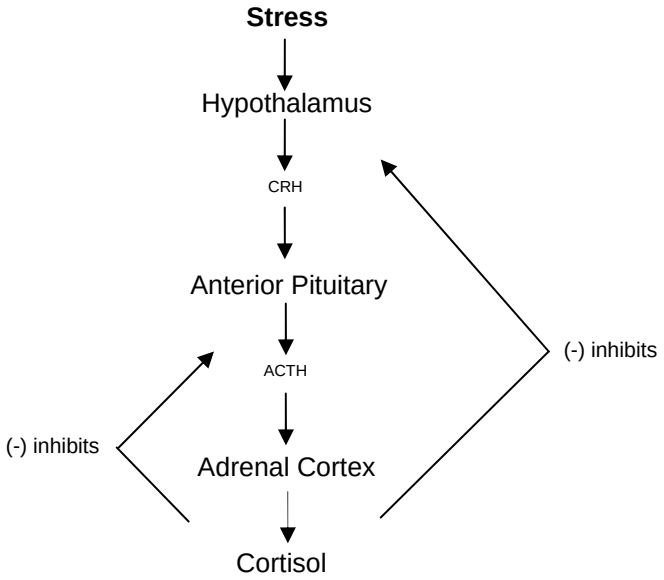


Figure 1-1. Components of the HPA axis and how it interacts. CRH – corticotrophin releasing hormone, ACTH – adrenocorticotropin stimulating hormone.

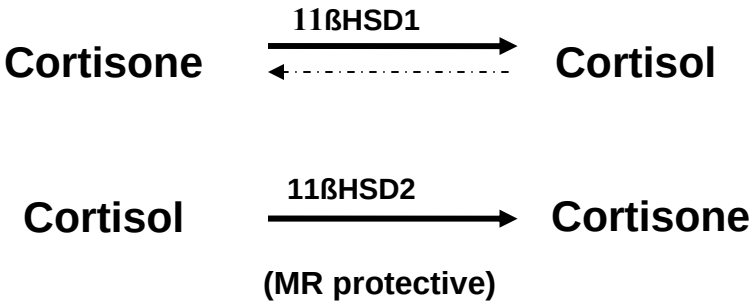


Figure 1-2. Different actions of 11βHSD1 and 2 and how they interact with cortisol.

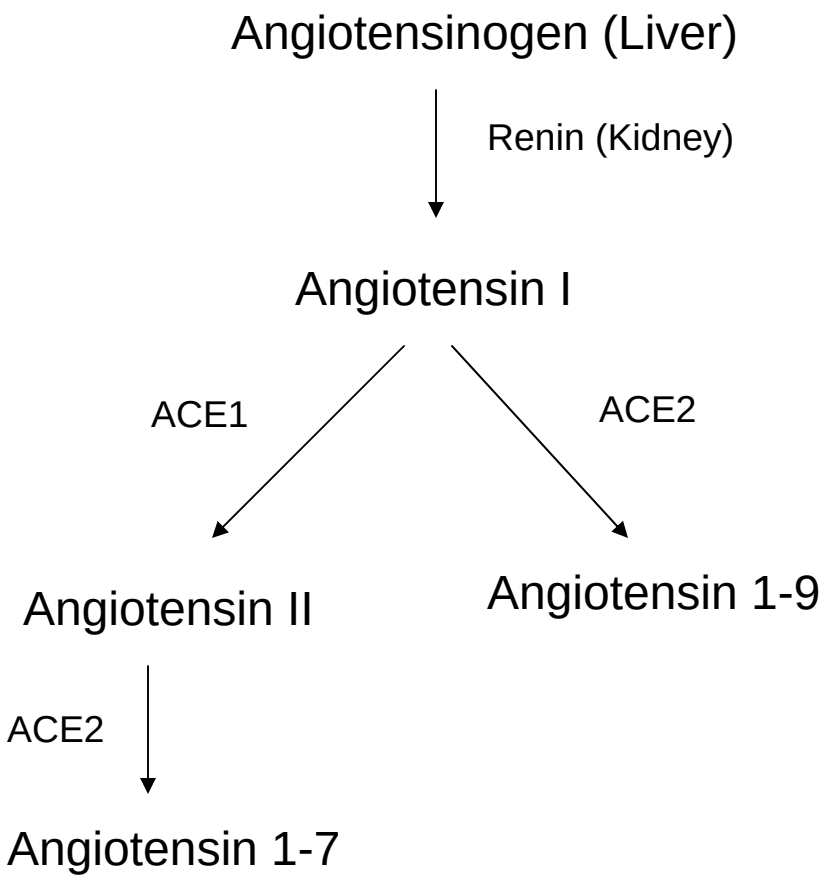


Figure 1-3. Components of the renin-angiotensin system.

CHAPTER 2
[1]INCREASED MATERNAL CORTISOL IN LATE GESTATION EWES DECREASES FETAL CARDIAC EXPRESSION OF 11B-HSD2 MRNA AND THE RATIO OF AT1 TO AT2 RECEPTOR MRNA

Introduction

Regulation of maternal cortisol levels during pregnancy is important for maintenance of fetal cardiovascular homeostasis and normal fetal growth. Previous studies in this laboratory have demonstrated that reduction of maternal cortisol levels in late gestation ewes results in reduced maternal plasma volume and uteroplacental blood flow, altered placental morphology and reduced fetal growth (Jensen *et al.* 2002, Jensen *et al.* 2005). The fetal consequences are similar to those observed with maternal hypovolemia (Daniel *et al.* 1989), suggesting that one of the effects of reduced maternal cortisol is mediated by reduced placental perfusion.

Elevations in maternal cortisol levels in late gestation also have an adverse effect on the fetus. Studies have indicated that maternal, but not fetal, glucocorticoid infusions reduce the rate of fetal growth (Newnham *et al.* 1999, Sloboda *et al.* 2000). Even modest chronic increases in maternal cortisol levels increase fetal heart growth while causing a reduction in overall fetal growth rates (Jensen *et al.* 2002). This finding is particularly interesting because it has been suggested that exposure of the fetus to glucocorticoids may have an adverse effect on postnatal cardiovascular health by preprogramming for hypertension or diabetes later in life (Clark *et al.* 1998, Roghair *et al.* 2005, Seckl *et al.* 1998). In rats, short-term prenatal treatment resulted in programming effects, including increased postnatal plasma corticosterone levels and blood pressure (Levitt *et al.* 1996). In sheep, postnatal hypertension results after glucocorticoid

[1]Reproduced with permission from Reini SA, Wood CE, Jensen E, & Keller-Wood M 2006. Increased maternal cortisol In late gestation ewes decreases fetal cardiac expression of 11{beta}-HSD2 mRNA and the ratio of AT1 to AT2 receptor mRNA. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology **291** 1708-1716.

treatment only when it is administered in early or mid gestation, and does not occur after synthetic glucocorticoid treatment in late gestation (Figueroa *et al.* 2005, Wintour *et al.* 2003). Acute glucocorticoid treatment in the late gestation ewe also does not appear to increase fetal heart weight (Newnham *et al.* 1999). The mechanism(s) by which chronically elevated maternal cortisol levels cause fetal heart enlargement is not known, but may require chronic corticosteroid exposure rather than acute glucocorticoid treatment, or may require the presence of agonists of the mineralocorticoid receptor (MR) rather than, or as well as, agonists of the glucocorticoid receptor (GR).

The purpose of this study was to investigate gene expression in the fetal hearts in which ventricular enlargement was measured in response to chronically elevated maternal cortisol concentrations in a previously published study (Jensen *et al.* 2005). I hypothesized that cortisol acts on mineralocorticoid receptors (MR) or glucocorticoid receptors (GR) in the fetal heart to induce genes involved in cardiac growth. In this study I used quantitative real-time PCR to test for changes in genes mediating cortisol action, MR and GR, as well as the 11β hydroxysteroid dehydrogenases (11β-HSD1 and 11β-HSD2), and genes suspected to be involved in growth: insulin-like growth factors (IGFs and their receptors), nitric oxide synthase (NOS-3), vascular endothelial growth factor (VEGF), myotrophin, angiotensin receptors, angiotensinogen, and angiotensin converting enzymes.

Materials and Methods

Experimental Design

RNA was extracted from the left ventricles taken from three groups of sheep fetuses from a previous study (Jensen *et al.* 2005). In that study one group of ewes was treated with cortisol (1 mg/kg/day) between 115-130 days of gestation ("high cortisol" group), a second group of ewes was adrenalectomized and treated with cortisol (0.5 mg/kg/day) between 115-130 days of

gestation ("low cortisol" group), and a third group of normal ewes had no alterations of cortisol between 115-130 days of gestation ("control group"). The "high cortisol" treatment regime produces circulating cortisol levels that are chronically elevated, but are within the range of maternal cortisol levels measured with mild maternal stress. The treatment regime in the "low cortisol" group produces maternal cortisol concentrations similar to those in nonpregnant ewes. Plasma hormone concentrations, organ weights and fetal growth rates for these studies have been previously published (Jensen *et al.* 2005). Fetal arterial and venous catheters were placed at the time of surgery; fetal and maternal plasma ACTH and cortisol concentrations were measured in samples collected on approximately days 120, 125 and 130 of gestation and blood pressure was measured on days 120 and 130 of gestation. Although there was no overall effect of maternal cortisol manipulation on maternal cortisol concentrations (maternal plasma cortisol concentrations, as previously reported (23) at 130d were 7.0 ± 1.0 ng/ml in controls, 10 ± 1 ng/ml in the high cortisol and 7.1 ± 0.3 in the low cortisol groups), maternal ACTH concentrations were increased ten-fold in the low cortisol group and were decreased by approximately 75% in the high cortisol group. The fetal cortisol concentrations at 130 days gestation were 5.7 ± 0.9 ng/ml in the control group, 7.4 ± 1.0 ng/ml in the high cortisol group, and 11 ± 3 ng/ml in the low cortisol group. Fetal ACTH levels were increased in the low cortisol group and decreased in the high cortisol group. These changes in ACTH indicate that the average cortisol levels over the day must be significantly altered in both ewes and fetuses. Further it is likely that the increase in plasma cortisol by 130d in fetuses in the low cortisol group result from the premature increase in plasma ACTH in these fetuses. We reported significant increases in fetal heart weight and left ventricular wall diameter in the fetuses from the high cortisol group compared to those in the control group; heart weight was increased by 25% and left ventricular

wall diameter was increased by 38%. There was no significant effect of increased maternal cortisol manipulation on fetal body weight, crown to rump or whole sternal girth measurements at necropsy, although there was a reduced rate of fetal sternal girth growth in the last 7 days of study (Jensen *et al.* 2005).

Real-Time PCR

Total RNA was extracted (Trizol; Invitrogen, Carlsbad, CA) from 0.2 - 0.3g of left ventricular free wall of fetal sheep in the control (n=6), low cortisol (n=4), and high cortisol group (n=5). All sheep were euthanized and tissues were collected at ~130 (129-132) days of gestation. Total RNA, as well as the RNA to DNA ratio, was measured spectrophotometrically to identify quantity and quality of RNA. RNA was checked for genomic DNA contamination using real-time PCR with the RNA as a template in place of cDNA and using probes and primers for GR (which produces a product within exon 2). RNA was then reverse transcribed into cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA).

Gene expression was measured using quantitative real-time PCR. The genes analyzed in this study were MR, GR, 11β-HSD1 and 2, IGF-I and II, IGF-1R and 2R, NOS-3, VEGF, myotrophin, angiotensinogen, ATR1 and ATR2, and ACE1 and ACE2. For measurement of mRNA for MR and GR (Keller-Wood *et al.* 2005), IGF-I (Meinel *et al.* 2003), NOS-3 (Wood *et al.* 2005), angiotensinogen (Burrell *et al.* 2003), 11β-HSD2, AT1R and AT2R (Dodic *et al.* 2002, Jensen *et al.* 2005), we used previously published sequences for ovine probes and primers. The primers for MR and GR were designed in the 3' untranslated region of the MR gene and in exon 2 of the GR gene and therefore detect α and β isoforms of MR and GR respectively (Kwak *et al.* 2003, Lu *et al.* 2006). Probes and primers used for IGF-1R, IGF-II, IGF-2R, ACE 1 and 2, and 11β-HSD1 were designed using Primer Express 2.0 (Applied Biosystems, Table 2-1) based on ovine sequences in the NCBI database or previously published by others. For the IGF-1R

probe/primer design, the ovis aries IGF-1R sequence (accession number AY162434) was used; the amplified sequence corresponds to base pairs 319-380. The IGF-II ovine sequence (accession number M89788) was used for IGF-II probe/primer design (base pairs 385-463) while the IGF-2R ovine sequence (accession number AF327649) was used for IGF-2R probe/primer design (base pairs 163-223 of published sequence). For ACE1, primers were designed using the ovine sequence (accession number AJ920032) between the base pairs of 662-726, while ACE2 primers were designed using an ACE2 bovine sequence (accession number BT021667) between the base pairs of 1245-1327. For both ACE1 and ACE2 SYBR Green (Bio-Rad, Hercules, California) was used instead of probes. The primers and probe for 11β-HSD1 were designed using the ovine sequence published by Yang et al. (base pairs 664-737 of published sequence) (Yang *et al.* 1992). The VEGF probe and primers were designed from the sequence published by Cheung and Brace (Cheung *et al.* 1998); the primers will detect the portion of the VEGF gene that encodes for the splice variants VEGF 120, VEGF 164, VEGF 188, and VEGF 205.

Because there were no published sequences for ovine myotrophin, we used PCR to amplify a portion of ovine myotrophin from adult heart RNA using primers designed from the published bovine sequence (accession number NM 203362; forward primer 221-240, reverse 555-574). PCR reactions were then carried out in an UNO II thermocycler (Biometra, Goettingen, Germany) using a PCR amplification kit (ABI, Foster City, CA). The PCR product was purified using a DNA purification kit (Promega, Madison, WI) and cloned into a TOPO vector (Invitrogen, Carlsbad, CA). The size of the product was confirmed on an ethidium bromide gel, and sequenced at The University of Florida MCBI DNA Sequencing Core Laboratory. The resulting ovine myotrophin partial gene sequence is shown in Table 2-2. The sequence obtained was 94% homologous to the corresponding bovine sequence (accession NM

203362.2; bp 321-674) and 90% homologous to the corresponding human sequence (accession NM 145808.1; bp 286-639). Probe and primers were then designed using Primer Express 2.0 (Applied Biosystems, Table 1).

Real time PCR reactions were performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). 25 µl reaction volume was used and contained 20 or 100 ng of cDNA for all genes except 18S, for which 1 ng was used. All probe and primer sets were checked for efficiency and for linearity of the relation between increasing concentrations of cDNA and Ct. Samples from all groups were analyzed in triplicate in the same 96 well plate. 18S expression was unchanged between the groups and all genes were normalized to 18S gene expression by calculating ΔCt. ΔCt is calculated as the difference between mean Ct of the gene of interest and mean Ct of 18S for the same cDNA sample; Ct is the cycle number at which the threshold amplitude is achieved.

Radioimmunoassay

Fetal plasma angiotensin II levels were measured by radioimmunoassay after extraction of the peptide from plasma using previously described methods (Pecins-Thompson *et al.* 1997). The lower limit of this assay is 1.9 pg/mL, as previously described. Fetal angiotensin II levels were measured in plasma collected at 120, 125, and 130 days of gestation from fetuses in the high maternal cortisol group (n=4), fetuses in the control maternal cortisol group (n=7), and fetuses in the low maternal cortisol group (n=5).

Immunohistochemistry and Collagen Staining

To determine localization of MR, GR, and 11β-HSD1 and 11β-HSD2 in fetal heart, untreated fetal sheep hearts of 126-128 days gestation were sacrificed, and the left ventricles were removed and fixed with 4% buffered paraformaldehyde. The tissues were dehydrated with increasing concentrations of reagent alcohol followed by xylene, and embedded in paraffin wax.

Five μm sections were cut by a Zeiss rotary microtome and placed onto poly-l-lysine coated slides. Deparaffinization and rehydration were performed using standard methods; following rehydration endogenous peroxide was quenched using incubation in hydrogen peroxide (0.3%; Fisher Scientific, Fair Lawn, NJ). Antigen retrieval was then performed by immersion into sodium citrate buffer at 95 degrees for 30 minutes.

The anti-MR monoclonal antibody G1-18 (provided courtesy of Dr. Elise Gomez-Sanchez, University of Mississippi Medical Center) and polyclonal antibodies GR M-20, 11β-HSD1 H-100, and 11β-HSD2 H-145 (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) were used to localize MR, GR, 11β-HSD1 (Schmidt *et al.* 2005), and 11β-HSD2 (Kadereit *et al.* 2005) in the sections. Immunohistochemistry for MR was performed following the methods described by Gomez-Sanchez (Gomez-Sanchez *et al.* 2006) except for the use of biotinylated goat anti-mouse secondary antibody (Zymed, San Francisco, CA). For immunohistochemical localization of GR, 11β-HSD1 and 11β-HSD2, tissue sections were blocked for one hour with 0.05M Tris pH 7.6, 5% milk, 5% goat serum, and 0.2% SDS, followed by incubation with primary antibody in blocking solution for one hour, and incubation with biotinylated secondary antibody (goat anti-rabbit; Zymed) for one hour. As a tertiary agent, streptavidin-peroxidase (Zymed, San Francisco, CA) was used. Metal enhanced diaminobenzidine (DAB; Pierce) was used as the chromogen. Control sections were similarly treated, but were incubated in blocking solution without primary antibody.

Some sections were stained with picrosirius red (Electron Microscopy Sciences, Hatfield, PA) which stains for collagen. Sections were hydrated as mentioned before and treated with 0.2% phosphomolybdic acid. The sections were immersed in sirius red (0.1% in saturated picric acid). Finally, the sections were washed with 0.01 N hydrochloric acid, rinsed in 70% alcohol,

dehydrated, and mounted in permount. All images were visualized using a Zeiss Axioplan 2 microscope and a SPOT Advanced digital imaging system (McKnight Brain Institute, University of Florida).

Data Analysis

Changes in gene expression among groups were analyzed by one way analysis of variance (ANOVA) using the ΔCt values. For graphical purposes, fold changes of the genes were calculated using the expression $2^{\wedge -\Delta\Delta Ct}$ in which ΔΔCt is the difference between ΔCt for the sample and mean ΔCt for the same gene in the control group (Livak *et al.* 2001). Comparisons of MR, GR 11βHSD1, and 11 βHSD2 gene expression were made by comparison of the ΔCt values for each gene relative to the 18S value; statistical analysis used paired t-test for comparison of two genes within the same tissue sample and t-test for comparison of the same gene in heart vs kidney.

Values for plasma angiotensin II concentration were compared by two-way ANOVA to determine significance across the cortisol treatment groups and gestational ages.

Linear regression analyses were performed to assess the relation between left ventricular wall thickness and fetal plasma angiotensin II concentrations levels, fetal plasma cortisol concentrations levels, AT1R mRNA, AT2R mRNA, IGF mRNA, IGF–R mRNA, 11β-HSD1 mRNA , 11 β-HSD2 mRNA, angiotensinogen mRNA, MR mRNA and blood pressure. Backward stepwise multiple linear regression was also performed to identify significant relationships between a series of possible independent variables and left ventricle wall thicknesses.

Results

Real-Time PCR Analysis

Expression of MR and GR

Real-time PCR analysis demonstrated expression of both MR and GR in the ovine fetal heart at 130 days. There was no significant difference in MR or GR gene expression between the high, low, and control maternal cortisol groups (Figure 2-1). GR expression relative to 18S was significantly greater than that of MR relative to 18S in the control fetal hearts (by 13 fold). However MR expression relative to 18S was significantly greater in fetal heart relative to that in fetal kidneys from the same fetuses (mean 2.6 fold difference).

Expression of 11β-HSD1 and 2

Real-time PCR analysis demonstrated expression of both 11β-HSD1 and 11β-HSD2 in fetal hearts. 11β-HSD2 expression relative to 18S in control hearts was significantly lower (by 5.5 fold) than 11β-HSD1 expression relative to 18S in the same hearts. 11β-HSD2 expression relative to 18S in heart was significantly lower than 11β-HSD2 in kidney (by 750 fold). No significant change in 11β-HSD1 expression was demonstrated in response to high or low maternal cortisol levels. 11β-HSD2 expression, however, was significantly lower in the high cortisol as compared to the control and low cortisol groups (Figure 2-1).

Expression of myotrophin, NOS-3, and VEGF

Expression of myotrophin, NOS-3, and VEGF mRNA in left ventricle were all unchanged in response to high or low maternal cortisol levels (Figure 2-2).

Expression of IGF-I and II, IGF-1R and 2R

No significant change in expression of IGF-I, IGF-II, or IGF-2R was found among the cortisol groups. IGF-1R expression significantly decreased in response to high maternal cortisol levels as compared to control and low maternal cortisol levels (Figure 2-3).

Expression of angiotensinogen, AT1R, AT2R, ACE1, and ACE2

I found that there were no significant differences in angiotensinogen, AT1R, AT2R, ACE1, or ACE2 gene expression in the fetal left ventricle among the maternal cortisol groups (Figure 2-4). However, I found that AT1R mRNA tended to decrease, whereas AT2R mRNA tended to increase, in fetuses of ewes with increased cortisol as compared to controls. There was a statistically significant increase in the ratio of AT2 to AT1 receptor expression in the high cortisol group (Figure 2-4, C).

Immunohistochemistry and Collagen Staining

MR, GR, and 11β-HSD1 staining was found in both cardiac blood vessels and myocytes in normal fetal hearts, while 11β-HSD2 showed very limited staining in myocytes and slightly more in blood vessels (Figure 2-5). GR positive cells were found in all layers of the blood vessel; MR, 11β-HSD1, and 11β-HSD2 appeared to be localized in both endothelial cells and the underlying smooth muscle cells. MR and 11β-HSD1 staining was more marked in the endothelial cells than in the underlying layers, and 11β-HSD2 staining was more marked in the vascular smooth muscle layer than in endothelial cells. MR, 11β-HSD1 and 11β-HSD2 positive cells did not appear to co-localize with picrosirius red staining regions (collagen containing regions surrounding the blood vessels), whereas there were some GR-positive cells apposing the collagen-rich regions, suggesting GR expression in fibroblasts.

Plasma Angiotensin II

Fetal plasma concentrations of angiotensin II tended to increase from day 120 to day 130 in response to the high cortisol as compared to the angiotensin II levels in the control group, which appear to remain relatively constant between days 120-130 (Table 2-3). The rise in fetal plasma angiotensin II levels in the high maternal cortisol group, however, was not statistically significant.

Regression Analysis

Linear regression analysis revealed a significant negative relationship between 11β-HSD2 mRNA in the heart and left ventricular wall thickness ($r=0.624$, $P<0.02$; Figure 2-6). There was no correlation between left ventricular wall thickness and either fetal blood pressure ($r=0.015$, $p=0.96$) or fetal plasma cortisol on day 130 ($r=0.214$, $p=0.48$), however fetal cortisol was elevated in the low cortisol as well as the high cortisol group by 130d. There were also no correlations between left ventricular wall thickness and either MR ($r=0.113$, $p=0.71$) or GR expression ($r=.065$, $p=0.83$) in the hearts. Negative relationships of left ventricular wall thickness with 11β-HSD1, AT1R and IGF-1R which did not reach statistical significance were also noted (11β-HSD1: $r=-0.544$, $p=0.054$; AT1R: $r=-0.458$, $p=0.058$; IGF-1R: $r=-.0541$, $p=0.056$). A weak positive relation between left ventricular wall thickness and plasma angiotensin II concentration on day 130 ($r=0.468$, $p=0.063$) was also found.

Backward stepwise multiple regression was used to assess the correlation between left ventricle wall thickness (dependent variable) to a series of independent variables: left ventricular AT1R and AT2R mRNA expression, 11β-HSD1 and 11β-HSD2 mRNA expression, angiotensinogen mRNA expression, MR mRNA expression, IGF-I and II mRNA expression, and IGF-1R and IGF-2R mRNA expression, as well as fetal plasma angiotensin II levels at 130 days, and blood pressure on day 130. Backward stepwise multiple regression using left ventricle thickness as the dependent variable identified a significant relationship (overall $r = 0.897$, $p<0.01$) between left ventricular wall thickness and AT2 to AT1 mRNA ratio, 11β-HSD1 mRNA, and 11β-HSD2 mRNA.

Discussion

In this study I found that both MR and GR are expressed in the fetal heart, as in adult myocytes in many species, including humans (Lombes *et al.* 1999). I found that MR and

11βHSD1 gene expression are relatively abundant in ovine fetal heart. The results suggest that small increases in cortisol could influence fetal heart size via action at the MR or GR receptors in the fetal heart. These results further suggest that changes in the ratio of angiotensin receptors (AT1 and AT2) may be a downstream mechanism for the effect of cortisol.

Role of Corticosteroids Acting at MR or GR

Action of cortisol in tissues depends on the expression of MR and/or GR and activity of 11βHSD1 and 11βHSD2. Whereas 11β-HSD1 primarily converts cortisone into cortisol in most tissues (Seckl *et al.* 2001), 11β-HSD2 converts cortisol into cortisone, which is inactive at MR and GR (Mihailidou *et al.* 2005). Further, action of 11β-HSD2 alters intracellular redox state, which may reduce the ability of cortisol to activate MR after binding. Thus in epithelial tissues such as kidney, high levels of 11β-HSD2 co-expressed with MR results in a "MR protective" effect which reduces basal MR activation by cortisol or corticosterone, but permits aldosterone action at the MR receptors (Mihailidou *et al.* 2005). However in normal hearts, MR are expressed during fetal life (by E13.5 in murine heart), but there is relatively little expression of 11β-HSD2 in prenatal mouse hearts nor is11β-HSD2 appreciably expressed in cardiomyocytes postnatally (Brown *et al.* 1996, Thompson *et al.* 2004)

It has also been suggested that low 11β-HSD2 activity in adult hearts allows cortisol as well as aldosterone to have detrimental effects on the heart (Funder *et al.* 2005, Glorioso *et al.* 2005). Evidence for both MR-mediated and GR–mediated effects on the heart have been found. In adult animals, aldosterone action at MR is thought to cause cardiac hypertrophy and fibrosis after ischemia (Fiebeler *et al.* 2003, Fraccarollo *et al.* 2004). In humans with severe heart failure, there is a reduction in severity of cardiac hypertrophy after blockade of the MR receptor (Pitt *et al.* 1999). On the other hand activation of either MR or GR alone have little effect on hypertrophy in cultures of neonatal myocytes, but GR have been shown to potentiate the effect of

phenylephrine on hypertrophy in neonatal myocytes (Lister *et al.* 2006). Interestingly, in 11β-HSD2 knockout mice postnatal mortality is high, but surviving mice have enlarged hearts without evidence of cardiac fibrosis (Kotelevtsev *et al.* 1999), suggesting that fibrosis only occurs in adult hearts, or when hearts have been subjected to ischemic damage. Overexpression of 11 β-HSD2 in murine cardiomyocytes, however, results in cardiac hypertrophy, interstitial fibrosis, and heart failure (Qin *et al.* 2003). This effect is markedly reduced by treatment with the MR blocker eplerenone; the effect is thought to be mediated by an increased access of aldosterone to MR in the myocytes with high levels of 11βHSD2, and suggests that MR binding with aldosterone produces a greater hypertrophic effect than does corticosterone binding at MR and GR.

Recently it was shown that cortisol stimulates cell cycle activity in cardiomyocytes of near term fetal sheep infused with cortisol into the circumflex artery. These results suggest that cortisol can act directly on the fetal heart to stimulate hyperplastic, but not hypertrophic, growth (Giraud *et al.* 2006). This suggests that the effects seen in our study, with even lower levels of circulating cortisol in the fetus, may be due to hyperplasia rather than hypertrophy.

Although I cannot determine from these studies whether MR or GR are responsible for the observed effects, the relative levels of MR and of 11βHSDs suggest that action at MR as well as at GR could be involved. In ovine fetal heart both MR and GR were expressed in myocytes, and 11βHSD1 appears to predominate over 11βHSD2 expression, particularly in the myocytes. In sheep as in other species, MR has greater affinity for cortisol than does GR (Richards *et al.* 2003), and MR affinity for cortisol and aldosterone is similar. In fetal sheep plasma aldosterone concentrations are relatively low, so that basal occupancy of MR by either cortisol or aldosterone is expected to be much less than 100%. However the increase in fetal plasma cortisol occurring

with maternal cortisol infusion in this study would be expected to cause a substantially greater change in MR occupancy than in GR occupancy because of the difference in affinity of cortisol for these two receptor subtypes. Left ventricular wall thickness was negatively correlated to abundance of 11β-HSD2 mRNA (Figure 5), suggesting that decreased inactivation of cortisol in the fetuses of the ewes treated with cortisol might play a role in chronic stimulation of cardiac growth.

Additionally, my data show that while MR, GR, and 11β-HSD1 appear to be localized to blood vessels and myocytes in the fetal heart, 11β-HSD2 seems to be more highly expressed in blood vessels than in myocytes, and in vascular smooth muscle than in endothelial cells. In the vasculature, 11β-HSD2 is thought to modulate vascular reactivity and may limit cortisol activation of MR (38). I do not have any data regarding distribution of 11βHSD2 in tissues from the treated fetuses, and so I cannot speculate on whether the decrease in 11βHSD2 with maternal cortisol infusion altered myocyte or vascular expression of the protein.

These data suggest that the heart enlargement is not an indirect effect of cortisol via changes in fetal blood pressure. In the previous publication from this study it was reported that fetal blood pressure was not significantly elevated by the chronic maternal infusion of cortisol (Jensen *et al.* 2005); fetal mean arterial pressure at 130d was 50.4 ± 1.5 mmHg in the high cortisol group, and 47.8 ± 2.1 mmHg in the control group. Neither linear regression nor backward stepwise multiple regression analysis showed a significant relationship between change in blood pressure from day 120-130 and left ventricle wall thickness. Studies in several animal models of hypertrophy, including hypertrophy induced by deoxycorticosterone (DOC) or carbenoxelone (an 11β-HSD inhibitor), have shown that the MR blocker eplenerone inhibits the effect on cardiac hypertrophy and inflammatory markers without altering blood pressure (Young

et al. 2003). These data suggest that the increase in left ventricular wall thickness was not secondary to increases in fetal arterial pressure, but by other steroid receptor mediated mechanisms.

Role of Growth-Related Genes: VEGF, eNOS, Myotrophin and IGFs

Several genes that might be expected to be related to growth were not found to be increased. I reasoned that since the fetal hearts were enlarged in the high cortisol group, perhaps angiogenesis was being stimulated by VEGF and NOS-3 in these hearts; however neither of these genes was significantly increased in fetal left ventricle among the maternal cortisol treatment groups (Figure 2). Myotrophin was also not increased in the enlarged hearts (Figure 2). Myotrophin has been suggested as a causal agent in cardiac hypertrophy in both humans and in rodents (Anderson *et al.* 1999, Sarkar *et al.* 2004, Shanmugam *et al.* 1996). In mice, over-expression of myotrophin causes cardiac hypertrophy, and pressure overload causes a ventricle-specific increase in myotrophin as well as wall thickness. Treatment of cultures of neonatal myoctes with myotrophin increased the size of the myocytes and stimulated protein synthesis without increasing DNA synthesis. The absence of a change in myotrophin mRNA in our study suggests, therefore, that the increased size of the fetal heart may result from hyperplasia rather than hypertrophy.

IGF-I stimulates proliferation of cardiomyocytes in cultures from fetal sheep hearts (Sundgren *et al.* 2003), and increased IGF protein has been implicated in the increase in heart size in fetuses of undernourished ewes (Dong *et al.* 2005). However, our findings suggest that the insulin-like growth factors may not be important in the cortisol-induced heart enlargement effect, as high cortisol levels appear to have a negative influence on IGF-1R mRNA expression in the heart. This should not be surprising as increases in cortisol at term have been shown previously to be responsible for IGF-I and IGF-II down-regulation in skeletal muscle in fetal

sheep (Li *et al.* 2002, Li *et al.* 1993); glucocorticoids have also been shown to decrease IGF-2R in fetal rat osteoblasts in culture (Rydziel *et al.* 1995). However, IGFs may play a role in the enlargement through differential regulation of the IGF actions by the IGF binding proteins; more studies regarding the IGF and IGF binding proteins concentrations in fetal plasma and fetal heart in this model are needed before it can be concluded that IGFs do not play any role in the enlargement.

Role of the Renin-Angiotensin System

The observation regarding the relative mRNA expression of AT2 to AT1 receptors in the fetal hearts suggest that the renin-angiotensin system could play a role in cortisol-induced fetal heart enlargement. In adult hearts, angiotensin appears to cause fibrosis and hypertrophy (Zhu *et al.* 2003); whereas infusion of angiotensin II in fetal sheep stimulates left ventricular growth (Segar *et al.* 2001), in cultures of ovine fetal cardiomyocytes, angotensin II stimulates hyperplastic growth (Sundgren *et al.* 2003). Although these results indicate that fetal plasma angiotensin II concentrations tend to increase in response to the chronic cortisol infusion when compared to the saline infused control group, it is not likely that the increase in plasma angiotensin *per se* stimulates the increase in wall thickness. Fetal plasma angiotensin II levels also tend to increase in the low maternal cortisol group; this increase in the fetuses of the ewes with reduced cortisol probably results from the dramatic increase in fetal ACTH and cortisol levels by 130d (Jensen *et al.* 2005). I found no correlation between plasma angiotensin II levels and the increase in left ventricular wall thickness, also suggesting circulating angiotensin is not the direct mediator of this effect.

Local production of angiotensin has been implicated in a previous study of cortisol-induced fetal heart growth in fetal sheep. A recent study (Lumbers *et al.* 2005) demonstrated that a more acute treatment with much larger doses of cortisol (approximately 72 mg/d for 2-3 days)

directly into the fetus late in gestation caused left ventricular hypertrophy and increased angiotensinogen gene expression in the heart. However the mechanism for the effect of these much larger doses of cortisol is likely to be different than the mechanism in our study. In the present studies we found that expression of angiotensinogen in the left ventricle tended to decrease. The differences between these studies could be related to the cortisol levels produced (> 300 ng/ml) which would maximally activate both MR and GR, or secondary to the larger increase in blood pressure produced by the greater dose of cortisol (46.7 ± 1.5 control mean arterial pressure increased to 59.7 ± 2.0 in cortisol treated). As dexamethasone treatment results in increased expression of angiotensinogen in cultured neonatal myocytes (Dostal *et al.* 2000), the effect on angiotensinogen may require higher concentrations of cortisol exerting effects via the GR. Nevertheless, it is possible that there was an initial transient rise in cardiac angiotensinogen in our chronic model.

The enzymes ACE1 and ACE2 can also regulate local levels of angiotensin II in the heart. ACE1 is an enzyme that converts angiotensin I into angiotensin II and has been found to augment cardiac hypertrophy when over-expressed in rat hearts (Tian *et al.* 2004) and induce cardiac arrhythmia, enlargement of the atria, and sudden death when over-expressed in mouse hearts (Xiao *et al.* 2004). ACE2, however, is thought to be cardio-protective because it converts angiotensin I into angiotensin 1-9 and angiotensin II into angiotensin 1-7, thereby limiting the amount of angiotensin II that is produced (Danilczyk *et al.* 2006). I observed no significant change in ACE1 or ACE2 mRNA expression between the cortisol groups, again suggesting local production of angiotensin II is not playing a major role in the enlargement.

In my study the magnitude of the increase in ventricular wall thickness was related to the relative expression of AT2 and AT1 receptors. My finding that the ratio of AT2 to AT1 receptor

mRNA ratio increases also differs from that of Lumbers; they found no significant change in expression in either of the receptors (Lumbers *et al.* 2005). In adult hearts, the hypertrophy caused by angiotensin II is thought to be mediated by AT1 receptors (Zhu *et al.* 2003). It has been observed in human hearts that are failing, AT2 receptor expression increases or remains constant while AT1 receptor expression decreases (Segar *et al.* 1997). The AT2 receptor is traditionally believed to have anti-AT1 receptor-mediated effects, whereas the AT1 receptor is known to be pro-growth (Dostal *et al.* 2000). Other studies in adult hearts have indicated that an increase in AT2 to AT1 receptor ratio in the heart is associated with an anti-growth effect in response to cardiac hypertrophy (Booz *et al.* 2004). It is therefore possible that the rise in the AT2R to AT1R ratio is simply a chronic response which limits cardiac growth. However it is also intriguing to hypothesize that the AT2 receptor has a different role prenatally and performs different actions in the period of normal heart growth in fetal life than in the response to hypertrophy in adult life. In support of this hypothesis is the observation that AT2 receptors are more highly expressed in many tissues in fetal or neonatal life relative to adult life. The role of AT2 receptors in fetal heart is not clear; in mice disruption of AT2 receptors does not result in histologic changes in the heart (Hein *et al.* 1995), and in rats there is no expression of AT2 receptors in myocytes at any age (Shanmugam *et al.* 1996). In the fetal sheep, AT2 receptors are more abundant in the heart than in other tissues and are much more abundant than in adult heart; in fact AT2 receptors rapidly decrease in expression at birth (Burrell *et al.* 2003). Although AT2 receptors do not appear to be involved in right ventricular hypertrophy after pulmonary artery banding, or to be involved in basal growth of the left or right heart (Segar *et al.* 1997), more studies are needed regarding the balance between actions mediated by AT1 and AT2 receptors on the late gestation myocyte and their role in cortisol-mediated heart growth in late gestation.

In conclusion, these data suggest the possibility that enlargement of the fetal heart can be induced by direct actions of cortisol on MR and/or GR in fetal cardiac myocytes. An action of cortisol in the fetal heart is supported by the relatively low expression of 11β-HSD2, which would allow the relatively low circulating concentrations of cortisol in fetal plasma to activate MR, and to a lesser extent, GR. Furthermore, our data suggests that genes related to cardiac hypertrophy are not stimulated and that the growth is independent of changes in blood pressure, but that local changes in myocyte and/or coronary vasculature activation by cortisol are involved. My data also suggest that changes in the renin-angiotensin system may play a role in the ventricular growth through changes in relative expression of AT1 to AT2 receptors. Further studies will be required to test these hypotheses. It is important to note that the observed changes in cardiac size and in gene expression occur with relatively small increases in maternal cortisol, well within the range measured in response to rather modest stress in the ewe, and with fetal cortisol levels within the range which will be produced later in gestation as the fetus matures. Although the increase in size of the fetal heart may reflect premature activation of left venticular growth, the maternal cortisol infusion also reduces thoracic girth of the fetus. Thus the elevation of maternal cortisol produces cardiac growth which is disproportionate to overall fetal growth.

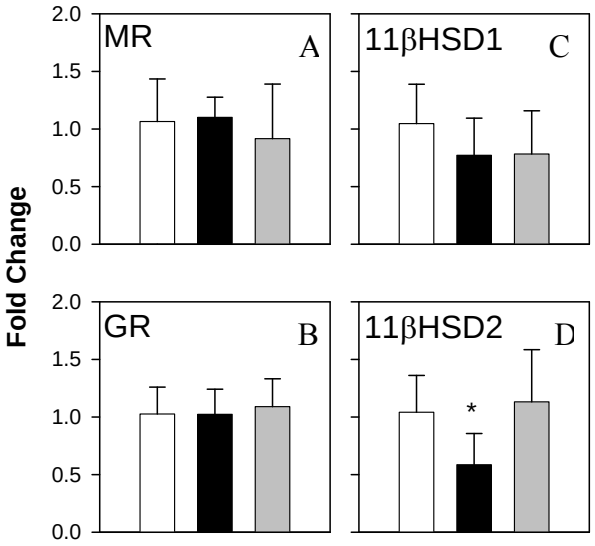


Figure 2-1. Gene expression of corticosteroid receptors and 11β-HSDs in the LV. Expression of mRNA for MR (A), GR (B), 11β-HSD1 (C), and 11β-HSD2 (D) in left ventricles from fetuses of the control (open bars), high (gray bars), and low maternal cortisol (black bars) groups. Fold changes of the genes were calculated using the expression 2^-ΔΔCt with respect to the control group and are expressed as mean fold change ± SEM.. *$p<0.05$ vs control

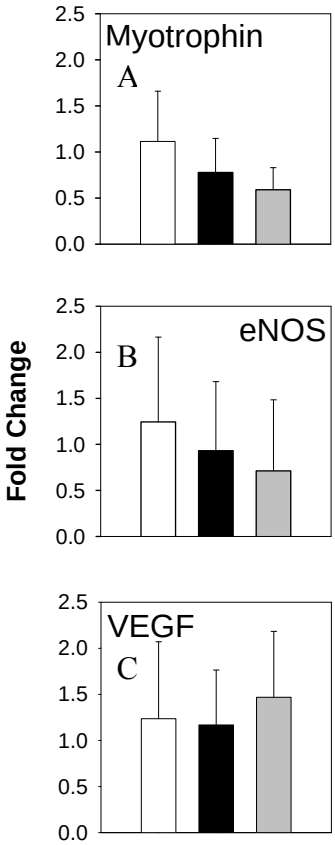


Figure 2-2. Gene expression of Myotrophin and vasculogenesis related genes in the LV. Expression of mRNA for myotrophin (A), eNOS (NOS-3; B), and VEGF (C) in left ventricles from fetuses of the control (open bars), high (gray bars), and low maternal cortisol (black bars) groups. Data are expressed as in Figure 2-1. *$p<0.05$ vs control

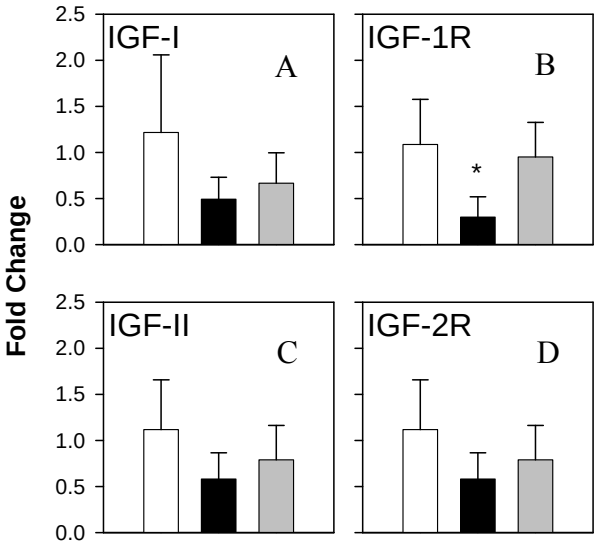


Figure 2-3. Gene expression of IGFs and IGF receptors in the LV. Expression of mRNA for IGF-I (A), IGF-1R (B), IGF-II (C), and IGF-2R (D) in left ventricles from fetuses of the control (open bars), high (gray bars), and low maternal cortisol (black bars) groups. Data are expressed as in Figure 2-1. *$p<0.05$ vs control

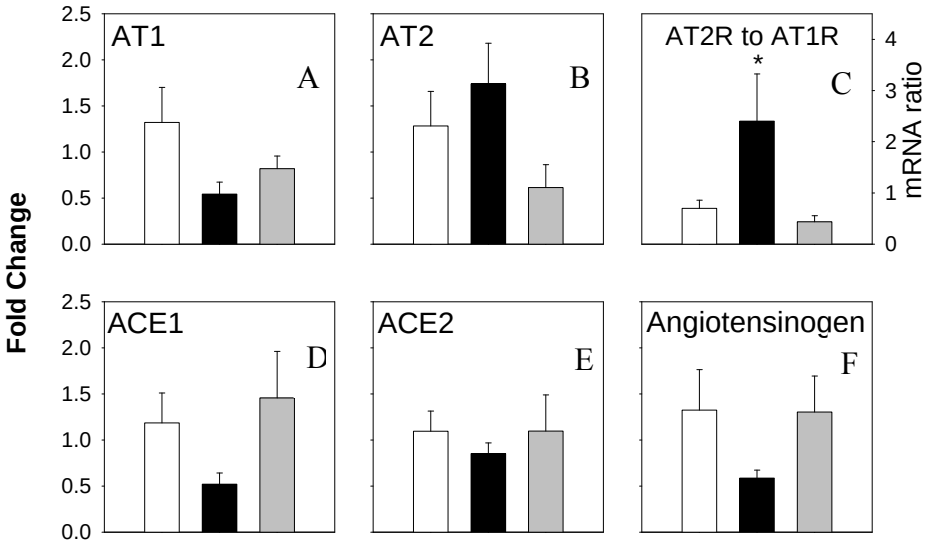


Figure 2-4. Gene expression of the RAS in the LV. Expression of mRNA for AT1R (A), AT2R (B), ACE1 (D), ACE2 (E) and angiotensinogen (F) in left ventricles from fetuses of the control (open bars), high (gray bars), and low maternal cortisol (black bars) groups. Data are expressed as fold changes as in Figure 2-1. The ratio of AT1R to AT2R mRNAs in left ventricles from each group are shown in Panel C. *$p<0.05$ vs control

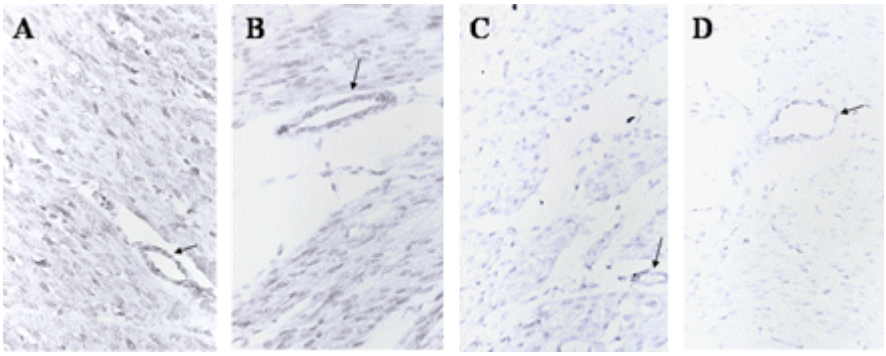


Figure 2-5. Localization of corticosteroid receptors and 11β-HSDs in the LV. Immunohistochemical localization of MR (A), GR (B), 11β-HSD1(C) and 11β-HSD2 (D) in hearts of untreated fetal sheep; 40x power. Arrow indicates location of blood vessel with positive staining.

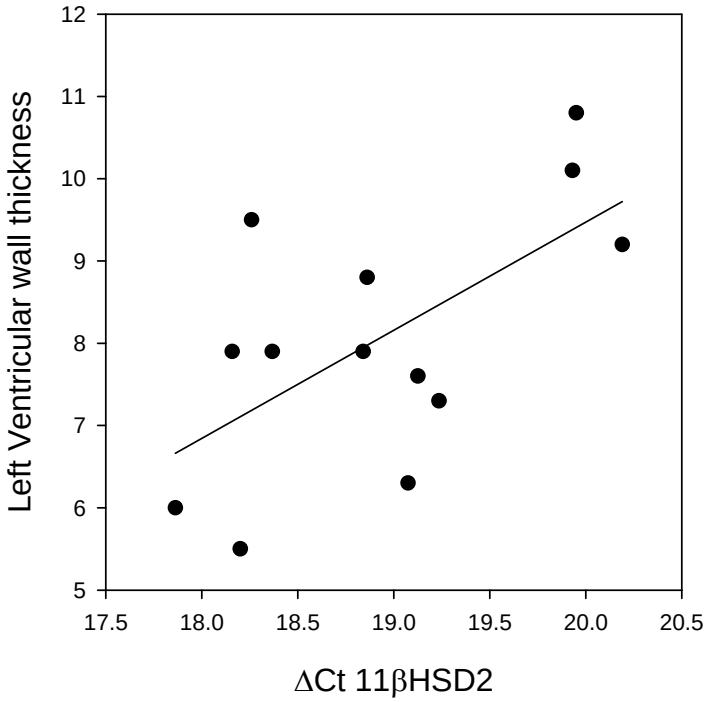


Figure 2-6. Linear regression correlation of 11β-HSD2 mRNA and left ventricular wall thickness. There was a significant negative relationship between the expression of 11β-HSD2 mRNA and left ventricular wall thickness ($r=0.624$, $P<0.02$). 11β-HSD2 mRNA levels are expressed as ΔCt using ribosomal RNA as the reference; higher ΔCt indicates relatively lower expression of 11β-HSD2.

Table 2-1. Primers and Probes used in real-time PCR assays.

GENE	Forward Primer Nucleotide Sequence (5'-3')	Reverse Primer Nucleotide Sequence (5'-3')
IGF-II	CTGCCTCTACGACCGTGCTT	TGCTTCCAGGTGTCAGATTGG
IGF-1R	AACTACACAGCCCGGATCCA	ACACAGGCTCCGTCCATGAC
IGF-2R	CTCACGGACGAGCAGCTGTAC	CGGGTCACCTTGAAGGTGTT
VEGF	GCTCTCTTGGGTGCATTGGA	TGCAGCCTGGGACCACTT
Myotrophin	GGCGCCGATAAGACTGTGA	CCTGGTTGTCAGTGGCTTCA
11β-HSD1	GGAATATGAGGCGACCAAGGT	TGGCTGTGTCTGTGTCGATGA
ACE1	CCAAATATGTGGAGCTCACCAA	GGAGTCCCCGCCATCC
ACE2	GCAGCCACACCTCACTATTTGA	AGGAAGTTTATTTCTGTTTCATTGTCTTC
	Probe Nucleotide Sequence (5'-3')	
IGF-II	TCACAGCATACCCCGTGGGCAAG	
IGF-1R	CCACCTCTCTCTCTGGG	
IGF-2R	TTCAACCTGTCCAGCCTCTCCAA	
VEGF	CCTTGCCTTGCTGCTCTACCTTCACCA	
Myotrophin	CCCCGATGGGCTGACTGCCC	
11β-HSD1	ATGTGTCAATCACCCTCTGTATTCT	
ACE1	(SYBR)	
ACE2	(SYBR)	

Table 2-2. Partial sequence of ovine myotrophin.

GACTATGTGGCCAAGGGAGAAGATGTCAACCGGACACTAGAAGGTGGAAGAAAGC
CTCTTCATTATGCAGCAGATTGTGGACAGCTTGAAATCCTGGAATTTCTGCTGCTGA
AAGGAGCAGATATTAATGCTCCAGATAAACATCATATCACACCTCTTCTGTCTGCCG
TCTATGAAGGTCATGTTTCCTGCGTGAAATTGCTTCTGTCAAAGGGCGCCGATAAGA
CTGTGAAAGGCCCCGATGGGCTGACTGCCCTTGAAGCCACTGACAACCAGGCGATC
AGAGCCCTCCTTCAGTGACGGACGGCGGGCTGACGGCTCTGGAAGGGTGGCTCTCC
TGTGCCTTCACACTG

Table 2-3. Fetal plasma angiotensin II levels (pg/ml) in fetuses in the high, control, and low maternal cortisol groups at 120, 125, and 130 days gestation.

	120 Days	125 Days	130 Days
Control (n=7)	88 ± 19	95 ± 18	95 ± 10
High (n=4)	71 ± 17	87 ± 13	131 ± 28
Low (n=5)	70 ± 7	112 ± 17	117 ± 15

Data are expressed as mean ± SEM.

CHAPTER 3
ONTOGENY OF GENES RELATED TO OVINE FETAL HEART GROWTH: IMPLICATIONS FOR GROWTH SECONDARY TO INCREASED CORTISOL

Introduction

There is a pronounced increase in fetal heart growth in the last third of gestation, paralleling a similar exponential growth of the fetus (Burrell et al. 2003, Jonker et al. 2007) At the same time as the heart is increasing in both total weight and left and right ventricle wall mass, an increasing number of myocytes terminally differentiate. This process results in binucleate or multinucleate myocytes which are unable to undergo further cell division (Burrell et al. 2003, Jonker et al. 2007). A similar pattern of decreasing proliferative activity near term has also been described for the human fetus (Huttenbach et al. 2001). Several factors have been identified as regulators of proliferation in the fetal heart in late gestation, these include cortisol (Giraud et al. 2006),IGFs (Liu et al. 1996, Sundgren et al. 2003), and angiotensin (Sundgren et al. 2003). My laboratory has found that small increases in fetal cortisol increase fetal heart weight and wall thickness (Jensen et al. 2005) and that this effect can be blocked by intracardiac administration of corticosteroid receptor antagonist (Reini et al. 2008).

Fetal secretion of cortisol increases exponentially before birth in humans and in sheep (Liggins et al. 1974), and induces maturation of intestine (Arsenault et al. 1985, Galand et al. 1989), lung (Ballard et al. 1996, Liggins et al. 1972) and liver (Fowden et al. 1995, Fowden et al. 1993). The role of cortisol in the fetal heart, however, remains unclear. In a previous study, I investigated the expression levels of a series of genes thought to be potential influential factors in cortisol-induced fetal heart enlargement (Reini et al. 2006). mRNA Expression of the 11 beta hydroxysteroid dehydrogenase, 11β-HSD2, was decreased in the fetuses of cortisol-treated, whereas the ratio of angiotensin type 2 receptor mRNA was increased relative to that of the angiotensin type 1 receptor.

The objective of this study was to determine the expression levels of the genes relating to actions of corticosteroids, angiotensin and IGFs in fetal left (LV) and right ventricles (RV) during normal development in late gestation and into early postnatal life. For this I used quantitative real-time (qrt) PCR to quantify gene expression levels of MR, GR, 11β-HSD1, 11β-HSD2, the IGFs, their receptors and binding proteins, and the components of the angiotensin system (angiotensin type 1 receptor, AT1R, and the angiotensin type 2 receptor, AT2R, angiotensin converting enzyme 1 and 2, and angiotensinogen) during late gestation and early extra-uterine life. . Jonker and coworkers, have demonstrated that the development of the left and right ventricle in fetal sheep differs slightly during the last third of gestation in that the right ventricle contains myocytes with a larger volume, and a higher percentage of myocytes in the cell cycle compared to the left ventricle (Jonker et al. 2007). These ventricle specific differences in development observed within the heart led us to also hypothesize that the changes in gene expression may differ in the LV compared to the RV, reflecting differences in myocytes proliferation or perhaps in anticipation of the greater work load after parturition. We hypothesized that MR, GR, 11β-HSD2, IGF2, IGF-2R, and AT2R (mRNA and protein) would decrease as pregnancy progressed while IGF1, IGF-1R, and AT1R would increase as pregnancy progressed, particularly in the right ventricle.

Materials and Methods

Real-Time PCR

RNA was extracted from left ventricles (LV) and right ventricles (RV) from time-dated pregnant ewes at 80 (n=4), 100 (n=4), 120 (n=4), 130 (n=4), and 145 (143-146) (n=5) days of gestation and from newborn lambs (days 1, 2 and 7) (n=8 for LV, n=4 for RV). RV was not extracted from the 130 day heart. RNA was extracted using Trizol according to the manufacturer's directions. LV RNA samples were checked for genomic DNA contamination

using real-time PCR with the RNA as a template in place of cDNA and using probes and primers for GR (which produces a product within exon 2). LV samples did not contain genomic DNA contamination. Genomic DNA was removed from RV samples using RNeasy Plus Mini Kit (Qiagen Inc., Valencia Ca). Total RNA was measured spectrophotometrically to measure the quantity and quality of RNA. Reverse transcription of the RNA into cDNA was then performed using a high capacity cDNA archive kit (Applied Biosystems; Foster City, CA) and aliquots for cDNA were stored at -20^{o}C until used.

Qrt PCR was utilized to measured gene expression. The genes analyzed in this study for the LV were MR, GR, 11β-HSD1 and 2, IGF-I and II, IGF-1R and 2R, IGF binding proteins 2 and 3 (IGFBP2 and, IGFBP3), angiotensinogen, ATR1 and 2, and angiotensin converting enzymes (ACE1 and ACE2). The same genes were studied in RNA from RV except for IGFBP3. Probe and primer sequences were based on previously published sequences: MR and GR (Keller-Wood *et al.* 2005), IGF-I (Meinel *et al.* 2003), angiotensinogen (Burrell *et al.* 2003), 11βHSD2, AT1R and AT2R (Dodic *et al.* 2002), IGFBP2 and IGFBP3 (Bloomfield *et al.* 2006), and for 11βHSD1, IGF-II, IGF-1R, IGF-2R, ACE1 and ACE2 (35). For ACE1 and ACE2, SYBR Green (Bio-Rad) was used instead of probes. An ABI PRISM 7000 Sequence Detection System (Applied Biosystems) was used to carry out the qrt PCR reactions. Reactions were carried out using 20 or 100 ng of template cDNA, except in the case of ribosomal RNA, for which 1 ng of template was used. All genes were normalized to 18s ribosomal RNA, and data was analyzed using the ΔCt method (Livak *et al.* 2001). 18s expression was unchanged between all the groups within each tissue.

Data Analysis

Changes in gene and expression among groups were analyzed by one way analysis of variance (ANOVA) using the ΔCt values. When data was not normally distributed, the Kruskal-

Wallis one way analysis of variance on ranks was utilized. Duncan's test or Dunn's test were used as appropriate for comparing differences between ages, and $p<0.05$ was used as the standard for significance in all statistical tests. For graphical purposes, fold changes of the genes in the heart ontogeny study were calculated using the expression $2^{\wedge -\Delta\Delta Ct}$ with respect to the mean value of delta Ct in the 80d fetal group.

Results

Expression of MR, GR, and 11β-HSD1 and 2 mRNA

There were significant ontogenetic changes in MR, GR, and 11β-HSD1 mRNAs in fetal left ventricles (Figure 3-1) ; MR and 11β-HSD1 and 2 mRNAs also changed ontogenetically in the right ventricle, but GR did not significantly change as a function of age in the right ventricle (Figure 3-1). MR mRNA expression was greatest in fetal LV at 80d and is significantly decreased at 130 days of gestation and in newborns. GR mRNA was also greatest at 80d, and decreased at 120, 130 d and in the newborn LV. This pattern differed from that in RV; MR mRNA in RV was significantly decreased at 100d compared to all other ages. In LV 11β-HSD1 mRNA expression was significantly decreased at 120 days gestation compared to 80 days and 145 days gestation. 11β-HSD2 mRNA expression in LV did not change throughout gestation. In RV, 11β-HSD1 and 11β-HSD2 mRNA expressions were highest in the newborns. The ratio of mRNA expression of 11β-HSD1 to 11β-HSD2 was unchanged throughout the ages studied in LV, but was significantly decreased in the RV at 145 days compared to 100 days gestation (Table 3-1).

Expression of IGF1, IGF-1R, IGF2, IGF-2R, and IGFBP2

There were ontogenetic changes in expression of IGF2, IGF1R, IGF2R, and IGFBP-2 mRNAs in the LV and IGF2, IGF2R, and IGFBP2 mRNAs in the RV (Figure 3-2). IGF1 mRNA expression did not change in either RV or LV over the ages studied. In contrast, IGF2

mRNA expression was greatest at 80-100 days in both LV and RV, and significantly decreased in the LV and RV from older animals; IGF-2 mRNA expression was lowest in the newborn LV or RV. The mRNA for IGF type 1 receptor was also decreased in LV after 120days, but did not change in RV. The mRNA for IGF type 2 receptor was also decreased after 120d in LV, but was significantly decreased only after 130d in RV. As a result of these changes in IGF2 mRNA, the ratio of expression of IGF1 to IGF2in the LV and RV were increased in late gestation (Table 3-1). In the LV the ratio was increased on day 120 compared to day 100 and on day 145 compared to 80 and 120 days, and was markedly increased in postnatal hearts compared to all gestational ages. In the RV the ratio of the expression of IGF1 to IGF2 was increased at 130 and 145 days gestation compared to the earlier gestational ages and the ratio in the postnatal RV was elevated compared to all gestational ages except for 145 days.

The ontogenetic pattern of IGFBP2 mRNA in both LV and RV were decreased after 100d with more dramatic decreases at 145d and in newborns. IGFBP3 mRNA expression in LV on the other hand, is not significantly changed over time, although the level at 100d was significantly lower at 120d than at 80d or 145d (Figure 3-2).

Expression of Angiotensinogen, AT1R, AT2R, ACE1, and ACE2 mRNA

Although expression of angiotensinogen mRNA did not change significantly in LV, angiotensinogen expression in RV is significantly decreased at 100 days compared to 80, 130, and 145 days gestation (Figure 3-3). Expression of ACE1 mRNA was dramatically increased in LV and RV at 145 days gestation and in the newborn. In contrast, expression of ACE2 mRNA in both LV and RV was greatest at 80 days and decreased at later ages (Figure 3-3). Therefore the ratio of ACE1 mRNA to ACE2 mRNA was increased in LV from 120d on, and in RV from 100d onward (Table 3-1).

AT1R mRNA expression was significantly decreased in LV at 120 and 130 days gestation and at 1 day postnatally. AT1R mRNA expression in RV at day 100 was significantly less than expression at day 80. Expression of AT2R mRNA did not significantly change in LV throughout the study, although AT2R expression was significantly higher at 130 days gestation than in newborn hearts in the RV. There was little change in the ratio of AT1R to AT2R mRNAs in LV, although the ratio in LV was significantly higher at 120 days when compared to postnatal lambs (Table 3-1). In the RV, the ratio was greater at 80d and in the newborn than in 100 or 130 fetuses, and at 145d was greater than 130d (Table 3-1).

Discussion

This study revealed ontogenetic patterns of expression of several genes implicated in growth or hypertrophy in neonatal and adult hearts. Previous studies of the ovine fetal heart have found that the number of mononuclear myocytes declines over the time period we studied (80 days to the early postnatal period), and the number of binucleate myocytes increases over this period (Burrell *et al.* 2003, Jonker *et al.* 2007). However, the number of proliferating myocytes in the left ventricle is approximately 50% at 100 days and decreases to 15% by 145d, whereas only 15-25% of right ventricular myocytes are proliferating over this time period (Jonker *et al.* 2007). In contrast, the number of myocytes that are enlarged due to terminal differentiation is greater in the right ventricle than in the left ventricle, particularly from day 130 to term, and right ventricular myocytes are on average greater in volume than are those in the left ventricle. Results from this study suggest that decreases in IGF2 and IGF-2R are associated with fetal cardiac maturation in both left and right ventricle, and are consistent with roles of the angiotensin and IGF1 in the transition of the left and right ventricle from fetal to postnatal life.

Role of Corticosteroids in the Heart

Previous studies suggest that even small increases in fetal cortisol can alter heart mass (Jensen *et al.* 2005, Jensen *et al.* 2002), suggesting an action of cortisol at MR and/or GR in fetal myocytes. Both MR and GR are abundantly expressed in the fetal heart (Reini *et al.* 2006), as is the case in many adult species, including humans (Lombes *et al.* 1995). While GR is relatively abundant in the fetal heart, in sheep as in other species MR binds cortisol with greater affinity than GR (Richards *et al.* 2003). The ability of cortisol to bind at MR and/or GR, however, depends in large part on the activity of 11β-HSD1 relative to that of the cortisol inactivating enzyme 11β- HSD2 (Mihailidou *et al.* 2005, Seckl *et al.* 2001). MR and GR follow a similar pattern of expression in the LV throughout late gestation with significant decreases from the expression levels at 80 days gestation occurring by 130 days gestation and postnatally in both MR and GR. There was no significant overall ontogenetic change in expression of MR or GR in the RV, although the MR levels at 100 days were lower than at other times. 11β-HSD1 and 11β-HSD2 both show relatively consistent expression levels in the LV throughout late gestation and early extrauterine life, while both 11β-HSD1 and 11β-HSD2 increase ~2-3 fold in expression in the RV after birth compared to earlier points in fetal life. While there are no major changes in the ratio of expression, 11β-HSD1 maintains a higher level of expression than 11β-HSD2 within both ventricles of the heart throughout all of late gestation, indicating the potential role of cortisol within the heart in the late gestation fetus, but suggesting that proliferative effects of cortisol are reduced in left ventricle as the heart matures.

Insulin-Like Growth Factors

In the current study, LV IGF1 mRNA expression did not significantly change throughout late gestation or neonatally, while IGF1R levels decrease in left ventricle by 120 days gestation and maintain that lower level of expression through birth. IGF2 mRNA and IGF2R mRNA are

decreased in both LV and RV after 120 days and remained low postnatally. In contrast, neither IGF1 nor IGF1R significantly changed in RV. The IGF2 results agree with previous observations of decreased IGF2 mRNA expression in at 133 days gestation as compared to 75d (Delhanty *et al.* 1993) by northern blot, and a decrease in IGF2 mRNA from 60d to 141d (Cheung *et al.* 1996) by in situ hybridization. However in these studies, investigators also found a decrease in IGF1 expression in the left ventricle from 100 days gestation toward term, whereas we did not observe a significant decrease in expression.

The pattern of decreased IGF1R in LV parallels the reduced number of LV myocytes entering the cell cycle in late gestation in LV, whereas the dramatic decrease in IGF2 from day 120 of gestation to parturition in both LV and RV parallels the reduction in mononuclear myocytes in both ventricles (Jonker *et al.* 2007). IGF2 and IGF1 both appear to stimulate myocyte proliferation in vitro. Liu *et al.* found that IGF2 stimulated an increase in proliferation of prenatal rat myocyte cultures, but did not stimulate proliferation in neonatal myocytes (Liu *et al.* 1996). Sundgren et al. have shown that infusion of an IGF1 analog to 124 day fetal sheep results in decreased numbers of binucleated cells, but increased percentages of monucleated myocytes; IGF1 administration in cultured fetal cardiomyocytes stimulated proliferation of the myocytes mediated by ERK and PI3K (Sundgren *et al.* 2003). Because *in vivo* both IGF1 and IGF2 act can act via binding at IGF1R, the decrease in IGF1R expression may limit the proliferative effects of both IGFs as the heart matures. Alternatively, the decrease in expression of IGF2 or IGF1R may reflect the decrease in mononuclear myocytes as terminal differentiation proceeds.

As a result of the decrease in IGF2 mRNA, the ratio of IGF1 to IGF2 mRNAs increased near term and postnatally (Table 1). This change in ratio of IGF1 to IGF2 mRNA expression is

consistent with the hypothesis that IGF2 is less important to postnatal growth than to prenatal growth. It is interesting to speculate that IGF2 may play a role in mononuclear myocyte proliferation, accounting for the gradual decrease in proliferation observed throughout the last third of gestation as IGF2 expression within the heart decreases.

One of the possible key regulators of both systemic and local IGF concentrations in fetuses is thought to be cortisol. Fetal skeletal muscle IGF1 mRNA expression decreases at the same time as the prepartum rise in ovine plasma cortisol levels, and appears to be cortisol-dependent (Li *et al.* 2002). This laboratory has found that moderately elevated cortisol levels late in gestation reduce IGF-1R mRNA levels in the heart, but did not alter IGF-1 mRNA (Reini *et al.* 2006). Increases in fetal cortisol decrease circulating IGF1 at the same time as increasing fetal heart weight (Jensen *et al.* 2002); the decrease in cardiac IGF1 expression in the late gestation fetal heart also occurs at a time of increased circulating cortisol. Although in previous studies we did not find a decrease in cardiac IGF1 mRNA with small increase in cortisol in the 130d fetus, we did find a reduction in IGF1R mRNA, similar to the finding in this study that IGF1R deceases at a time that the fetal adrenal begins to secrete low concentrations of cortisol, and at a time of relative abundance of MR expression in LV.

The biological actions of IGFs are in part regulated by IGF binding proteins 1-6 *in vivo*, which function to prolong the half life of IGFs in plasma. IGFBPs have the ability to modulate the actions of IGF through regulating transport, turnover, and tissue distribution (Jones *et al.* 1995). The ontogenetic pattern of IGFBP2 mRNA in both LV and RV were decreased after 100d with more dramatic decreases at 145d and in newborns. IGFBP3 mRNA expression in LV on the other hand, is not significantly changed over time, although the level at 100d was significantly lower at 120d than at 80d or 145d (Figure 2). Previous studies have shown IGFBP2

and IGFBP3 play roles in fetal development. It has been reported that over-expression of IGFBP2 (Hoeflich *et al.* 1999) and IGFBP3 (Modric *et al.* 2001) in mice leads to a ~10% decrease body weight, although there is no change in heart weight with over-expresssion of either binding protein. Maternal nutrient restriction leads to an increase in plasma IGFBP2 levels within the fetus between 90 and 135 days gestation (Osgerby *et al.* 2004), but did not alter fetal heart weights.. In contrast, Greenand coworkers found that umbilical cord occlusion for four days (107-108 d fetuses) led to no change in plasma IGFBP2 or IGFBP3, but did lead to an increase in RV mRNA expression of IGFBP2 (Green *et al.* 2000); there was no change in either body weight or heart weight with this 4 days of manipulation. This study reveals a progressive decrease in IGFBP2 mRNA expression in the LV and RV starting at 120 days gestation and continuing on until postnatal life; IGFBP3 mRNA expression did not change in LV. Although the role of IGFBPs in mediating IGF effects in the fetal heart are not known, these results suggest that the decrease in IGFBP2 mRNA within the fetal heart as gestation progresses may reduce IGF1 mediated proliferative effects in both LV and RV.

Renin-Angiotensin System

Infusion of angiotensin II into fetal sheep stimulates left ventricular growth (Segar *et al.* 2001), and in cultures of ovine fetal cardiomyocytes, angiotensin II has been shown to stimulate hyperplastic growth (Sundgren *et al.* 2003). I previously found that the elevated cortisol levels in sheep during late gestation produced enlarged hearts with AT2 to AT1 mRNA ratios (Reini *et al.* 2006), although this infusion does not appear to increase genes associated with hypertrophy. Infusion of very high doses of cortisol in the sheep fetus causes hypertension, left ventricular (LV) hypertrophy and increased cardiac expression of angiotensinogen mRNA (Lumbers *et al.* 2005), and in the adult heart the local RAS has been implicated in playing a major role in cardiac

hypertrophy and fibrosis. In murine hearts local over-production of angiotensin II, without involvement of the systemic RAS, causes interstitial fibrosis within the heart (Xu *et al.* 2007).

This study indicates that changes in the expression of mRNAs for RAS components correspond to changes in proliferative activity and differentiation in the maturing heart. ACE1, which converts angiotensin I to angiotensin II, increased ~5-fold in the LV and RV at term. ACE1 is known to augment cardiac hypertrophy in rat hearts when over-expressed (Tian *et al.* 2004), and the pattern of expression in late gestation is consistent with expression in terminally differentiated myocytes. ACE2, which converts angiotensin I into angiotensin 1-9 and angiotensin II into angiotensin 1-7, limits the amount of angiotensin II that is thought to be cardio-protective (Danilczyk *et al.* 2006). In this study, ACE2 mRNA expression significantly decreased in the LV by 120 days gestation and remained low, while expression in RV did not significantly change. Interestingly, the ACE1 to ACE2 mRNA ratio increases ~15-fold by 145 days gestation compared to 80 days in both the LV and RV. This increase in the ratio suggests that local angiotensin II production may be associated with the terminal maturation of the myocytes. Although high doses of cortisol stimulates angiotensinogen expression in the fetal sheep heart (Lumbers *et al.* 2005), the physiologic increase in cortisol that occurs at term in fetal sheep did not increase angiotensinogen mRNA expression, suggesting that changes in cardiac angiotensin II production are related to a decrease in ACE2 expression and increase in ACE 1 expression, rather than by a local increase in transcription of the gene for the precursor protein.

In this study, AT2R mRNA expression remained relatively constant throughout gestation in both the LV and RV, while AT1R mRNA expression significantly decreased at 120-130 days gestation and in the newborn compared to expression at 80 days in the LV. These results are in contrast to the increase in AT2R protein in the heart in late gestation observed by others (Burrell

et al. 2001). This decrease in expression of AT2R mRNA in the LV at 120 days coincides with the decrease in ACE2 mRNA expression, whereas the drop in expression in the postnatal LV of AT1R mRNA coincides with the increase in ACE1 mRNA expression, suggesting that the decreased expression of the AT1R mRNA may be in response to an increase in local angiotensin II production. In the adult heart, the AT1Rs are thought to be responsible for hypertrophic effects, while actions at AT2R are hypothesized to counteract the AT1R (Booz *et al.* 2004, Zhu *et al.* 2003). Thus the relative increase in AT1R to AT2R may be consistent with increased capacity for myocyte hypertrophy in differentiated myocytes. In the RV the daily increase in ventricular mass is primarily due to enlargement with terminal differentiation (Jonker *et al.* 2007); the greater increase in AT1R to AT2R ratio in RV appears to correlate to this, suggesting a greater stimulation of myocyte volume is associated with greater relative AT1R expression in RV.

In conclusion, our results suggest that changes in gene expression in the RV and LV is associated with the changes in proliferative activity of mononuclear myocytes, and with terminal differentiation of binucleate myocytes, which increase in number near birth.

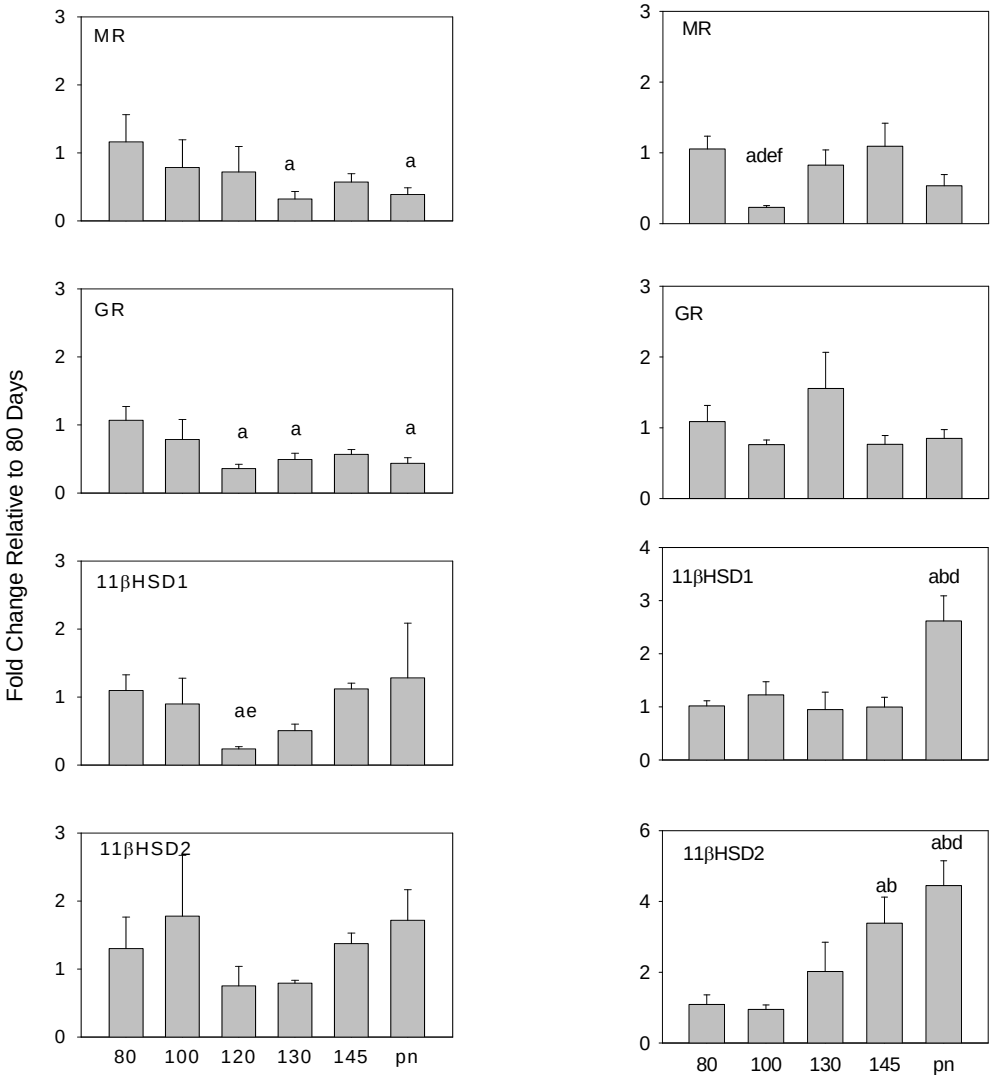
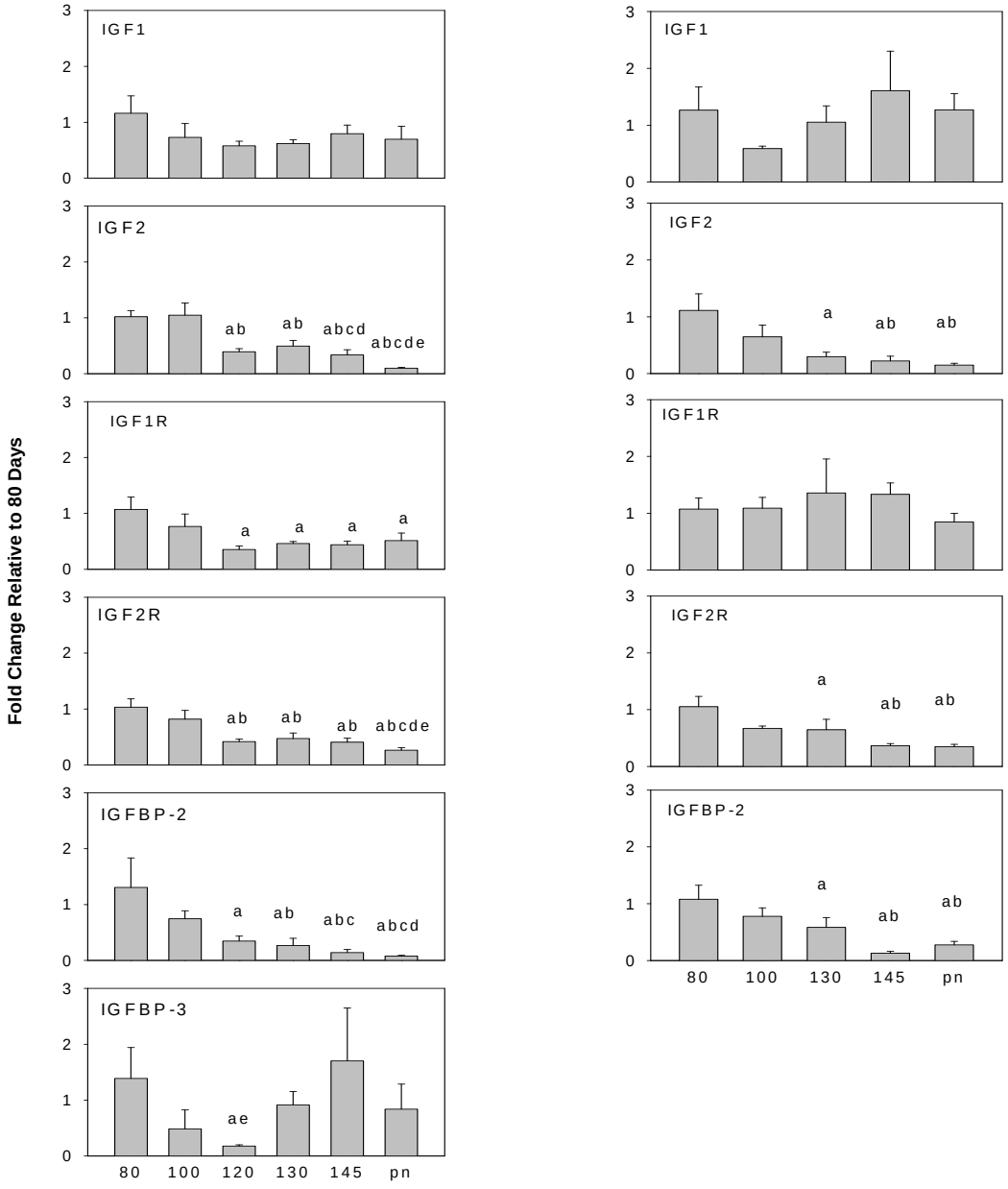
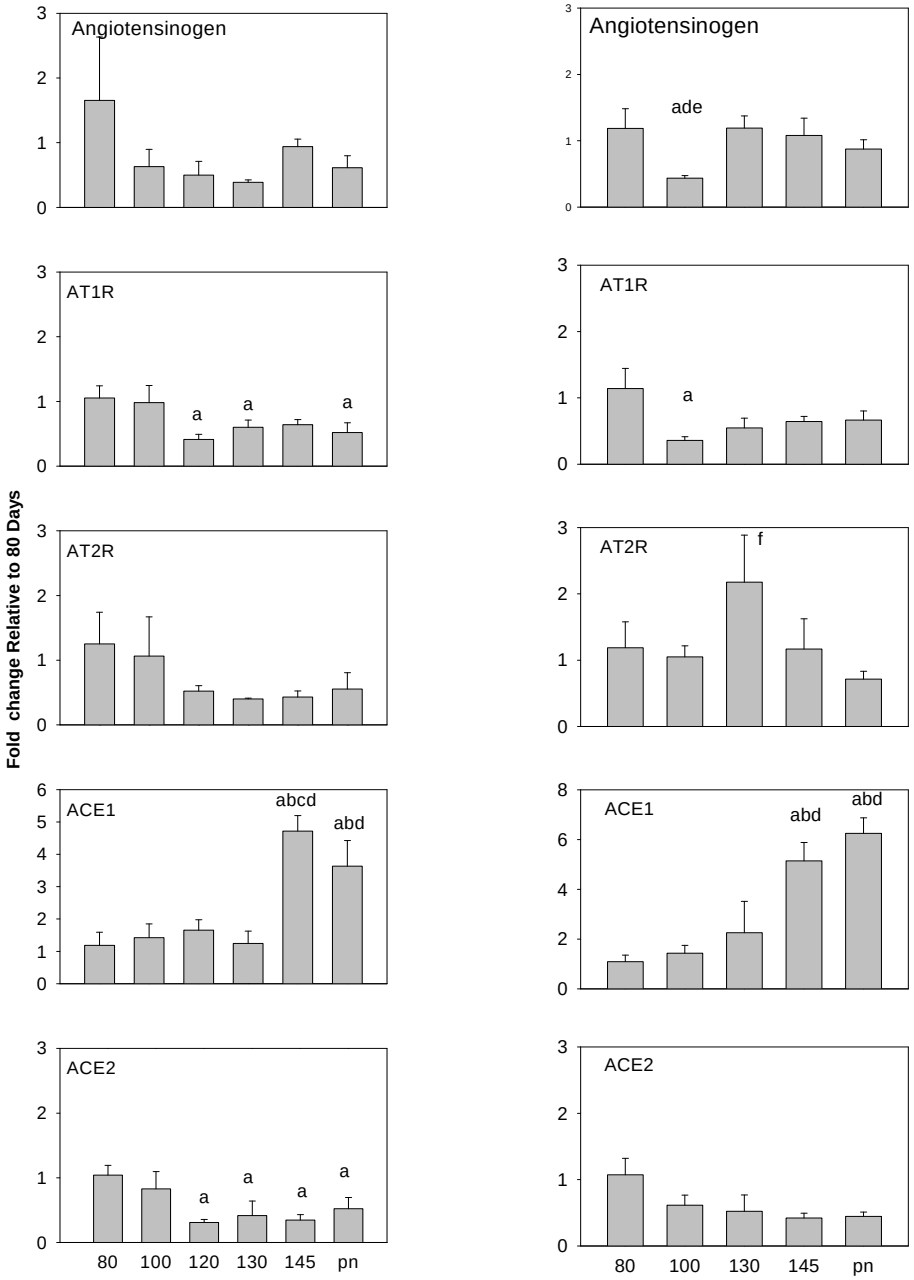


Figure 3-1. Ontogenetic expression of corticosteroid receptors and 11β-HSDs in the LV and RV. Expression of mRNA for MR, GR, 11β-HSD1, and 11β-HSD2 in left ventricles of 80, 100, 120, 130, and 145 day fetuses and postnatal lambs (pn) and in right ventricles of 80, 100, 130, 145 and postnatal lambs. Data are depicted as mRNA fold changes relative to 80d calculated using the expression 2^-ΔΔCt and expressed as a mean fold change ± SEM. Letters indicate significant differences (p<,0.05) a: 80d, b: 100d, c:120d, d: 130d, f: 145d, g: newborn.



Left Ventricle **Right Ventricle**

Figure 3-2. Ontogenetic expression of IGFs, IGF receptors, and binding proteins in the LV and RV. Expression of mRNA for IGF1, IGF2, IGF1R, IGF2R and IGFBP2 from left and right ventricles of fetal and newborn lambs. IGFBP3 were measured in left ventricle only. Ages and significance are as indicated in legend to Figure 3-1.



Left Ventricle **Right Ventricle**

Figure 3-3. Ontogenetic expression of the RAS in the LV and RV. Expression of mRNA for Angiotensinogen, AT1R, AT2R, ACE1 and ACE2 in left and right ventricles of fetal and newborn lambs. Ages and significance are as indicated in legend to Figure 3-1.

Table 3-1. Expression ratio of 11β-HSD1 to 11β-HSD2, IGF2 to IGF1, AT1R to AT2R, and ACE1 to ACE2 in LV and RV mRNA

Age (days gestation)	11βHSD1/ 11βHSD2		IGF2/ IGF1		AT1R/ AT2R		ACE1/ ACE2	
	LV	RV	LV	RV	LV	RV	LV	RV
80	62 ± 21	13 ± 3	241 ± 62	978 ± 276	1.4 ± 0.4	4.8 ± 1.6	14 ± 4	2.5 ± 0.2
100	36 ± 11	16 ± 4 *f	433 ± 116	949 ± 265	1.7 ± 0.4	1.5 ± 0.3 *ag	23 ± 2.8	6.0 ± 0.7 *a
120	25 ± 6.4	nm	151 ± 11 *b	nm	0.9 ± 0.1	nm	64 ± 6.0 *a	nm
130	33 ± 4.5	6.1 ± 1.8	176 ± 25	268 ± 37 *ab	1.8 ± 0.4	1.3 ± 0.5 *afg	78 ± 31 *a	9.6 ± 1.6 *a
145	45 ± 5.0	5.7 ± 2.1	88 ± 10 *ab	146 ± 48 *ab	2.1 ± 0.5	3.3 ± 1.1	212 ± 66 *abd	31 ± 6 *abd
postnatal	31 ± 9.0	6.7 ± 0.5	41 ± 7 *abcdf	105 ± 4 *abd	1.4 ± 0.2	3.8 ± 0.3	133 ± 41 *ab	37 ± 6 *abd

Letters indicate significant differences (p<,0.05) a: vs 80d, b: vs 100d, c: vs120d, d: vs 130d, f: vs 145d, g: vs postnatal lambs; nm, not measured.

CHAPTER 4
[2]CARDIAC CORTICOSTEROID RECEPTORS MEDIATE THE ENLARGEMENT OF THE OVINE FETAL HEART INDUCED BY CHRONIC INCREASES IN MATERNAL CORTISOL

Introduction

In late gestation, normal fetal growth and fetal cardiovascular homeostasis is dependent on the proper regulation of maternal cortisol levels. Although reductions in maternal cortisol prevent the normal increases in maternal plasma volume and uteroplacental blood flow and reduce fetal growth (Jensen *et al.* 2002a; Jensen *et al.* 2005), increases in maternal cortisol also alter fetal growth. Chronically increased maternal cortisol levels, within the range that occurs with maternal stress, reduce fetal growth rates while increasing heart growth in fetal sheep (Jensen *et al.* 2002b; Jensen *et al.* 2005).

The mechanisms by which chronically elevated maternal cortisol levels increase the size of the fetal heart are not known. Giraud *et al.* have shown that cortisol chronically infused directly into the coronary artery increased cell cycle activity in myocytes of late gestation sheep fetuses, suggesting a direct induction by cortisol of hyperplastic growth rather than hypertrophic growth (Giraud *et al.* 2006). Conversely, it has been demonstrated that large doses of cortisol infused directly into the fetus in late gestation causes left ventricular (LV) hypertrophy along with an increase in fetal arterial pressure and cardiac expression of angiotensinogen mRNA (Lumbers *et al.* 2005). This laboratory has shown that maternal cortisol infusion in sheep during late gestation caused an increase in fetal heart size and wall thickness without increasing fetal arterial pressure or cardiac angiotensinogen; we found an increase in the ratio of angiotensin type 2

[2] Reproduced with permission from Reini S, Dutta G, Wood C, & Keller-Wood M 2008 Cardiac corticosteroid receptors receptors mediate the enlargement of the ovine fetal heart induced by chronic increases in maternal cortisol. The Journal of Endocrinology Epub May 21, 2008.

receptor (AT2 receptor) to angiotensin type 1 receptor (AT1 receptor) mRNA in the fetal heart, suggesting that the renin-angiotensin system (RAS) may play a key role in the enlargement process. Furthermore, in the same study it was observed that left ventricular expression of 11β-HSD2 mRNA, the enzyme that converts cortisol into cortisone, decreased in the fetal hearts in response to the elevated cortisol, suggesting that cortisol can act directly on mineralocorticoid (MR) or glucocorticoid (GR) receptors to induce the cardiac enlargement (Reini *et al.* 2006).

In adult hearts, both MR and the RAS have been implicated in cardiac fibrosis and hypertrophy after injury (Fraccarollo *et al.* 2003; Fraccarollo *et al.* 2005; Xiao *et al.* 2004). I propose that corticosteroid receptors also play a role in cardiac enlargement in the fetal heart, although by mechanisms independent of cardiac injury and fibrosis. The purpose of this study was to test the hypothesis that increase in fetal heart weight and wall thickness in response to increased maternal cortisol is mediated by cardiac corticosteroid receptors, MR and/or GR, and to determine if cardiac fibrosis accompanies the cardiac enlargement in response to cortisol. I hypothesized that cortisol acts within the myocardium on MR receptors, and to a lesser degree GR receptors, to induce enlargement of the fetal heart. I also hypothesized that cardiac fibrosis is not involved in the enlargement of the heart observed in the fetuses of cortisol-infused ewes.

Materials and Methods

Experimental Design

Ewes (*Ovis aries*) pregnant with single fetuses were studied. 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. Ewes and their fetuses were operated on between 118 and 123 days of gestation (term approximately 148 days). Animals were randomly assigned to one of four groups at the time of surgery. The first group consisted of six control animals; the second group consisted of five ewes to which cortisol

(hydrocortisone hemisuccinate; Sigma, St Louis, MO) was administered by continuous intravenous infusion (1 mg kg^{-1} day^{-1}; cortisol); the third group consisted of six ewes to which cortisol was infused, with infusion of the MR antagonist potassium canrenoate (Sigma; 600 μg day^{-1}; cortisol + MRa) directly into the pericardial space of the fetus; and the fourth group consisted of four ewes to which cortisol was infused, with infusion of the GR antagonist mifipristone (Sigma; 50 μg day^{-1}; cortisol + GRa) directly into the pericardial space of the fetus. For the control and cortisol groups, there were no infusions into the pericardial space. The intrapericardial infusions were performed by use of Alzet minipumps (2ML2; 5 μl·$hour^{-1}$; Cupertino CA) in order to achieve continuous infusion of the antagonists into the pericardial space without any appreciable increase in pericardial fluid volume (0.12 ml/day). The doses of MR and GR antagonists were calculated based on their effective systemic doses, and scaled to reflect the smaller distribution volume of the fetal heart (20g). Because these drugs are steroid (mifepristone) or lactone (canrenoate) derivatives, they are able to distribute throughout tissue over the 10 days of study after mixing in the pericardial fluid. Effects of the MR and GR antagonists were confirmed using immunohistochemistry to confirm the expected cellular redistribution of receptors with antagonist administration (see below)

The cortisol dose and the duration of cortisol infusion (10 days) were determined based on a previous study in this laboratory (Jensen *et al.* 2005) showing that infusion at this rate and duration produces levels similar to mild maternal stressors and results in enlargement of the fetal heart .

Surgical Procedures

Halothane (1.5-2.5%) in oxygen was used to anesthetize ewes during surgery. Fetal femoral tibial artery catheters and an amniotic fluid catheter were placed as previously described (Jensen *et al.* 2002a; Wood & Rudolph 1983). Catheters were also placed in the fetal pericardial

space for the delivery of drug as previously described (Wood 2002). In each case, an incision was made in the uterus over the left side of the fetal chest and an incison was made between the third and fourth fetal ribs. The fetal skin was marsupialized to the uterus to prevent leakage of amniotic fluid. The fetal heart was exposed and a small incision was made in the pericardium, through which a silastic catheter (0.76 mm id, 1.65 od; Dow Corning, Midland, Michigan) was placed and held in place with use of a purse-string suture (4-0 Tevdek; Teleflex Medical, Mansfield, MA). For infusion of potassium canrenoate, the silastic catheter was connected to a Tygon tubing connector (1.27 mm od; St Gobain Performance plastics; Akron, OH) which was connected at its other end to the the Alzet pump containing the drug (50 mg ml^{-1} in 0.9% saline). Because mifepristone is not soluble in aqueous solution and therefore cannot be directly loaded into the pump reservoir, mifepristone was dissolved in 47.5% ethanol in saline (0.42 mg ml^{-1} ethanol-saline); this solution was placed in a polyethylene tubing (1.40 mm id, 1.90 mm od) which was then connected to the silastic pericardial catheter on one end and to the Alzet minipump on the other end using smaller gauge polyethylene tubing. The Alzet pump, filled with saline, provided the flow to pump the mifepristone solution from the tubing into the pericardium. The pump was placed under the skin of the fetus near the scapula. In the control group, 5 of the 6 fetuses also had pericardial catheters placed, but no infusion was delivered; in the cortisol group, 3 of 5 fetuses had pericardial catheters placed, but no infusion was delivered.

After closure of the uterus, catheters were placed in the maternal femoral artery and vein and routed to the maternal flank. All ewes were treated with flunixamine (1 mg kg^{-1} im; Fort Dodge Animal Health, Fort Dodge, IA) at the end of the surgical procedure, before recovery from anesthesia.

Ewes were returned to their pen after recovery from anesthesia. At this time, the intravenous infusion of cortisol (1mg kg^{-1} day^{-1} cortisol as cortisol hemisuccinate in normal saline; Sigma) or infusion of saline to the ewe was initiated. Maternal infusions were delivered through a 0.22 μm filter (Fisher Scientific) via a syringe pump at the rate of 1.17 ml $hour^{-1}$. Animals were housed in individual pens with access to water, food, and salt blocks ad libitum. Ampicillin (500mg im bid; Webster Veterinary) was administered for 3 days postoperatively. Flunixamine was administered on the morning after surgery.

Experimental Protocol

Fetuses were studied from the day of surgery until death on 129-132 days gestation. All cortisol infused ewes and their fetuses were sacrificed on day 10 of infusion. Fetal and maternal blood samples were withdrawn on day 5 (124-126 days gestation) and day 10 (129-132 days gestation) after the start of the infusion for determination of blood gases, plasma cortisol and plasma ACTH 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 ACTH and cortisol. On day 10 of infusion, maternal and fetal blood pressure and heart rate 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 intra-arterial pressures in order to calculate fetal arterial pressure. In two animals, one in the cortisol group and one in the cortisol+MRa group, we were unable to reliably measure fetal heart rate; data from those two fetuses are excluded from analysis.

The ewe was euthanized on day 10 using an overdose of euthanasia solution containing pentobarbital, and the fetus was removed and weighed. The fetal heart was also immediately dissected, blotted to remove blood from the chambers, and weighed. Ventricular and septal wall

thicknesses were measured using a micrometer at a standardized site on the heart, taking care to exclude measurement at the level of the papillary muscles or valves.

Analysis

Blood gases and pH were measured with a blood gas/electrolyte analyzer (ABL77; Radiometer America, Westlake, OH). 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.

Plasma ACTH was measured by radioimmunoassay, using an antibody to 1-39 ACTH (Bell *et al.* 1991) and plasma cortisol concentration was measured using a commercially available enzyme immunoassay kit (EA 65, Oxford Biomedical, Oxford, MI) which has minimal cross-reactivity with cortisone (2.08%).

Immunohistochemical Localization of MR and GR

At the time of sacrifice, a section of the fetal heart was fixed in 4% buffered paraformaldehyde overnight. Hearts were dehydrated with increasing concentrations of reagent alcohol followed by xylene, embedded in paraffin wax, cut into 10-μm-thick sections on a Zeiss rotary microtome, and placed on poly-l-lysine coated slides. The sections were stained with anti-GR (Santa Cruz Bioreagents, M-20,) or anti-MR (M1-18, 6G1, gift of E. Gomez-Sanchez; (Gomez-Sanchez *et al.* 2006)) as previously described (Reini *et al.* 2006) This analysis was performed to assess the ability of the drugs to act in the heart and cause the expected changes in cytonuclear localization of the receptors. The MR antagonist canrenoate acts in a similar manner to spironolactone and would therefore be expected to prevent nuclear localization of MR (Fejes-Toth *et al.* 1998; Lombes *et al.* 1994); conversely the GR antagonist mifepristone (also known as

RU486) causes nuclear localization even in the absence of agonist (Jewell *et al.* 1995; Scheuer *et al.* 2004). The localization observed (Figure 4-1) is consistent with these effects. In the control fetuses GR were primarily located in the cytosol, whereas MR were apparent in cytosol and nucleus. A dramatic increase in MR localization to the nucleus was apparent in the cortisol-treated fetuses, indicating cortisol activation of MR. We did not find as dramatic an increase in nuclear GR with cortisol, indicating fewer GR are activated. In the case of MR antagonist administration, fewer MR were apparent in the nucleus than with cortisol alone, whereas with GR antagonist, equivalent MR localization to the nucleus occurred as with cortisol alone. Consistent with the known effect of mefipristone, in GR antagonist -treated ewes, there was more nuclear GR than in the case of cortisol alone or cortisol+ MRa.

Collagen Staining

Sections from each group (n = 4-6) were stained with picrosirius red (Sigma) in order to determine collagen content. Sections were hydrated and immersed in sirius red (0.1% in saturated picric acid). The sections were then washed in acidified water (0.5% glacial acetic acid), dehydrated, and mounted in permount. All images were visualized using an Olympus DP71 microscope and Olympus software. Ten pictures of LV, five of RV, and five of septum were taken from each heart in areas without large blood vessels so that primarily interstitial, rather than perivascular, collagen deposition could be quantified. Picrosirius red staining was quantified using Image J software (NIH, Bethesda, MD) by three different people who were blinded as to the experimental group. The average value of the percentage of the image that stained red from these three observations was calculated.

Data Analysis

Fetal heart weight was normalized to body weight. The heart weight to body weight ratio, LV, RV and septal thickness, fetal and maternal blood pressure and heart rate, as well as fetal

and maternal plasma ACTH and cortisol, sodium and potassium, and PCVs were analyzed by one way analysis of variance (ANOVA) with multiple comparison's using Duncan's method (Zar 1984). Plasma hormone (cortisol and ACTH) and protein concentrations were also analyzed by one-tailed t-test, comparing the data from all 3 groups of cortisol- treated ewes to the data from the control group (Zar 1984). Average cortisol values were calculated from the 5 day and 10 day values and were log transformed before analysis. The Mann-Whitney Rank Sum Test was used for maternal plasma protein analysis at 10d (Zar 1984).

Values for the picrosirius red staining were analyzed by two-way ANOVA in order to determine significance across the cortisol treatment groups and areas of the heart (LV, RV, and septum); the percent stained area data was transformed using arc sine prior to analysis to correct for heterscedascity (Winer 1971).

For all analyses, $p < 0.05$ was used as the criterion for statistical significance.

Results

Maternal Physiology

Maternal cortisol concentrations were significantly increased in the ewes treated with cortisol when compared to the non-treated ewes (5d and 10d day average, 9.0 ± 0.9 vs. 5.9 ± 1.4 ng ml^{-1}). When the four groups were compared individually, there was a trend for each cortisol treated group to have increased cortisol concentrations as compared to the control ewes (Table 4-1), but there were no differences among groups. ACTH levels were not significantly altered in response to cortisol treatment, although there was a trend for cortisol treated ewes to have lower ACTH concentrations than the control group (Table 4-1).

Maternal sodium, potassium, and packed cell volume values were not different between the groups at day 5 or at day 10 (data not shown). Maternal plasma protein concentrations were

significantly elevated in the cortisol treated ewes on days 5 (8.2 ± 0.2 vs. 7.6 ± 0.2 g $100ml^{-1}$) and 10 (7.9 ± 0.1 vs. 7.4 ± 0.1g $100ml^{-1}$) days as compared to the control ewes.

Maternal arterial pressures and heart rates were not different between the four groups (data not shown).

Fetal Physiology

The average plasma cortisol concentrations (5 and 10 days) were significantly elevated in the fetuses whose mothers were infused with cortisol compared to control (3.4 ± 0.6 vs.1.5 ± 0.6 ng ml^{-1}). There was a trend for each cortisol treated group to have increased cortisol concentrations as compared to the control fetuses when the four groups were compared individually (Table 4-1). ACTH levels were not significantly altered in response to cortisol treatment (Table 4-1).

There were no significant differences among the groups in the blood gas values or packed cell volume (Table 4-2), nor were there effects on fetal electrolytes (data not shown). There were also no effects of treatment on fetal heart rate and blood pressures (Table 4-3).

Fetal Heart Measurements

Heart weight was significantly greater in the cortisol group compared to the control group and cortisol + MRa group, but not the cortisol + GRa group (Figure 4-2). Left ventricular and right ventricular free wall thicknesses were significantly greater in the fetal hearts of the cortisol treated group as compared to the control group. Left and right ventricular free wall, as well as septum, thicknesses were greater in the fetal hearts of the cortisol group as compared to the cortisol + MRa group (Figure 4-2). Left ventricular free wall thickness and septum thickness were not different in the cortisol group as compared to the cortisol + GRa group (Figure 4-2). However, right ventricular wall thickness was greater in the cortisol group as compared to the cortisol+ GRa group.

Collagen Staining

Fetal heart sections were stained with picrosirius red in order to measure the amount of interstitial collagen deposition (Figure 4-3). The percentage of collagen staining in the left ventricle, right ventricle, septum, and whole heart was not significantly altered among the groups (Table 4-4, Figure 4-3).

Discussion

I conclude that blockade of corticosteroid receptors in the fetal heart prevents the enlargement of the heart observed when maternal cortisol concentrations are chronically increased. I found that blockade of the mineralocorticoid receptors blocked the increase in heart weight, as well as in wall thickness. Blockade of glucocorticoid receptors significantly reduced right ventricular enlargement, and produced smaller, insignificant effects on thickness of the left ventricular free wall and septum and on heart weight. Neither administration of MR or GR blocker into the pericardium resulted in increases in fetal ACTH or fetal blood pressure, suggesting that the infusions of antagonist did not produce systemic effects. The results indicate that small increases in cortisol increase fetal heart size via an intracardiac action at the MR and, to a lesser extent, GR receptors within the fetal heart. I also conclude that the increase in fetal heart weight in response to elevated cortisol occurs without an increase in collagen deposition.

Role of MR and GR in the Heart

This laboratory has previously shown that both MR and GR are abundantly expressed in the heart in the late gestation ovine fetus (Reini *et al.* 2006), suggesting a role for these receptors in fetal heart development *in vivo*. Other investigators have found that aldosterone directly stimulates myocyte surface area (Okoshi *et al.* 2004) and remodeling of myocyte membrane (Kliche *et al.* 2006) in cultures of neonatal myocytes, and effect presumed to be mediated by MR in the myocytes. Cortisol also increases expression of atrial natriuretic peptide in cultured

neonatal myocytes, and both cortisol and aldosterone potentiate the effect of phenylephrine on hypertrophy in these cultures (Lister *et al.* 2006), also indicating an intracardiac action at MR in these cultures.

One of the major factors influencing the ability of cortisol to activate MR and/or GR is the local activity of the 11hydroxysteroid dehydrogenase enzymes, 11β-HSD1 and 11β-HSD2. 11β-HSD1 primarily converts cortisone into cortisol, while 11β-HSD2 converts cortisol into cortisone, which is inactive at MR and GR (Krozowski *et al.* 1999). This laboratory has previously shown that mRNA expression of 11β-HSD2 mRNA is relatively low compared to 11β-HSD1 within the fetal heart (Reini *et al.* 2006). Using immunohistochemistry, I also found that although MR, GR, and 11β-HSD1 are abundantly expressed in both myocytes and blood vessels within the fetal heart, 11β-HSD2 seemed to be localized in blood vessels more abundantly than in myocytes. This suggested that cortisol has access to both MR and GR within the fetal heart, and that when plasma cortisol levels are increased, as in the present study, action of cortisol at MR and GR in the heart would also increase. My present study demonstrates that the effect of cortisol is blocked by antagonists of the MR and/or GR, suggesting a role of intracardiac corticosteroid receptors. This is consistent with the ability of cortisol to alter myocyte growth in cultured myocytes. I also hypothesized that blockade of MR would have a greater effect in inhibiting the effect of cortisol than would blockade of GR, because MR has been shown to have greater affinity for cortisol than GR (Reul & DeKloet 1985; Richards *et al.* 2003). Indeed this is what I observed in the present study: in the cortisol group, there was a 14% increase in heart weight relative to body weight as compared to the control group; this enlargement was completely blocked when MR antagonist was administered to the heart, whereas there was only 44% blockade of the increase in weight after administration of the GR

antagonist. Similarly, in the cortisol group, LV, RV, and septum thicknesses were approximately 20% thicker than control fetuses and the MR antagonist produced a 95%, 149%, and 114% reduction of this increase in thickness of the LV, RV, and septum respectively, whereas the GR antagonist group produced 63%, 110%, and 65% reductions of thickness. Overall, GR blockade was approximately half as effective as MR blockade in inhibiting the increase in heart weight or wall thickness.

The relative differences in effectiveness of MR and GR blockade are consistent with the expected relative binding of fetal cortisol at these receptors. The MR are higher affinity receptors with greater occupancy at low cortisol concentrations (Reul & DeKloet 1985), and therefore a greater effect would be expected after blockade of these receptors. Based on the expected free fraction of cortisol in the fetuses, I calculate that the free cortisol concentrations would be approximately 0.8 nM in the control fetuses and 1.9 nM in the fetuses of the cortisol-infused ewes. Based on previous studies of cortisol binding at ovine MR and GR (Richards *et al.* 2003), I would predict that these free concentrations would result in approximately 65% occupancy of MR and 35% occupancy of GR in the control fetuses, and 85% occupancy of MR and 60% occupancy of GR in the cortisol-infused fetuses. Thus, these levels would be expected to exert more effects via MR than via GR activation if both act at GRE to induce genes responsible for cardiac growth.

It should be noted that mifipristone is also an antagonist of the progesterone receptor (PR). In this study, circulating progesterone levels were not measured, however, an increase in circulating progesterone levels would not be expected in response to cortisol manipulation, suggesting mifipristone infusion most likely resulted in blockade of baseline progesterone action in the fetal heart. While the relative expression levels of PR have not been elucidated in the fetal

heart, I would not expect PR antagonism to greatly affect heart growth since growth of the heart appears to be primarily stimulated by an increase in plasma cortisol concentrations. It is possible, however, that blockade of PRs within the heart contributed to the reduction in heart mass observed with mifipristone infusion in the cortisol + GRa group.

Role of MR in Hypertrophy in the Adult Heart

In adult rats the mineralocorticoid receptor is thought to induce cardiac hypertrophy and fibrosis occurring in response to ischemia; systemic administration of MR blockers have been shown to reduce markers of inflammation and fibrosis in hearts of adult rats (Brilla *et al.* 1993; Fraccarollo *et al.* 2005; Sun *et al.* 2002). It has been established that in adult humans with severe heart failure, there is a reduction in the severity of cardiac hypertrophy and an increase in survival rate after treatment with the MR receptor antagonists eplenerone or spironolactone (Pitt *et al.* 1999; Pitt *et al.* 2001). The effect of MR blockers on survival rate appears to be the result of a decrease in cardiac fibrosis (Fraccarollo *et al.* 2004); increases in interstitial collagen content are a feature of adult cardiac hypertrophy (Pearlman *et al.* 1981), particularly in the case of hypertension or myocardial infarction (Young *et al.* 2007). The mechanism for the *in vivo* effect of MR in contributing to inflammation and subsequent fibrosis is not clear. It has been suggested that the effect is through a nongenomic action, and that the effect in ischemic tissue is predominately on vascular cells expressing MR, rather than on fibroblasts or on myocytes (Young *et al.* 2007; Mihailidou & Funder 2005). It is generally assumed that the protective effect of the MR antagonists results from blocking the action of aldosterone at MR. It has been suggested, however, that many heart failure patients without elevated plasma aldosterone levels still benefit from MR blockade, indicating aldosterone may not be the only relevant MR ligand (Young *et al.* 2007). Since plasma cortisol concentrations are much higher than aldosterone, and since there is not a significant amount of 11β-HSD2 expressed within the heart, it is

reasonable to propose that cortisol may be playing a role in the fibrosis that is observed in heart failure patients.

In this study the effects of cortisol do not appear to involve increase in fibrosis, as there was no increase in collagen content with maternal infusion of cortisol, nor were there any effects of either MR or GR blockade. This suggests that the mechanism of the enlargement of the fetal heart in the current study may be fundamentally different from what is observed in adult rat models or human pathology, in which ischemia is a contributing component.

Mechanisms of Enlargement of the Fetal Heart

Due to the unique ability of the fetal heart to grow through both hyperplasia and hypertrophy, either mechanism could account for the cortisol-induced increases in fetal heart weight and wall thickness in our model. In early gestation, cardiac growth is mostly a result of the production of new myocytes originating through cell division and proliferation (Smolich 1995). After approximately day 115 of gestation in sheep, however, cardiac growth results primarily from increases in myocyte size (Jonker *et al.* 2007). Myocytes lose their ability to divide and proliferate shortly after birth in an event in which there is nuclear division without subsequent cell division (Oparil *et al.* 1984). In fetal sheep the number of terminally differentiated or binucleate myocytes increases from ~115 days of gestation through term, and heart growth during this period is due to both increases in myocyte size and myocyte proliferation (Jonker *et al.* 2007). Theoretically, cortisol could be stimulating growth through either hypertrophy or hyperplasia, or possibly even both.

Rudolph *et al.* showed that cortisol (1.2 μg min^{-1}) infusion for 72-80 hours directly into the left coronary artery of the ovine fetus (124-131d) decreased left ventricular DNA content (Rudolph *et al.* 1999). This was interpreted as cortisol-induced inhibition of myocyte proliferation in preparation for life after birth. The fetal blood pressures from that study were not

reported. In a study by Lumbers *et al* ., high dose infusion of cortisol (72.1mg d^{-1} for ~60h) increased left ventricular myocyte size and increase cardiac angiotensinogen mRNA (Lumbers *et al.* 2005), suggesting an induction of hypertrophy. However, there was also a significant increase in blood pressure in these fetuses, suggesting that the cardiac hypertrophy may have resulted from elevated blood pressure.

Conversely, maternal dexamethasone administration (48 μg d^{-1} from E17) increased relative heart weight and increased myocyte proliferation in the fetal and newborn rat heart (Torres *et al.* 1997). In agreement with this, Giraud *et al.* (Giraud *et al.* 2006) showed that subpressor doses of cortisol (0.5 μg $kg^{-1}min^{-1}$ for 7 days) infused directly into the circumflex coronary artery of the fetus led to an increase in Ki-67 stained myocytes in both the left and right ventricles; as Ki-67 is expressed only in cells in the proliferative phase, this suggested that cortisol stimulated proliferation in these hearts. Hearts infused with cortisol weighed more than control hearts in this study, but there were no changes in myocyte size or percent binucleation. Interestingly, there were also no differences in aortic, right atrial, systolic, and diastolic pressures between the groups. These studies suggest that elevated fetal cortisol concentrations directly stimulate cardiomyocyte proliferation in the late-term fetus.

The current study does not provide direct evidence for cardiomyocyte proliferation as a means of cardiac enlargement in response to cortisol. It is important to note that in this study a subpressor dose of cortisol was used, as in the study by Giraud *et al.*(Giraud *et al.* 2006). I did not observe an increase arterial pressure in response to the moderately elevated cortisol levels indicating that the fetal hearts in this study were not subjected to chronically increased systolic load, a possible trigger to myocyte hypertrophy seen in some other studies. Although in the present study blood pressure was only measured at 10 days of cortisol infusion, a previous study

in this laboratory (Jensen *et al.* 2005) showed fetal arterial blood pressure was not elevated at either 5 or 10 days of maternal cortisol infusion. The doses of cortisol administered in the present study resulted in relatively small increases in fetal cortisol, well below those that have been shown to increase fetal blood pressure in other studies (Unno *et al.* 1999; Tangalakis *et al.* 1992; Wood *et al.* 1987). Furthermore, in this study I observed no evidence within the fetal heart in support of interstitial collagen deposition, a symptom of cardiac hypertrophy in response to hypertension within the adult human heart (Diez 2007).

Conclusions

The data suggest that the enlargement of the fetal heart in response to a modest and chronic rise in maternal cortisol levels is mediated by MR receptors, and to a lesser extent, GR receptors within the fetal heart. Intrapericardial infusion of an MR antagonist completely prevented the increase in wall thickness and heart weight. GR blockade was less effective, although GR blockade prevented the increase in RV free wall thickness, and tended to attenuate the increase in left ventricular wall thickness and whole heart weight. The cortisol-induced enlargement is not accompanied by an increase in interstitial collagen deposition within the fetal heart. This indicates the possibility of a different mechanism for the enlargement observed in the fetal heart than that observed in adult cardiac hypertrophy and fibrosis.

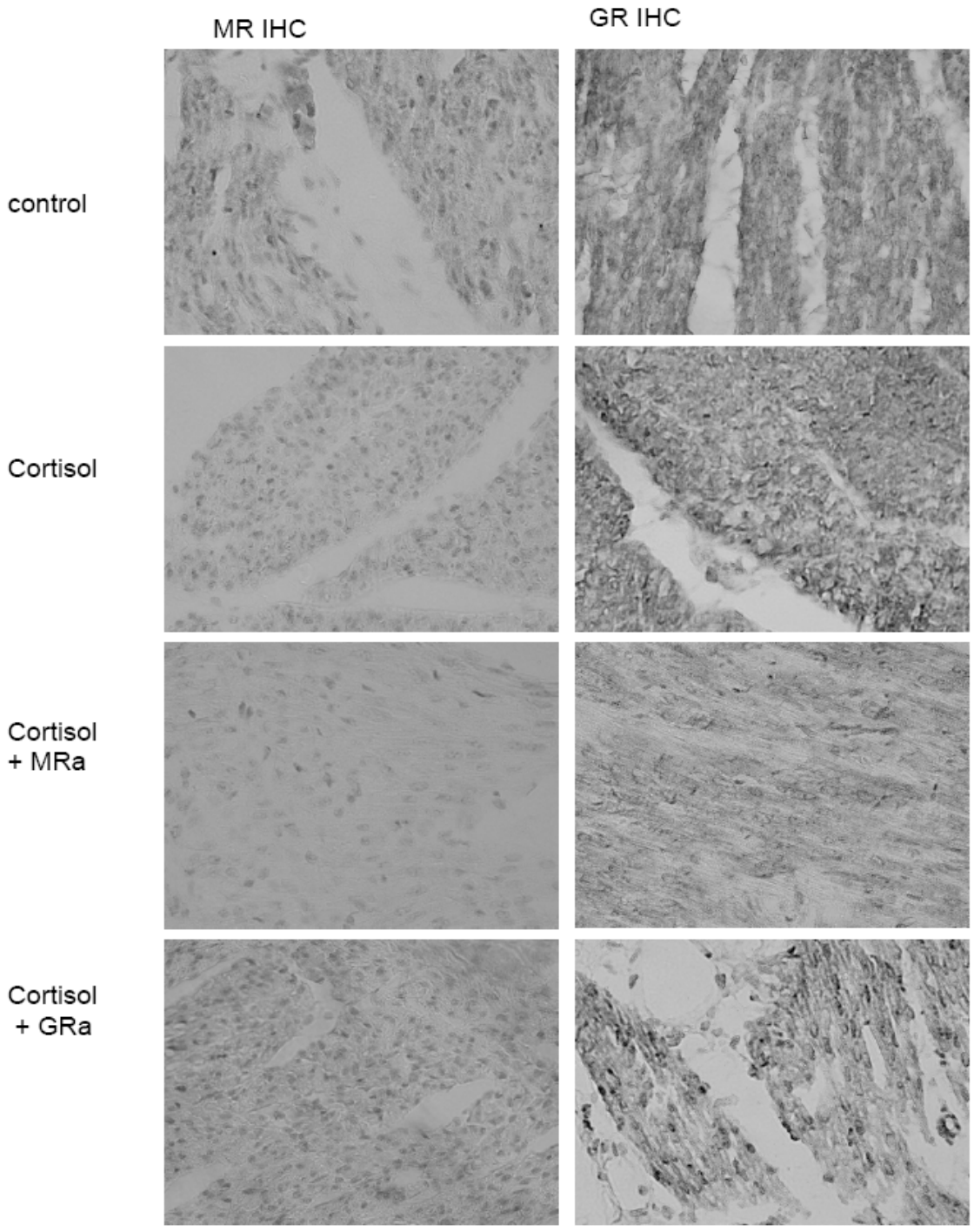


Figure 4-1. Immunohistochemical localization of MR and GR in representative hearts from fetuses of control , cortisol , cortisol +MRa and cortisol+GRa groups. All photos at 40x power.

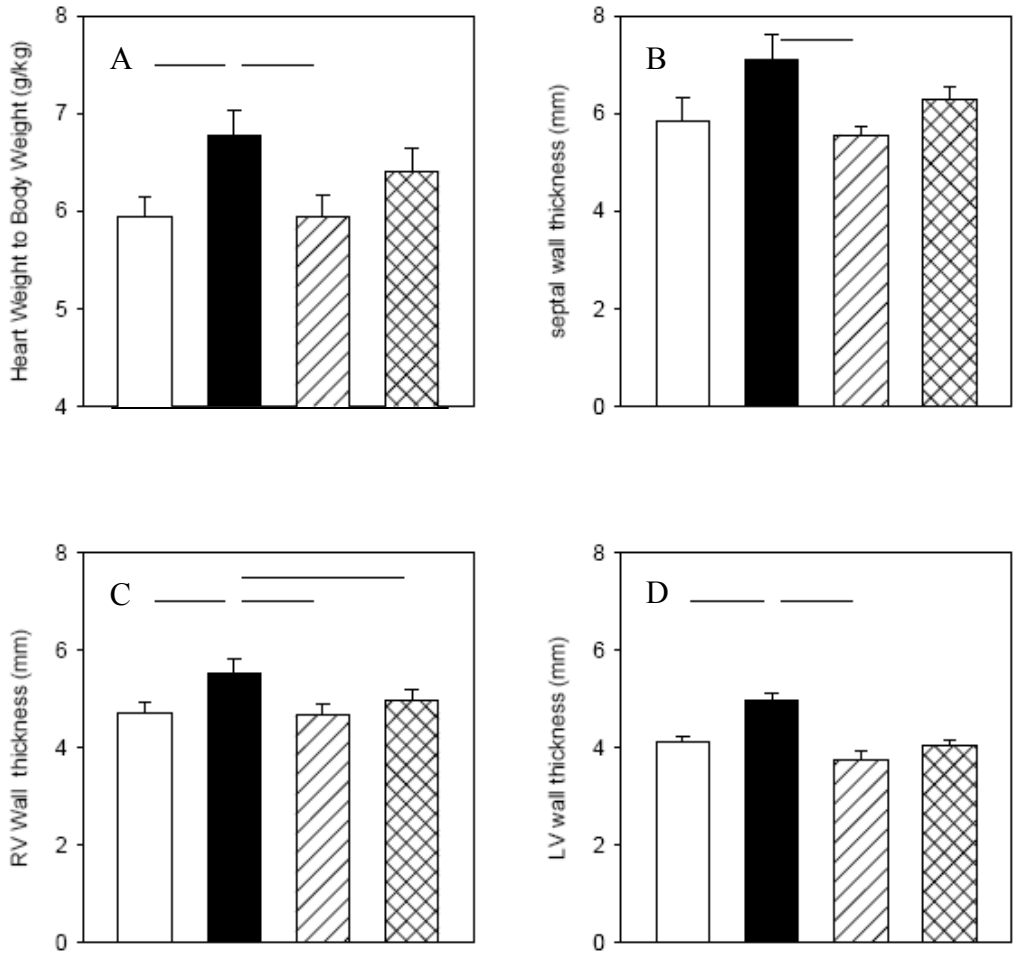


Figure 4-2. Fetal heart measurements in response to manipulations. Mean fetal heart measurements from control (open bars), cortisol (solid bars), cortisol+MRa (diagonal striped bars) or cortisol+GRa (cross-hatched bars) groups taken at time of sacrifice: heart to body mass ratio (A), left ventricular (LV) septal wall thickness, (B)wall thickness (C), and right ventricular (D) wall thickness (lower left). Data are expressed as mean ± SEM. Horizontal lines between groups indicate differences are statistically significant, $p < 0.05$.

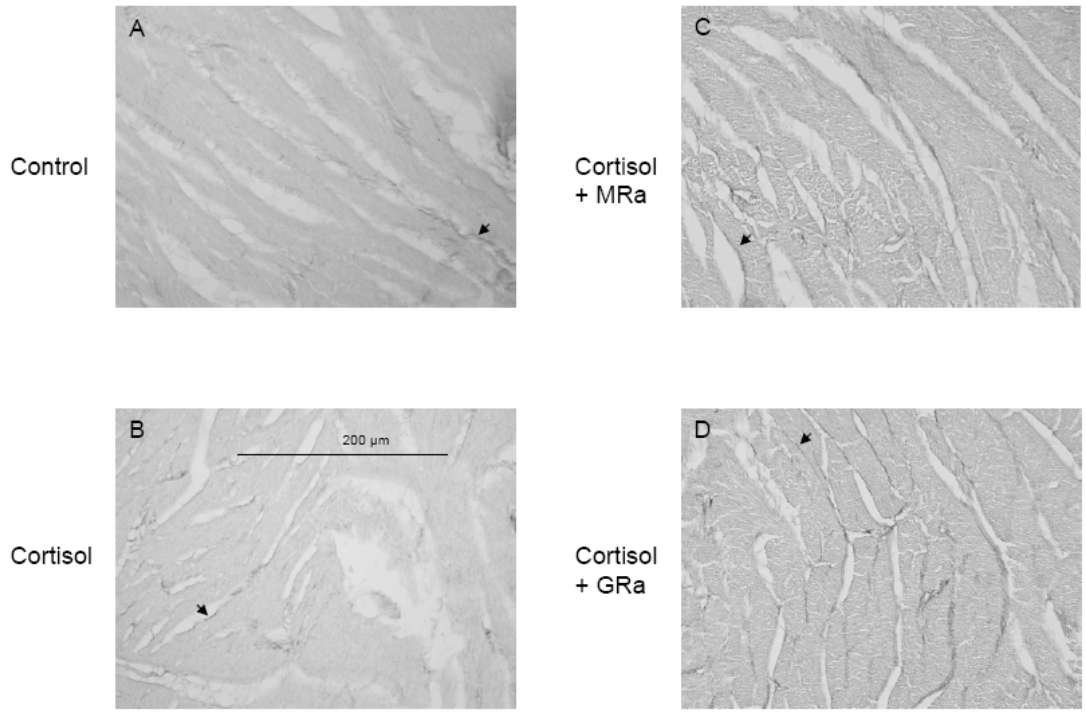


Figure 4-3. Collagen staining of fetal hearts. Representative pictures showing picrosirus red staining of collagen in left ventricular wall of fetal hearts from the (A) control, (B) cortisol, (C) cortisol + MRa, and (D) cortisol + GRa groups; 40x power. Bar indicates 200um. Arrows indicate the dark staining corresponding to positive Sirius red staining.

Table 4-1. Fetal and Maternal Cortisol concentrations (average of days 5 and 10) and ACTH concentration on day 10.

	Maternal Cortisol (ng/ml)	Fetal Cortisol (ng/ml)	Maternal ACTH (pg/ml)	Fetal ACTH (pg/ml)
Control	5.9 ± 1.4	1.5 ± 0.6	37 ± 8	36 ± 8
Cortisol	9.6 ± 2.3	2.7 ± 0.5	20 ± 1	27 ± 5
Cortisol + MR antagonist	8.7 ± 0.4	3.6 ± 1.0	31 ± 11	38 ± 6
Cortisol +GR antagonist	8.3 ± 1.5	3.9 ± 1.9	21 ± 1	57 ± 33

Data are expressed as mean ± SEM.

Table 4-2. Fetal blood gas and packed cell volume.

	Fetal PO2 (mmHg)	Fetal PCO2 (mmHg)	Fetal pH	Fetal Packed cell Volume (%)
Control	21.7 ± 1.0	56 ± 1	7.34 ± 0.01	0.313 ± 0.007
Cortisol	21.5 ± 1.0	53 ± 2	7.35 ± 0.01	0.326 ± 0.013
Cortisol + MR antagonist	20.7 ± 1.1	54 ± 2	7.30 ± 0.03	0.348 ± 0.007
Cortisol +GR antagonist	21.9 ± 0.4	55 ± 1	7.32 ± 0.02	0.325 ± 0.009

Data are expressed as mean ± SEM.

Table 4-3. Fetal arterial pressure and fetal heart rate on day 10.

	Fetal Arterial pressure (mmHg)	Fetal Heart Rate (beats per minute)
Control	47.5 ± 2.7	170 ± 6
Cortisol	46.9 ± 2.7	168 ± 11
Cortisol+MR antagonist	43.0.± 0.9	172 ± 4
Cortisol+GR antagonist	46.0 ± 1.0	164 ± 7

Data are expressed as mean ± SEM

Table 4-4. Collagen content determined by picrosirius red staining (fraction of total area) in left ventricle (LV), right ventricle (RV), and septum.

	LV	RV	Septum
Control	.037 ± 0.005	.039 ± 0.004	.037 ± 0.006
Cortisol	.050 ± 0.009	.057 ± 0.009	.052 ± 0.010
Cortisol + MR antagonist	.049 ± 0.011	.037 ± 0.007	.043 ± 0.008
Cortisol +GR antagonist	.060 ± 0.012	.058 ± 0.013	.059 ± 0.013

Data are expressed as mean ± SEM.

CHAPTER 5
ANALYSIS OF PROLIFERATION MARKERS AND EXPRESSION LEVELS OF POTENTIAL GROWTH PROMOTERS WITHIN THE FETAL HEART

Introduction

Evidence from multiple studies has supported the idea that elevations in cortisol levels late in gestation induces cardiac enlargement of the ovine fetus (Reini *et al.* 2008, Giraud *et al.* 2007, Lumbers *et al.* 2005, Jensen *et al.* 2005). Whereas cardiac growth in early gestation is mostly a result of the production of new myocytes originating through cell division and proliferation (Smolich *et al.* 1995), it has been demonstrated in sheep that hearts can grow by both cell hypertrophy and cell proliferation throughout the last third of gestation (Jonker *et al.* 2007). The ability of myocytes to divide and proliferate, however, comes to an end shortly after birth in an event in which there is nuclear division without subsequent cell division (Oparil *et al.* 1984). This means that cortisol could theoretically be stimulating growth through either hypertrophy or hyperplasia, or possibly even both.

Lumbers *et al.* performed a study in which high dose infusion of cortisol (72.1mg d^{-1} for ~60h) increased cardiac angiotensinogen mRNA and increased left ventricular myocyte size (Lumbers *et al.* 2005), suggesting an induction of hypertrophy. However, there was also a significant increase in blood pressure in these fetuses, implying the cardiac hypertrophy may have resulted from elevated blood pressure. Additionally, Rudolph *et al.* showed that left ventricular DNA content was decreased following cortisol (1.2 μg min^{-1}) infusion for 72-80 hours directly into the left coronary artery of the ovine fetus (124-131d; Rudolph *et al.* 1999). The interpretation of this study was that cortisol functioned to induce inhibition of myocyte proliferation in preparation for life after birth.

Conversely, maternal dexamethasone administration (48 μg d^{-1} from E17) increased relative heart weight and increased myocyte proliferation in the fetal and newborn rat (Torres *et*

al. 1997). Furthermore, Giraud *et al.* showed that physiologically relevant doses of cortisol (0.5 μg $kg^{-1}min^{-1}$ for 7 days) infused directly into the circumflex coronary artery of the fetus led to an increase in heart mass without an increase in blood pressure; it also led to an increase in Ki-67 stained myocytes in both the left and right ventricles (LV and RV) indicating cortisol stimulated proliferation in these hearts (Giraud *et al.* 2006).

Previously in this laboratory, it was shown that fetal cardiac enlargement in response to chronically elevated maternal cortisol levels can be prevented by mineralocorticoid receptor (MR) blockade, and diminished by glucocorticoid receptor (GR) blockade (Reini *et al.* 2008). No change in blood pressure or cardiac collagen deposition was observed in the hearts exposed to elevated cortisol in that study, however, no direct evidence for cell proliferation was obtained either. It has also been previously shown in this laboratory that hearts enlarged from increased cortisol exposure exhibited a decrease in 11β- hydroxysteroid dehydrogenase 2 (11β-HSD2) and insulin-like growth factor type 1 receptor (IGF1R) mRNA expression and also an increase the angiotensin type 2 receptor (AT2R) to angiotensin type 1 receptor (AT1R) ratio of mRNA expression within the left ventricle (LV; Reini *et al.* 2006). However, whether these alterations in gene expression are negated with MR or GR blockade has yet to be determined.

The purpose of this study was to investigate if fetal cardiac growth in response to elevated cortisol levels is due to increased cell proliferation, and to also determine if expression levels of genes that are changed within the enlarged heart are still altered when cortisol action at MR or GR is blocked. I hypothesized that heart enlargement primarily occurred through an increase in cell proliferation and that the changes in LV gene expression observed in the enlarged hearts exposed to elevated cortisol levels would be prevented in hearts where cortisol action at MR was blocked, and lessened in hearts where cortisol binding at GR was prevented.

Materials and Methods

Experimental Design

At the time of sacrifice during a previous study (Reini *et al.* 2008), fetal hearts were fixed with 4% buffered paraformaldehyde for future immunohistochemical analyis and chunks of the LV were frozen in liquid nitrogen and then stored at -80ºC for RNA and protein analysis at a later time. In that study one group of ewes was treated with cortisol (1 mg/kg/day) between ~120-130 days of gestation ("cortisol" group), a second group of ewes was treated with the same amount of cortisol but fetal cardiac MR was chronically antagonized in this group ("cortisol + MRa" group), a third group contained ewes administered cortisol but fetal cardiac GR was chronically blocked in this group ("cortisol + GRa" group), and a fourth group of ewes in which no maternal or fetal manipulations occured ("control group"). Treatment with cortisol in the manner done in that study produces circulating cortisol levels that are within the range measured with mild maternal stress, but are also known to induce fetal heart enlargement (Jensen *et al.* 2005). Fetal arterial and venous catheters were placed at the time of surgery; blood pressure and heart rate was measured on day 130 of gestation and maternal plasma ACTH and cortisol concentrations were measured in samples collected at ~125 and ~130 of gestation. Significant increases in fetal heart weight along with LV and RV increases in the fetuses from the cortisol group compared to those in the control group were reported in that study, but it was also observed that MR blockade prevented the increase in relative heart mass and hearts in that group contained significantly thinner LVs, RVs, and septums compared to the cortisol group (Reini *et al.* 2008). GR blockade lessened the increase in relative heart mass along with LV and septal thickening, and completely prevented the increase in RV thickness.

Immunohistochemistry

To determine percentage of myocytes, Ki67 staining of heart sections from each animal was performed, as Ki67 is a protein only expressed by cells in the S-phase of the cell cycle. Hearts were taken at the time of sacrifice (~130 days gestation) and fixed with 4% buffered paraformaldehyde. The hearts were dehydrated with increasing concentrations of reagent alcohol followed by xylene. The hearts were then embedded in paraffin wax. Ten μm sections were cut by a Zeiss rotary microtome and placed onto poly-l-lysine coated slides. Standard methods were used for deparaffinization and rehydration. Endogenous peroxide was then quenched using incubation in hydrogen peroxide (0.3%; Fisher Scientific, Fair Lawn, NJ). Antigen retrieval was performed by immersion into sodium citrate buffer at 95 degrees for 30 minutes. The section was blocked for one hour with 5% non-fat dry milk in phosphate buffered saline (PBS), followed by anti-Ki67 monoclonal antibody (dilluted 1:100 in blocking solution; Dako, Glostrup, Denmark) addition for overnight incubation at 4ºC, and incubation with biotinylated goat anti-mouse secondary antibody (Zymed, San Francisco, CA) for one hour. As a tertiary agent, streptavidin-peroxidase (Zymed, San Francisco, CA) was used and metal enhanced diaminobenzidine (DAB; Pierce) was used as the chromogen. Lastly, hematoxylin (Fisher Scientific) staining of the nuclei for 45 seconds was utilized in order to co-localize with the Ki67 staining. The stained sections were then dehydrated and a cover-slip was mounted onto the section.

All images were visualized using an Olympus DP71 microscope and Olympus software, and pictures were taken at 40x for analysis. For each heart sample, six pictures were taken of each ventrcle; 3 in the middle of the ventricle, and 3 in the inner area of the ventricle. The nuclei from each picture were then counted along with the number of Ki67 positively stained nuclei. For each ventricle the percentage of Ki67 stained nuclei was then calculated.

Real-Time PCR

RNA was extracted from left ventricles (LV) of each heart using RNeasy Plus Mini Kit (Qiagen Inc., Valencia Ca). Total RNA was measured spectrophotometrically to measure the quantity and quality of RNA. RNA was reverse transcribed into cDNA using a high capacity cDNA archive kit (Applied Biosystems; Foster City, CA) and aliquots for cDNA were stored at -20^{o}C until used.

Qrt PCR was utilized to measured gene expression. The genes analyzed in this study were MR, GR, 11β-HSD1 and 2, IGF-1R, ATR1 and 2, glucose transporter 1 (GLUT1). All probe and primer sequences except for GLUT1 were based on previously published sequences: MR and GR (Keller-Wood *et al.* 2005), 11βHSD2, AT1R and AT2R (Dodic *et al.* 2002), and 11βHSD1 and IGF-1R (Reini *et al.* 2006). GLUT1 primers and probe were designed using Primer Express 2.0 (Applied Biosystems) based on an ovine sequence in the NCBI database (accession number U89029; base pairs 334-396). The forward primer, reverse primer, and probe used for GLUT1 were CTGCTCATTAACCGCAACGA, GGTCCCACGCAGCTTCTTC, and AGAACCGGGCCAAGAGCGTGC respectively. An ABI PRISM 7000 Sequence Detection System (Applied Biosystems) was used to carry out the qrt PCR reactions. Reactions were carried out using 20 or 100 ng of template cDNA. All genes were normalized to β-actin mRNA.

Western Blotting

Immunoblot detection with antibodies to AT1R (sc-579; Santa Cruz, Sant Cruz, CA), AT2R (a generous gift from Dr. Ian Bird, University of Wisconsin, Madison, WI), and proliferating cell nuclear antigen (PCNA; Santa Cruz; sc-7907) was performed on protein isolated from LVs of each of the hearts. Protein was isolated using the DC Protein Assay (BioRad, Hercules, CA) and each sample was measured spectrophotometrically to identify the quantity of protein present. For AT1R and AT2R, 105 μg of protein was loaded into each well

and separated by size using a 10% Tris-HCL gel (BioRad) by SDS-PAGE. The proteins were electrophoretically transferred to 0.45-μm nitrocellulose membranes for 1 hour at 100 V. The same was done for PCNA except only 30 μg of protein was added to each well. Following protein separation, the membranes were washed once with tris-buffered saline with 0.5% Tween-20 (TBST) and then stained with Ponceau S (Fisher Scientific) for normalization purposes. They were then washed again with TBST and left to dry until wetted with TBST the day of staining.

On the day of staining, membranes were blocked with 5% non-fat dried milk in TBST for two hours. Primary antibodies were then dilluted in blocking solution (1:750 for AT1R, 1:2,000 for AT2R, and 1:500 for PCNA) and incubated with the blot overnight at 4°C. After washing twice for 5 minutes in TBST, the membranes were incubated with peroxidase-linked secondary antibody (1:16,000; Sigma, St. Louis, MO; A0545) for one hour at room temperature. The blots were then washed with TBST and the bands were visualized with a chemiluminescence kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's directions. Films (Kodak Biomax XAR Film, Sigma) were developed and bands were quantified using image analysis software (BioRad ChemiDoc XRS). Probing for the AT2R followed stripping of the developed AT1R blot using stripping solution (2% SDS, 62.5 mM Tris pH 6.8, and 100 mM β-mercaptoethanol) at 50°C for 30 minutes. The membrane was then probed for AT2R using the same method as AT1R and PCNA.

Data Analysis

Changes in the percentage of Ki67 positive nuclei were calculated using two way analysis of variance in order to look for differences between the groups and between the middle and inner areas of the ventricle.

Changes in gene expression among groups were analyzed by one-way analysis of variance (ANOVA) using the ΔCt values. For graphical purposes, fold changes of the genes were calculated using the expression $2^{\wedge -\Delta\Delta Ct}$ in which ΔΔCt is the difference between ΔCt for the sample and mean ΔCt for the same gene in the control group (Livak *et al.* 2001). For ratio comparisons of 11βHSD1 and 11 βHSD2, and AT1R and AT2R, Ct values were compared directly to the Ct values of the other gene without β-actin normalization.

Differences in protein expression were calculated by dividing the band density value by the total protein for that lane as measured by Ponceau S staining. Calculation of the AT1R to AT2R ratio was carried out by directly comparing band densities of each sample without normalizing to total protein.

Results

Immunohistochemistry

The percentage of nuclei positively stained for Ki67 was increased in the cortisol group and cortisol + GRa group compared to the other groups in the LV (Table 5-1; Figure 5-1). In the RV, only the cortisol group had significantly more Ki67 stained nuclei than the control and cortisol + MRa groups (Table 5-1).

Real-Time PCR analysis

Expression of MR, GR, 11β-HSD1, and 11β-HSD2

MR LV mRNA expression decreased in the cortisol + GRa group compared to control hearts while GR mRNA expression tended to the same. 11βHSD1 mRNA expression did not change between the groups whereas 11βHSD2 mRNA expression tended to go down in response to elevated cortisol, but this tendency was blocked with MR and GR antagonism. Also, the

11βHSD1 to 11βHSD2 mRNA ratio tended to increase in the cortisol group compared to the other groups (Figure 5-2).

Expression of IGF1R, AT1R, AT2R, and GLUT1

No differences in mRNA expression were observed between the groups in IGF1R, AT1R, or AT2R. The AT1R to AT2R mRNA ratio also did not change. GLUT1 mRNA expression was similar in the control and cortisol groups but was significantly increased in the cortisol + MRa and cortisol + GRa groups (Figure 5-3).

Western Blot Analysis

Expression of PCNA

PCNA protein expression in the LV did not change between the groups although there was a tendency for expression to be decreased in both the cortisol + MRa and cortisol + GRa groups (Figure 5-4).

Expresssion of AT1R and AT2R

AT1R protein expression in the LV was relatively consistent between the groups whereas AT2R expression tended to decrease in the cortiol group compared to the other groups. The AT1R to AT2R protein ratio tended to increase in the cortisol group (Figure 5-5).

Discussion

This study provides potential evidence that fetal heart enlargement in response to moderately elevated cortisol levels late in gestation may be due to an increase in myocyte proliferation, although the results are conflicting. Additionally, these results provide further confirmation that local RAS activity and decreasing 11β-HSD2 expression levels may be involved in the cardiac enlargement. This study also shows that GLUT1 expression in the LV is unchanged by moderate increases in cortisol, but also indicates that basal cortisol activity at MR and at GR are required for proper GLUT1 expression.

Cortisol Stimulation of Myocyte Proliferation

Previously, this laboratory has shown that fetal cardiac enlargement results from chronically elevated cortisol levels (Jensen *et al.* 2005, Reini *et al.* 2008), but the method of enlargement has not been elucidated. At this point in gestation (~130d), the fetal heart has the unique ability to grow through both hyperplasia and hypertrophy (Jonker *et al.* 2007), so cortisol could be stimulating growth through either hypertrophy or hyperplasia, or possibly even both. The current study provides evidence for cardiomyocyte proliferation as a means of cardiac enlargement in response to cortisol. Ki67 is a protein only expressed by cells in the S-phase of the cell cycle and is therefore a marker of cell proliferation. Similar to what Giraud *et al* found (Giraud *et al.* 2006), I observed an increase in Ki67 staining in both the right and left ventricles of fetuses with cortisol infused mothers compared to the control and cortisol + MRa groups. I also observed an increase in Ki67 staining in LVs of the cortisol + GRa group. Interestingly, this Ki67 pattern of expression closely resembles the pattern of increases in ventricular wall thicknesses within these hearts with the LV exhibiting a significant increase in thickness in the cortisol group only when compared to the control and cortisol + MRa groups, whereas the RV exhibited a thickness increase in the cortisol group compared to all other groups.

It is also important to point out that the hearts in this study were merely exposed to a subpressor dose of cortisol and were not subjected to an increase arterial pressure or an increase in cardiac fibrosis, suggesting hypertrophy may not be involved. This is similar to the findings of Giraud et al. in which hearts infused with cortisol weighed more than control hearts due to an increase in myocyte proliferation, but no differences in aortic, right atrial, systolic, and diastolic pressures were observed between the groups. The studies that implicate hypertrophy as the method of enlargement seem to involve larger, more acute doses of cortisol (Rudolph *et al.* 1999,

Lumbers *et al.* 2005). With this in mind, it is interesting to speculate that the mechanism of enlargement may differ based on delivery method, amount, and duration.

In contrast to the Ki67 staining results, quantification of PCNA protein in the LV was not suggestive of an increase in proliferation accounting for the increase in ventricular thickness in response to elevated cortisol. No significant difference was observed in PCNA expression between the groups, but expression did tend to decrease in both the cortisol + MRa group and the cortisol + GRa group. One explanation for the contradiction is that Ki67 staining may be a more dependable marker of proliferation than quantification of PCNA expression. It has been reported that PCNA is more abundant and potentially less specific to the cell cycle when compared to KI67 in the same tissue (Ekramullah *et al.* 1996, Aoyagi *et al.* 1995, Dierendonck *et al.* 1991). It is also possible that western blot may not be sensitive enough to elucidate differences in expression when only ~1-2% of the total number of cells are in the cell cycle.

Expression of IGF1R, AT1R, and AT2R

Both IGF2 and IGF1 have been shown to stimulate myocyte proliferation in vitro. Liu *et al.* found that IGF2 stimulated an increase in proliferation of prenatal rat myocyte cultures, but did not stimulate proliferation in neonatal myocytes (Liu *et al.* 1996). Similarly, it was shown in a previous study that infusion of an IGF1 analog into fetal sheep at 124d gestation results in decreased numbers of binucleated cells, but increased percentages of monucleated myocytes; and it was also demonstrated that IGF1 administration in cultured fetal cardiomyocytes stimulated proliferation of the myocytes mediated by ERK and PI3K (Sundgren *et al.* 2003). Previously in this laboratory it was shown that IGF1R mRNA expression decreased in response to elevated maternal cortisol late in gestation (Reini *et al.* 2006). Because both IGF1 and IGF2 act via binding at IGF1R *in vivo*, we interpreted the decrease in IGF1R mRNA expression in response to

elevated cortisol as a mechanism to limit growth following cardiac enlargement. However, IGF1R mRNA expression was not decreased in the LV in this study.

We also observed an increase in the AT2R to AT1R mRNA ratio in a previous study (Reini *et al.* 2006). In the ovine fetus, angiotensin II has been implicated in stimulating growth of the heart. Segar *et al.* showed that infusion of angiotensin II into fetal sheep stimulates left ventricular growth (Segar *et al.* 2001), and Sundgren *et al.* demonstrated in cultures of ovine fetal cardiomyocytes that angiotensin II stimulates hyperplastic growth (Sundgren *et al.* 2003). Furthermore, Lumbers *et al.* demonstrated that high-dose infusion of cortisol into the ovine fetus (72.1mg d^{-1} for ~60h) increased cardiac angiotensinogen mRNA (Lumbers *et al.* 2005). In the adult heart, the AT1Rs are thought to be responsible for hypertrophic effects, while actions at AT2R are hypothesized to counteract the AT1R (Booz *et al.* 2004, Zhu *et al.* 2003). We therefore reasoned that the increase in the AT2R to AT1R ratio observed previously was a response to slow growth in the hearts exposed to elevated cortisol (Reini *et al.* 2006). However, we did not observe a change in receptor mRNA ratio in this study. A possible explanation for the lack of change in the AT1R to AT2R ratio, along with the lack of change in IGF1R expression, is that the cortisol hearts experienced a greater increase in mass in the previous study (~25%; Jensen *et al.* 2005) than did the cortisol hearts from this study (~13%; Reini *et al.* 2008), making it possible that the greater increase in mass is necessary for changes in expression of these genes.

Interestingly, western blot revealed a tendency for AT2R protein to be decreased in the cortisol group while the AT1R to AT2R protein ratio tended to increase in the cortisol group. The pattern of protein expression is opposite of the observation regarding mRNA expression of the angiotensin receptors from an earlier study in this laboratory (Reini *et al.* 2006). The

discrepancy between mRNA and protein could possibly be due to an attempt by the myocytes to slow heart enlargement by transcribing genes that discourage growth when expression of pro-growth proteins are elevated. Analysis of western blot results suggest cortisol may be increasing the pro-growth action of the RAS within the heart by changing the receptor ratio in order to favor growth.

Expression of MR, GR, 11βHSD1, and 11βHSD2

Cortisosteroid receptors have been implicated in both fetal and adult forms of cardiac enlargement. It has been shown previously that MRs, and to a lesser extent GRs, in the heart may play a primary role in cortisol-induced fetal cardiac enlargement (Reini *et al.* 2008). Similarly, it has been established that treatment with the MR receptor antagonists eplenerone or spironolactone leads to a reduction in the severity of cardiac hypertrophy and an increase in survival rate in adult humans with severe heart failure (Pitt *et al.* 1999, Pitt *et al.* 2001). Whereas MR has been shown to mediate an increase inflammation markers and cardiac fibrosis in the adult hypertrophied hearts (Fraccarollo *et al.* 2004, Brilla *et al.* 1993, Fraccarollo *et al.* 2005, Sun *et al.* 2002), MR and GR-mediated fetal cardiac enlargement caused by elevated cortisol is not accompanied by an increase in fibrosis (Reini *et al.* 2008). This suggests the mechanism of enlargement in the fetal heart may be fundamentally different from what is observed in adults.

Interestingly, this study provides further evidence that sub-pressor elevations in cortisol may lead to increased exposure of MR and GR to cortisol. I found that hearts exposed to elevated cortisol exhibited a tendency for reduction in 11βHSD2 mRNA expression in the LV, but this tendency is blocked with MR and GR antagonism. This is important because one of the major factors influencing the ability of cortisol to activate MR and/or GR is the local activity of the 11hydroxysteroid dehydrogenase enzymes, 11β-HSD1 and 11β-HSD2. 11β-HSD1 primarily

converts cortisone into cortisol, while 11β-HSD2 converts cortisol into cortisone, which is inactive at MR and GR (Krozowski *et al.* 1999). Additionally, I found that the 11βHSD1 to 11βHSD2 mRNA ratio tended to increase in the cortisol group compared to the other groups. I also observed that elevated cortisol had no effect on MR or GR expression, but I did find that MR mRNA expression decreased in the cortisol + GRa group compared to control hearts while GR mRNA expression tended to the same. This suggests that inhibition of cortisol binding at MR or GR in the heart reduces transcription of GRs in fetal myocytes. These findings agree with a previous study in which it was observed that elevated maternal cortisol levels did not alter LV mRNA expression of MR, GR, and 11βHSD1, but caused a decrease in 11βHSD2 expression (Reini, 2006).

Expression of GLUT1

GLUT1 is thought to be responsible for basal glucose uptake in cardiac myocytes. In rats, it was determined that the embryonic heart is rich in GLUT1 mRNA whereas the adult heart contains predominantly GLUT4 mRNA, making it appear as though the major type of glucose transporter in rat heart switches from GLUT1 to GLUT4 during development (Wang *et al.*1991). In adult rat myocytes, it was discovered via immunogold labelling that GLUT1 is predominantly (76%) localized in the capillary endothelial cells, with only 24% of total cardiac GLUT1 present in myocytes, suggesting a potential role in transporting glucose across the capillary wall before myocyte uptake via GLUT1 (Davey *et al.* 2007). Glucose metabolism is thought to be very important in hearts that have suffered an ischemic event. In rats with a large myocardial infarction, progression from compensated remodeling to overt heart failure is associated with upregulation of GLUT1 (Rosenblatt-Velin *et al.* 2001). Interestingly, there is evidence to support glucocorticoid regulation of GLUT1 expression in both skeletal and cardiac muscle in

the ovine fetus. Maternal antenatal dexamethasone (GR agonist) treatment given as a single course (4 doses), or multiple courses (20 doses), increased GLUT 1 protein concentrations in fetal skeletal muscle at 106 or 107 days gestation (Gray *et al.* 2006). Conversely, infusion of high doses of cortisol directly into the ovine fetus in late gestation decreased levels of GLUT1 mRNA in the fetal LV (Lumbers *et al.* 2005). The present study found that mRNA expression of GLUT1 did not change in the cortisol hearts compared to controls, but did increase significantly in both the cortisol + MRa group and the cortisol + GRa group. These results imply that modest increases in cortisol have no effect on GLUT1 expression within the fetal heart, but also that basal amounts of cortisol action at both MR and GR are required for proper GLUT1 expression within the fetal heart. An interesting observation is that dramatic increases in cortisol action at MRs and GRs in the fetal heart, such as in the study by Lumbers *et al.* (Lumbers *et al.* 2005), leads to decreased GLUT1 mRNA expression in the LV whereas complete blockade of cortisol action at MR or GR results in increased GLUT1 mRNA expression in the LV according to this study, suggesting cortisol action at MRs and GRs in the fetal myocytes directly regulates GLUT1 mRNA expression in the LV.

In conclusion, I observed an increase in the percentage of myocytes positively stained for Ki67 in the LV and RV of hearts from the cortisol group, suggesting myocyte proliferation is at least partially accountable for the cardiac enlargement in response to elevated cortisol. Quantification of PCNA via western blot, however, did not support this conclusion. I found that the ratio of AT1R to AT2R protein expression tended to increase in the LV with elevated cortisol, indicating a relative increase in angiotensin II action at the AT1R, which is thought to be the more growth-friendly receptor. I also observed a trend for 11βHSD2 mRNA expression to decrease in the LVs of the cortisol group, but this decrease does not occur with MR or GR

blockade in the heart. Lastly, I found that basal cortisol action at MRs and GRs may be required for proper maintenance of GLUT1 expression within the LV.

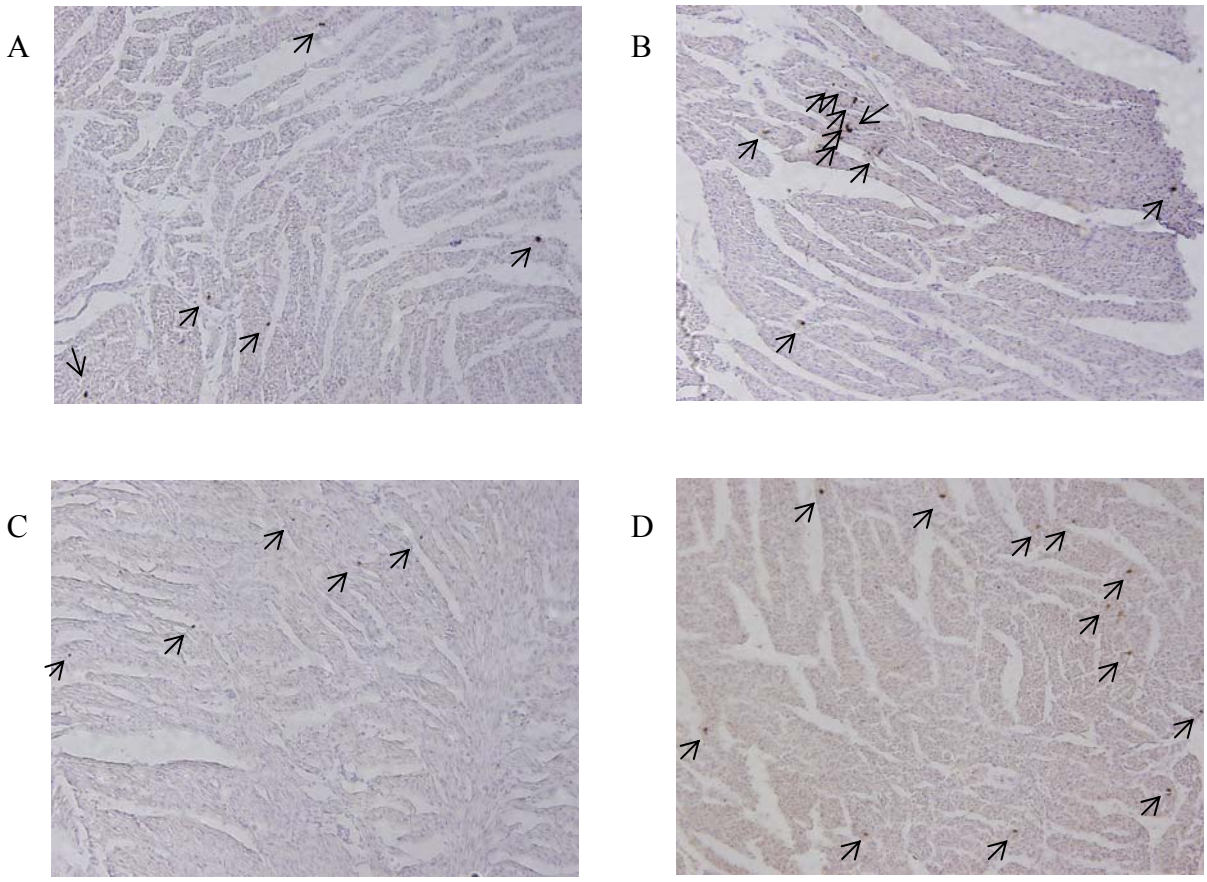


Figure 5-1. Immunohistochemical localization of Ki67 in representative hearts from fetuses of control (A), cortisol (B), cortisol +MRa (C) and cortisol+GRa (D) groups. All photos at 10x power.

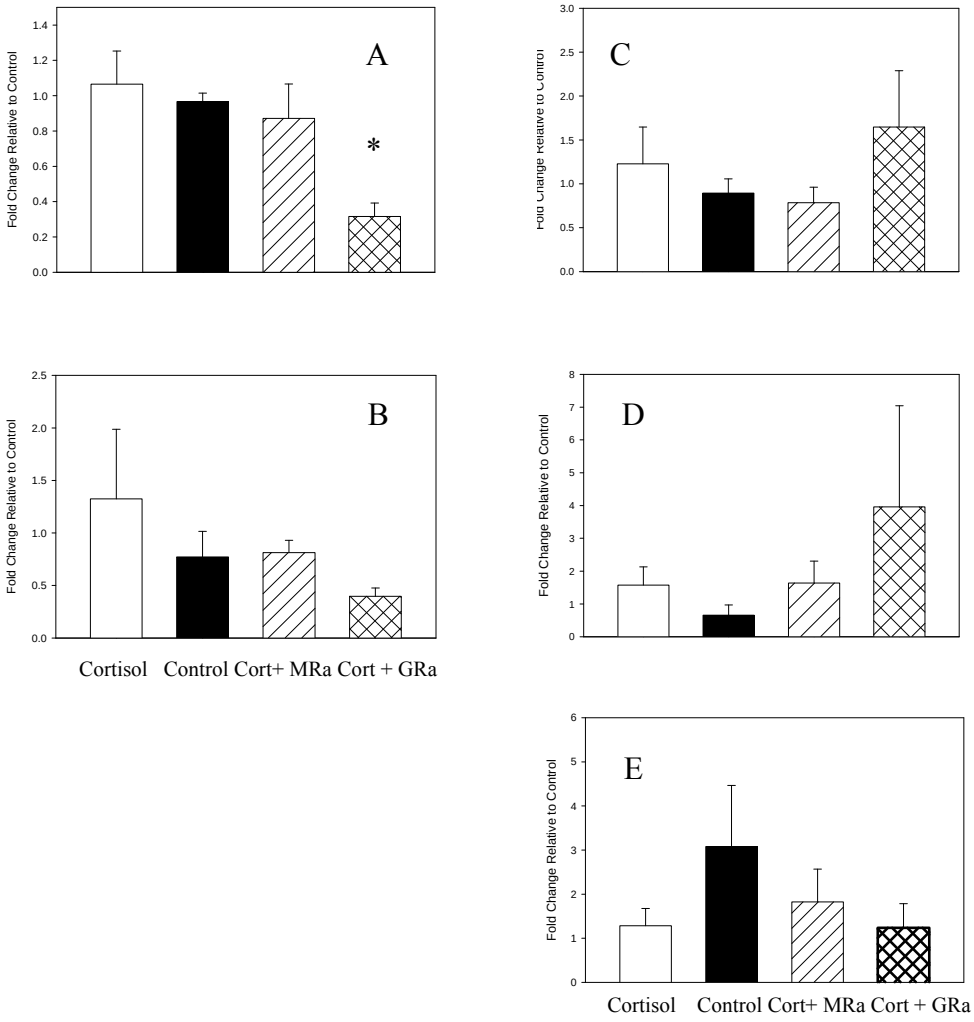


Figure 5-2. Gene expression of corticosteroid receptors and 11β-HSDs in the LV. Expression of mRNA for MR (A), GR (B), 11β-HSD1 (C), and 11β-HSD2 (D) in left ventricles from fetuses of the control, cortisol, cortisol + MRa, and cortisol + GRa groups. The ratio of 11β-HSD1 to 11β-HSD2 mRNAs in left ventricles from each group are shown in panel E. Fold changes of the genes were calculated using the expression 2^-ΔΔCt with respect to the control group and are expressed as mean fold change ± SEM. *$p<0.05$ vs control

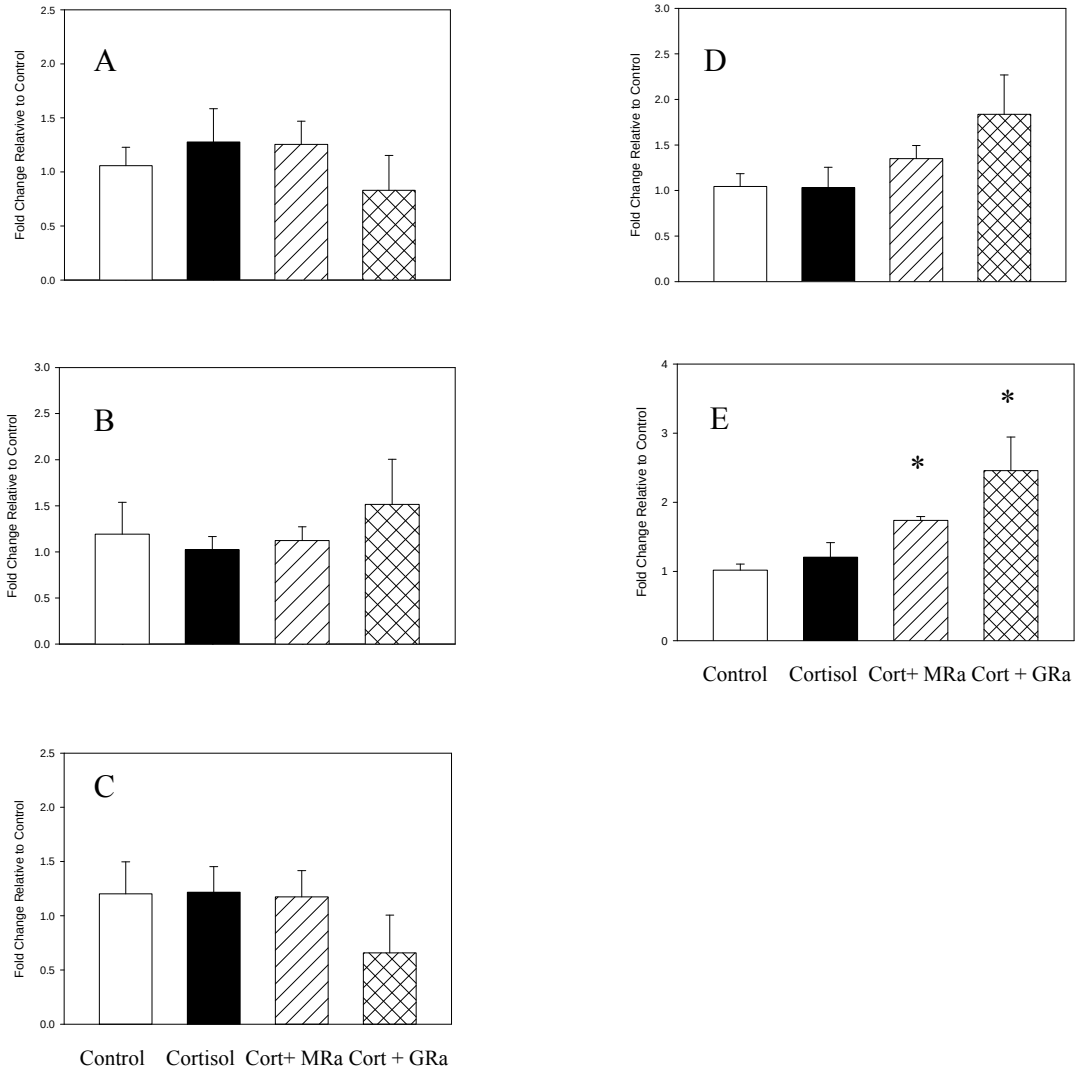


Figure 5-3. Gene expression of angiotensin receptors, IGF1R, and GLUT1 in the LV. Expression of mRNA for AT1R (A), AT2R (B), IGF1R (D), and GLUT1 (E) in left ventricles from fetuses of the control, cortisol, cortisol + MRa, and cortisol + GRa groups. Data are expressed as fold changes as in Figure 5-2. The ratio of AT1R to AT2R mRNAs in left ventricles from each group are shown in panel C. *$p<0.05$ vs control

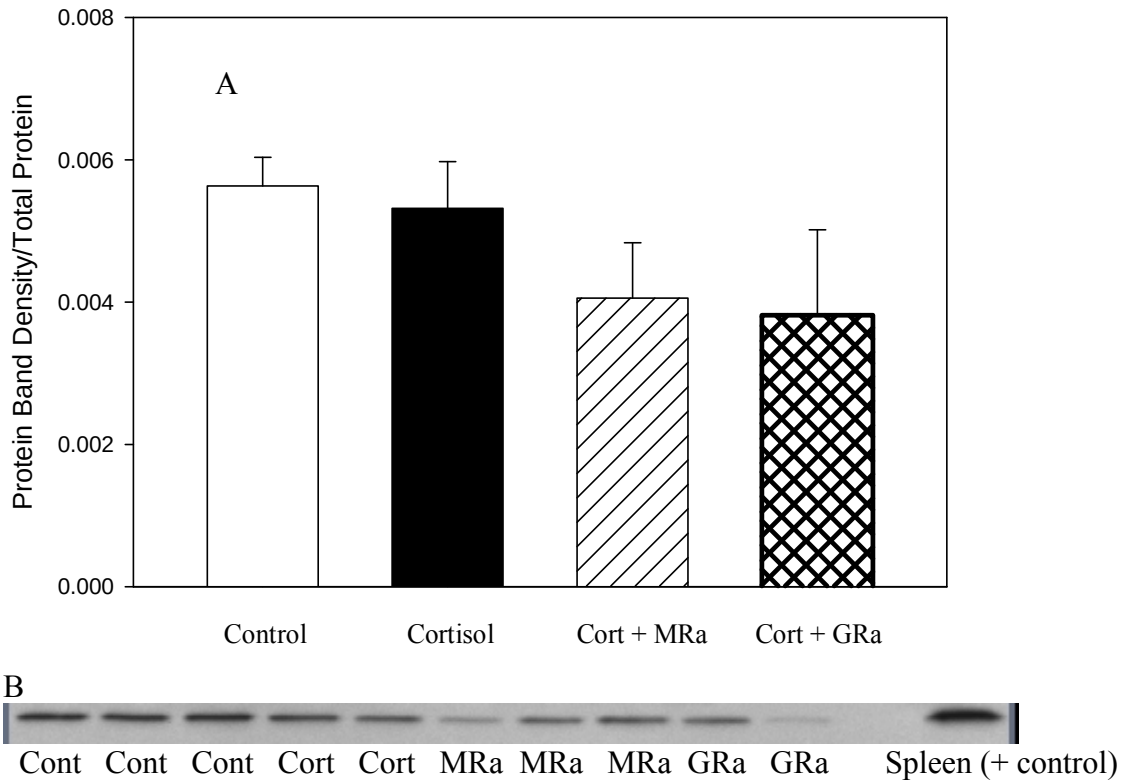


Figure 5-4. Protein expression of PCNA (A; 36 kDa) in control, cortisol, cortisol + MRa, and cortisol + GRa groups in LV. Representative bands are shown in panel B. Band density is normalized to total protein. Values are represented as mean ± SEM.

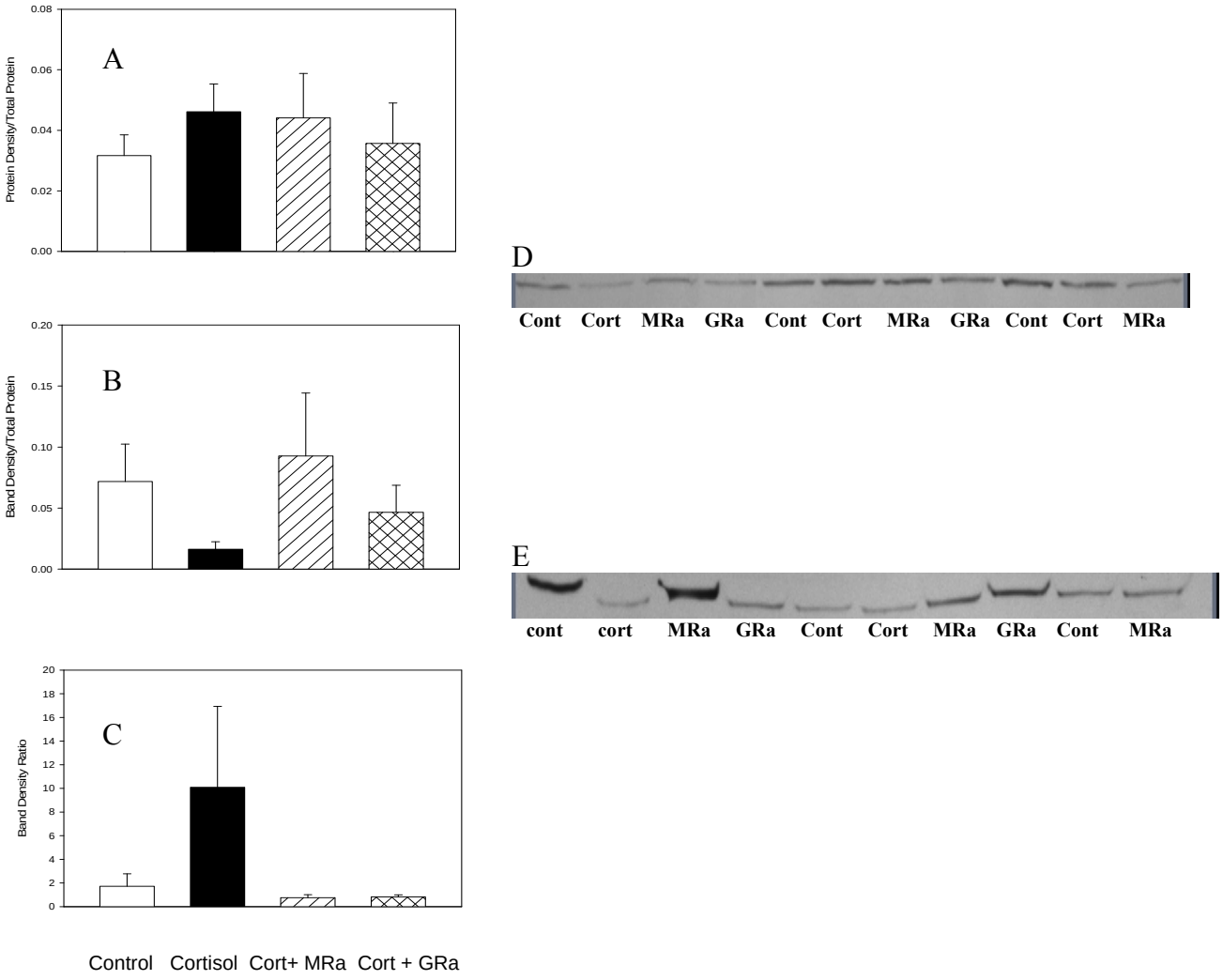


Figure 5-5. Protein expression of AT1R (67 kDa; A) and AT2R (68 kDa; B) in control, cortisol, cortisol + MRa, and cortisol + GRa groups. Band density is normalized to total protein. The ratio of AT1R to AT2R protein expression in each group is shown in the panel C. Representative bands are shown for AT1R (D) and AT2R (E). Values are represented as mean ± SEM.

Table 5-1. Percentage of nuclei positively stained for Ki67 in the LV and RV.

	Control	Cortisol	Cortisol + MRa	Cortisol + GRa
LV	0.9 ± 0.10	1.6 ± 0.19*#	0.9 ± 0.09	1.5 ± 0.4*#
RV	1.0 ± 0.08	1.5 ± 0.18*#	1.0 ± 0.12	1.3 ± 0.23

Data are expressed as mean ± SEM. * indicates significance compared to control, # indicates significance compared to cortisol + MRa.

CHAPTER 6
SUMMARY

Cardiovascular disease is one of the most challenging health concerns in the modern era. Mortality data from the year 2005 indicates cardiovascular disease contributes to 1 of every 2.8 deaths, and 1 death every 37 seconds, within the United States (Rosamond *et al.* 2008). There are many factors contributing to these staggering statistic including obesity, blood pressure, glucose tolerance, and lipid profile. Many of these factors can be attributed lifestyle and genetic predisposition; however we now know fetal growth and nutrition can also be a strong predictor of many of these risk factors for CVD (Roseboom *et al.* 2001). It is interesting that recent evidence has implicated overexposure of the fetus to glucocorticoids as having similar effects in predisposing the offspring to increased CVD risk later in life, a situation often referred to as "programming" (aghajafari *et al.* 2002, Newnham *et al.* 2001, Walfisch *et al.* 2001, Banks *et al.* 1999, French *et al.* 1999). Increased cortisol exposure to the fetus can happen in several ways including increased maternal stress, a natural over-production as seen in Cushing's disease, and administration of synthetic glucocorticoids during premature labor. While we are beginning to understand the initial causes of "programming" and its consequences, very little is understood about the direct effects of cortisol overexposure on organ development. Understanding the role of cortisol in organ maturation could give valuable insight into the mechanisms behind programming and lead to future prevention and treatments. Since different organs go through different developmental stages at different times, timing and duration of the increased exposure probably affects each organ differently. This dissertation focused on the importance maintaining proper cortisol levels late in gestation has on fetal heart development.

Fetal secretion of cortisol increases exponentially before birth in humans and in sheep (Liggins *et al.* 1974), and induces maturation of intestine (Galand *et al.* 1989, Arsenalt *et al.*

1985), lung (Ballard *et al.* 1996, Liggins *et al.* 1972) and liver (Fowden *et al.* 1993, Fowden *et al.* 1995). Studies conducted previous to the studies here suggested that heart mass can be altered by small increases in fetal cortisol (Jensen *et al.* 2002, Jensen *et al.* 2005), indicating possible action of cortisol at MR and/or GR in fetal myocytes. The experiments outlined in chapter 2 were designed quantify expression of genes in the LV, from a previous study in the laboratory (Jensen *et al.* 2005), potentially involved in stimulating growth of the heart in response to moderately elevated maternal cortisol late in gestation (1mg/kg/day infusion between ~120-130 days). I found an increase in the AT2R to AT1R mRNA ratio, indicating the RAS as an important contributor in the heart enlargement. I also observed a decrease in IGF1R mRNA expression in enlarged hearts. Since IGF1R is the primary ligand responsible for pro-growth effects of both IGF1 and IGF2, this indicated cortisol may regulate IGF action within the heart in a negative matter as is seen within skeletal muscle (Li *et al.* 2002). The most interesting observation from that study is that while MR, GR, and 11β-HSD1 were all found to be very abundant in mRNA expression within the heart, 11β-HSD2 mRNA decreased in its already very low expression within the LV in response to the elevated cortisol. This suggested cortisol is able exert direct actions on MR and GR within the developing heart in late gestation and that cortisol action increases in the heart in an elevated cortisol environment not only due to extra circulating cortisol, but also due to a decrease in conversion of cortisol into inactive cortisone within the heart. Furthermore, immunohistochemical staining showed that MR, GR, and 11β-HSD1 are all abundantly expressed in both myocytes and blood vessels within the fetal heart at 128 days gestation while 11β-HSD2 is primarily only expressed in blood vessels within the heart at this time, further indicating the ability of cortisol to act directly on corticosteroid receptors within myocytes at this time.

Since I found MR, GR, 11β-HSD 1 and 2, along with components of the RAS and IGF family to be important factors in determining proper heart growth in the study conducted in chapter 2, my next study outlined in chapter 3 focused on determining the ontogeny of each of these genes throughout late gestation and early postnatal life in both the LV and RV. This allowed me to look at ventricle specific alterations in gene expression. Interestingly, I found evidence for the RAS being an important influencer of heart growth in late gestation. While there were no significant overall patterns of change in expression of angiotensinogen mRNA in LV or RV, ACE1 mRNA increased ~ 5-fold in the LV and RV at term. ACE2 mRNA expression, on the other hand, significantly decreased in the LV by 120 days gestation and remained low, while expression in RV did not significantly change. The ACE1 to ACE2 mRNA ratio increased ~15-fold by 145 days gestation compared to 80 days in both the LV and RV, suggesting that local angiotensin II production may be associated with the terminal maturation of the myocytes in preparation for life outside of the womb. The local mRNA expression of the receptors of angiotensin II did not change dramatically in either ventricle, but AT1R mRNA expression did decrease slightly in the LV in conjunction with the rise of ACE1 mRNA around the time of parturition, suggesting a possible response to increased local angiotensin II levels. These findings also suggest that the enzymes responsible for angiotensin II production may be the primary modulators of the RAS in late gestation and early postnatal life rather than the precursor protein, angiotensinogen, or the receptors for angiotensin II.

The IGF family members also exhibit an expression pattern consistent with modulating growth of the heart in late gestation. LV IGF1 mRNA expression did not significantly change throughout late gestation or neonatally in either ventricle. IGF2 mRNA and IGF2R mRNA were decreased in both the LV and RV after 120-130 days and remained low postnatally. As a result

of the decrease in IGF2 mRNA, the ratio of IGF2 to IGF1 mRNA decreases near term and postnatally in both ventricles. This is interesting because IGF2 and IGF1 both appear to stimulate myocyte proliferation (Liu *et al.* 1996, Sundgren *et al.* 2003). The dramatic decrease in IGF2 mRNA from day 120 of gestation to parturition in both LV and RV parallels the reduction in mononuclear myocytes in both ventricles (Jonker *et al.* 2007). It is interesting to speculate that IGF2 may play a role in mononuclear myocyte proliferation, accounting for the gradual decrease in proliferation observed throughout the last third of gestation as IGF2 mRNA expression within the heart decreases. IGF1R mRNA levels are also decreased in the left ventricle by 120 days gestation and maintained that lower level of expression through birth. Because *in vivo* pro-growth actions of IGF1 and IGF2 are primarily mediated by IGF1R, the decrease in IGF1R mRNA expression may limit the proliferative effects of both IGFs as the heart matures.

Strong evidence for cortisol influencing heart growth throughout all of late gestation was also observed in the studies outlined in chapter 3. I found that MR mRNA was highly expressed at all points in both ventricles but expression is greatest in fetal LV at 80d and is significantly decreased at 130 days of gestation and in newborns. GR mRNA was also highly expressed at all points in both ventricles but is highest in the LV at 80d and decreased at 120, 130 d and in the newborn LV. I found 11β-HSD1 mRNA expression in the LV was significantly decreased at 120 days gestation compared to 80 days and 145 days gestation while 11β-HSD2 mRNA expression in LV did not change throughout gestation. However, in the RV 11β-HSD1 and 11β-HSD2 mRNA expressions were highest in the newborns. The ratio of 11β-HSD1 to 11β-HSD2 expression was unchanged throughout the ages studied in LV and was significantly decreased in the RV at 145 days compared to 100 days gestation, but at all points 11β-HSD1 expression was

far more abundant to 11β-HSD2. It is important to note that the ability of cortisol to bind at MR and/or GR depends in large part on the activity of 11β-HSD1 relative to 11β- HSD2 (Mihailidou 2005; Seckl 2001). The maintenance of high 11β-HSD1 mRNA expression relative to 11β-HSD2 mRNA expression within both ventricles of the heart throughout all of late gestation indicates a significant role for cortisol within the heart in the late gestation fetus. However, the decrease in MR and GR mRNA expression as plasma cortisol concentrations are increasing in vivo suggests that proliferative effects of cortisol may be reduced in left ventricle as the heart matures.

With evidence that cortisol has access to both MR and GR within the fetal heart, it was reasonable to hypothesize that when plasma cortisol levels are increased, action of cortisol at MR and GR in the heart would also increase. The purpose the experiments outlined in chapter 4 were designed to elucidate whether corticosteroid receptors mediate the enlargement of the fetal heart in response to elevated cortisol levels late in gestation. I also know, however, that MR is a higher affinity receptors with greater occupancy at low cortisol concentrations (Reul *et al.* 1985). I therefore reasoned that a greater effect may be expected with blockade of the MRs as compared to blockade of GRs.

In order to elucidate if the cardiac enlargement is mediated by either MRs or GRs, I designed an experiment in which we were able to block MRs and GRs within the fetal heart through administration of specific antagonists into the pericardial space while maternally infusing sub-presser doses of cortisol (1mg/kg/day). This study also involved examination of fetal hearts not administered corticosteroid blockers from maternal ewes that were either infused with cortisol (1mg/kg/day) or not infused with cortisol. As expected from findings in a previous study in this laboratory relative fetal heart mass, LV wall thickness, and RV wall thickness were

all increased in the high cortisol group as compared to controls despite no differences between any of the four groups in mean arterial pressure or heart rate. Interestingly, blockade of the MR within the heart resulted in complete negation of the increase in relative heart mass while GR blockade tended to decrease the enlargement. Furthermore, LV, RV, and septal thicknesses were significantly decreased in the group receiving cardiac MR antagonism compared with the cortisol group. GR blockade resulted in a significant reduction in RV wall thickness along with a tendency for reduction in LV wall thickness and septal thickness compared to the cortisol group. These results are consistent with our hypothesis that suggests cortisol acts directly on MRs, and to a lesser extent GRs, within the fetal heart to stimulate growth.

Within the studies in chapter 4, I also wanted to examine whether fetal cardiac enlargement stimulated by increased cortisol levels is accompanied by an increase in cardiac fibrosis. This is interesting because cardiac MRs have been implicated as playing a role in remodeling of the heart after injury or during heart failure in adult animals and humans. For instance, in adult rats evidence exists that the mineralocorticoid receptor induces cardiac hypertrophy and fibrosis occurring in response to ischemia while systemic administration of MR blockers reduce markers of inflammation and cardiac fibrosis (Brilla *et al.* 1993, Fraccarollo *et al.* 2005, Sun *et al.* 2002). It has been established in adult humans with severe heart failure that treatment with the MR receptor antagonists eplenerone or spironolactone reduces the severity of cardiac hypertrophy and increases the survival rate (Pitt *et al.* 1999, Pitt *et al.* 2001). While increases in interstitial collagen content are a feature of adult cardiac hypertrophy (Pearlman *et al.* 1981), particularly in the case of hypertension or myocardial infarction (Young *et al.* 2007), the effect of MR blockers on survival rate appears to be the result of a decrease in cardiac fibrosis (Fraccarollo *et al.* 2004). This suggests the possibility that cortisol-induced enlargement of the fetal heart may be similar

to that seen in adult cardiac injury. My studies indicate, however, that there was no increase in collagen content with maternal infusion of cortisol, nor were there any effects of either MR or GR blockade. This suggests that the mechanism of the enlargement of the fetal heart may be fundamentally different from what is observed in adult rat models or human pathology, in which ischemia is a contributing component.

In the last set of studies detailed in chapter 5, I wanted to further elucidate the mechanism by which cortisol induces fetal heart enlargement. In early gestation, cell proliferation is the main stimulus of cardiac growth (Smolich *et al.* 1995). It is known, however, that there is a pronounced increase in fetal heart growth in the last third of gestation, paralleling a similar exponential growth of the fetus. At the same time as the heart is increasing in both total weight and left and right ventricle wall mass, an increasing number of myocytes terminally differentiate. Binucleate or multinucleate myocytes are a result of this process and cells experiencing this are unable to undergo further cell division (Burrell *et al.* 2003, Jonker *et al.* 2007). In fetal sheep the number of binucleate myocytes increases from ~115 days of gestation through term, and heart growth during this period is due to both increases in myocyte proliferation and cell size (Jonker *et al.* 2007). This means that cortisol-induced fetal heart enlargement occurring between ~120 and ~130 days gestation could be accounted for by either hypertrophy or hyperplasia, or possibly even both. Since no difference in blood pressure or fibrosis staining between the groups had been observed, I hypothesized that cardiac growth was primarily due to cell proliferation.

In order to investigate this I decided to stain for Ki67 (only expressed in the nuclei of proliferating cells) in heart sections from each of the experimental groups. I found a higher percentage of positively stained nuclei in both the LV and the RV of the cortisol group compared

to control and cortisol + MRa hearts. I also observed a higher percentage of cells stained in the LV of the cortisol + GRa hearts compared to controls and cortisol + MRa hearts. These results indicate cell proliferation as a mode of cardiac enlargement in response to elevated cortisol. However, I found no change between the groups in protein expression of PCNA, another marker of cell proliferation, via western blot. One possible explanation for this contradicting result is that western blot may not be sensitive enough to elucidate differences in expression when only ~1-2% of the total number of cells are in the cell cycle. Another explanation is that PCNA has been observed to be generally more abundant and less specific to the cell cycle when compared to Ki67 in the same tissue, suggesting Ki67 staining may be more dependable when the percentage of cells in the cell cycle is low (Ekramullah *et al.* 2005, Aoyagi *et al.* 1995, Dierendonck *et al.* 1991).

In this study, I also wanted to examine the expression of genes and proteins known to be potentially important in fetal cardiac enlargement. MR mRNA expression decreased in the cortisol + GRa group compared to control hearts while GR mRNA expression tended to the same. This suggests that cardiac-specific inhibition of cortisol binding at MR or GR reduces the synthesis of GR transcripts being manufactured in fetal myocytes. 11βHSD2 mRNA expression tended to go down in the LV in response to elevated cortisol, but this tendency was blocked with MR and GR antagonism. This result agrees with what was observed in Chapter 2 where 11βHSD2 decreased in the LV in the high cortisol group. Also, the 11βHSD1 to 11βHSD2 mRNA ratio tends to increase in the cortisol group compared to the other groups. This further indicates that exposure of MR and GR to cortisol may increase in response to sub-pressor increases in cortisol, but this increase appears to be negated with MR or GR blockade.

IGF1R mRNA expression did not decrease nor did the AT2R to AT1R mRNA ratio increase in the LV of the elevated cortisol group in this study as they did in Chapter 2. The reason for examining these genes was to see if the decrease in IGF1R mRNA and increase in the AT2R to AT1R mRNA ratio seen in the Chapter 2 study was negated by MR and GR blockade, and so I was not anticipating observing no change between control LV expression and cortisol LV expression of these genes. This could be due to the fact that the cortisol hearts from the study in Chapter 2 experienced a greater increase in mass (~25%; Jensen *et al.* 2005) than did the cortisol hearts from this study (~13%; Reini *et al.* 2008), making it possible that the greater increase in mass is necessary for changes in expression of these genes.

Interestingly, the AT1R to AT2R protein ratio tended to increase in the cortisol group. While this does not match with the mRNA expression of the same study, and is the opposite trend of what was observed in the mRNA expression study in Chapter 2, this indicates that cortisol may be increasing the pro-growth action of the RAS within the heart by changing the receptor ratio in order to favor growth. Previously, the AT1R has been implicated in mediating proliferation of vascular smooth muscle cells (Kohno *et al.* 2000), so it is possible that the AT1R is mediating hyperplastic growth of the fetal heart is situations of elevated elevated cortisol. This study also underscores the importance of interpreting the physiologic consequences of mRNA expression increases and decreases with caution because it is not always reflective of protein expression. Whereas AT1 and AT2 receptor mRNA increases are often indicative of similar increases in protein expression in the heart, such as in cases of hypothyroidism in rats (Carneiro-Ramos *et al.* 2008), this study shows this is not always the case and that post-transcriptional modifications and protein turnover rates are also a major factor in determining physiologic outcomes.

Lastly, I found that mRNA expression of GLUT1 did not change in the cortisol hearts compared to controls, but did increase significantly in both the cortisol + MRa group and the cortisol + GRa group. This implies that moderate increases in cortisol have no effect on GLUT1 expression within the fetal heart, but it also implies that basal amounts of cortisol action at both MR and GR are required for proper GLUT1 expression.

The studies within this dissertation have added important insights to the field of fetal heart development and they have specifically investigated and provided potential mechanisms for fetal heart enlargement as a consequence of elevated cortisol exposure. Specifically, these studies have provided evidence that cardiac enlargement in response to elevated cortisol levels is corticosteroid mediated, leads to increased activity of the RAS and decreased activity of the IGF family, and is in part due to an increase in cell proliferation (Figure 6-1). These insights, however, have larger implications and lead to some important questions. Questions like: if cortisol-induced fetal cardiac enlargement is due to an increase in cell proliferation as the Ki67 staining evidence suggests, is the heart enlargement necessarily a negative consequence? Or could obtaining an increased number of myocytes be beneficial? And since evidence here indicates the heart enlargement is corticosteroid mediated, should better, more lung-specific, methods of administration of synthetic glucocorticoids be considered for cases of pre-term labor? Also, does fetal heart enlargement induced by elevated cortisol levels contribute to programming for cardiovascular disease later in life, or are the negative consequences of programming limited to other organs and not related to heart development? These are all important questions for scientists studying fetal heart development to keep in mind, and answering these questions could go a long ways towards reducing the risk for cardiovascular disease in individuals before even the first breath of life is taken.

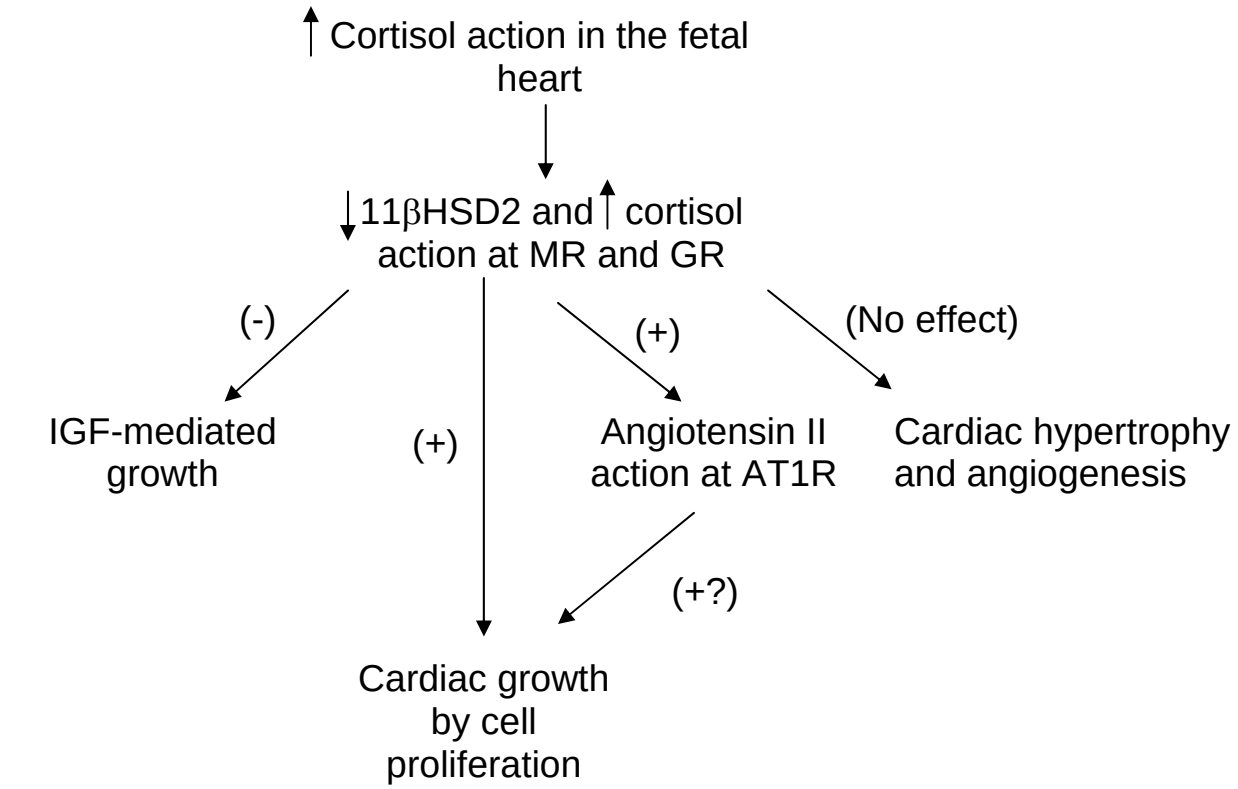


Figure 6-1. Effects of elevated cortisol on fetal heart growth. Cortisol acts directly on MR and GR in the heart to stimulate cardiac growth by cell proliferation, which may be mediated by local increases in angiotensin II action at AT1R.

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BIOGRAPHICAL SKETCH

Seth Andrew Reini was born in 1981, and lived in Lewiston, New York, until the age of 7. At this time his family relocated to Ohio, where he developed a strong interest in science during his junior high years. He graduated high school as valedictorian of his class in 1999. He graduated summa cum laude with a degree in biology, along with minors in chemistry and religious studies, from The University of Findlay in 2003. He also received the "Senior Science Award" from The University of Findlay in 2003. Seth began his graduate studies at the University of Florida College of Medicine in August 2003. His dissertation work was completed with Dr. Maureen Keller-Wood in the Department of Physiology, where he studied the mechanisms by which elevated cortisol levels enlarge the fetal heart. Seth was supported in his graduate studies by an American Heart Association pre-doctoral fellowship and by a scholarship through the Health Services Collegiate Program with the U.S. Navy. Seth has accepted a position to do stress-response physiology research for the U.S. Navy.