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The Effect of Adrenalectomy on the Cardiac Response to Subacute Fetal Anemia

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Abstract

The mechanisms that stimulate fetal heart growth during anemia are unknown. To examine the hypothesis that adrenal hormones contribute to this process, we determined the effects of adrenalectomy (Adx) on heart growth and the activation of cardiac mitogen activated protein kinases (MAPKs) in the presence and absence of fetal anemia. To identify mechanisms contributing to the initiation of cardiac growth, the duration of anemia was limited to a period shorter than that previously described to result in increased cardiac mass. Four groups of fetal sheep were studied (Adx-Anemic, Adx-Control, Intact-Anemic, Intact-Control). Anemia was created by daily controlled hemorrhage for 5 days; hearts were collected for analysis at 133 d gestation (term 145 d). Cardiomyocyte morphometry, immunohistochemistry for Ki-67 (proliferation marker) and Western blotting for protein levels of MAPKs and proliferating cell nuclear antigen (PCNA) were performed. Blood pressure, heart rate, heart weight-to-body weight ratio, and cardiomyocyte length and width remained similar among groups throughout the study. PCNA levels in the Adx-Anemic group were twice as high as any other group (both ventricles, $P < 0.05$). Levels of phosphorylated extracellular signal-regulated kinase (ERK) were ~60% higher in Intact-Anemic and Adx-Anemic compared to the Intact-Control and Adx-Control groups ($P < 0.02$). These results suggest that adrenal hormones may attenuate fetal cardiomyocyte proliferation in response to anemia (as evidenced by the increased PCNA in Adx-Anemic fetuses) and that phosphorylation of myocardial ERK results from fetal anemia, irrespective of the status of the fetal adrenal gland.

Keywords

MAPK; hyperplasia; cortisol; sheep; fetus; heart; cardiomyocyte

INTRODUCTION

To provide adequate nutrient delivery and meet the demand of increased systemic blood flow to support fetal tissue growth, fetal heart size and cardiac output increase as gestation advances. This relationship explains how in healthy fetuses, heart mass closely matches somatic mass across a wide range of body weights (Jonker 2007). However, this association between heart and body mass may be disrupted under a variety of pathophysiologic hemodynamic, hormonal and nutritional conditions. Chronic anemia is one such circumstance, as it serves as a major stimulant of cardiac growth in the fetus (Davis 1991; Davis 1999; Oberhoffer 1999; Olson 2006a).

Fetal anemia may be caused by feto-maternal hemorrhage, alloimmunization and malarial infection of the placenta or fetus, or as a result of other factors such as twin-to-twin transfusion or parvovirus (Ergaz 2006; Moise 2008; Uneke 2007; Wylie 2010). Fetal hemorrhage results in a redistribution of blood flow with preservation of flow to the heart, brain and adrenal glands (Fumia 1984), and increases fetal cortisol levels (Matsuda 1992). Chronically, anemia induces rapid heart growth such that stroke volume and cardiac output are increased but cardiac reserve is maintained (Davis 1991). Anemia-induced heart growth is accomplished largely by robust cardiomyocyte proliferation (a response unique to the fetus), although cellular hypertrophy also contributes (Jonker 2010).

The mechanisms that stimulate heart growth in fetal anemia are unknown, but cortisol may be a mediator. Cortisol levels are ordinarily low in mid- to late-gestation, rising during fetal stress and in the days immediately prior to birth. Unlike the classical growth-retarding effects of high-dose cortisol, direct coronary infusion of lower dose, non-pressor levels of cortisol induces *in vivo* fetal cardiomyocyte proliferation (Giraud 2006). Higher levels of exogenous cortisol induce cardiomyocyte cellular enlargement (Lumbers 2005), secondary to increased arterial pressures or activation of the renin-angiotensin system.

Investigation of intracellular signaling cascades involved in fetal heart growth has to date produced inconsistent and even contradictory results. *In vitro*, phosphorylation of extracellular signal-regulated kinase (ERK 1/2) and Akt (protein kinase B) have both been shown to be necessary for proliferation by fetal sheep cardiomyocytes (Sundgren 2003a). However, evidence of activation of the mitogen activated protein kinase (MAPK) pathway during stimulation of fetal growth *in vivo* has been mixed (Lumbers 2005; Olson 2006a; Olson 2006b). Possible explanations for these results include variable experimental ages, and differences in sampling during acute or chronic phases of growth responses.

This study was designed to examine the role of adrenal hormones *in vivo* on modulation of the fetal cardiac response during sub-acute anemic stress. Specifically, we determined the effects of adrenalectomy, in the presence and absence of fetal anemia, on activation of cardiac MAPK signaling pathways (ERK 1/2, c-Jun N-terminal kinases 1, 2, 3, (JNK1/2/3), p38, MAPK phosphatase 1 (MKP1) and cardiomyocyte dimensions, maturation and cell cycle activity. We previously found that cardiac enlargement was present after 7 days (Jonker 2010), but not 4 days (Jonker, unpublished observation) of progressive fetal anemia induced by daily blood withdrawal. To examine potential signaling mechanisms and whether endogenous adrenal hormones play a role early in the adaptive cardiac response to anemia, animals were studied at after 5 days of anemia. We chose this time point expecting no significant change in cardiac mass, but allowing for a stable period of anemia.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee approved all animal experiments, which were performed within the regulations of the Animal Welfare Act and the National Institutes for Health *Guide for the Care and Use of Laboratory Animals*. Time-bred ewes of mixed western breed were obtained from a local supplier and acclimatized to the laboratory. At 122 days gestational age (dGA; term being ~145 days), sterile surgery was performed to instrument fetuses. After 12 hours of fasting, ewes were given an intramuscular injection of 7.5 mg atropine to control salivation. Anesthesia was induced with an intravenous injection of thiopental sodium (12 mg/kg) (Abbott Laboratories, Chicago, IL) and maintained with isoflurane (2%) in a 30/70 mixture of oxygen/nitrous oxide. The uterus was exposed through a flank or midline incision, and the fetal hindquarters partially exteriorized. Indwelling catheters were placed in the fetal femoral arteries (PE-160, ID = 1.14 mm, OD = 1.57 mm; Intramedic, Franklin Lakes, NJ) and veins (PE-90, ID = 0.86 mm, OD = 1.27 mm) and

advanced to the great vessels. Fetal blood gases were monitored throughout the surgery following catheter placement. In some fetuses, a retroperitoneal approach was used to bilaterally isolate, ligate, and remove the fetal adrenals as has been previously described (Segar 2002). This procedure has previously been shown by us to result in non-detectable fetal serum levels of cortisol (Segar 2002). No more than one fetus per ewe underwent adrenalectomy. Fetal incisions were closed in layers, and a catheter with side-ports was anchored to the fetal skin to reference amniotic fluid pressure. Uterine and maternal incisions were closed in separate layers, and catheters were exteriorized through a subcutaneous tunnel and stored in a cloth pouch on the ewe's flank. At the end of surgery, and for 3 days post-operatively, ampicillin (Wyeth-Ayerst Laboratories, Philadelphia, PA) was directly infused into the amniotic cavity (2 g) and administered intramuscularly to the ewe (2 g). Butorphanol (0.1 mg/kg iv; Torbugesic, Fort Dodge Animal Health, Fort Dodge, IA) was given for 24 hr postoperatively for analgesia. In adrenalectomized fetuses, dexamethasone sodium phosphate (0.2 mg, American Pharmaceutical Partners, Schaumburg, IL) was administered intravenously to the fetus immediately following the surgery and 3 times at 12-hour intervals thereafter to prevent postoperative complications from adrenal insufficiency. Because the end point of the study was 10 days removed from the last dose of dexamethasone, direct effect of the steroid on measured outcomes are unlikely.

Physiological Studies

A 6-day recovery period was allowed before initiating the daily experimental protocol. Ewes were confined to stanchions during the course of the experiment, where they were afforded free access to food and water. Pressures were recorded with Transpac pressure transducers (Abbott, Abbott Park, IL) on a calibrated computerized system (MacLab, ADInstruments, Colorado Springs, CO; Apple, Cupertino, CA). Fetal arterial pressures were corrected relative to concomitant amniotic fluid pressure and reported as arithmetic mean from computer tracings. Arterial pressure tracings were used to calculate fetal heart rate. Fetal blood samples were obtained for pH and blood gases (GEM 3000, Instrumentation Laboratory, Lexington MA) hemoglobin and oxygen content (IL 682 co-oximetersystem (Instrumentation Laboratory) and hematocrit and cortisol.

Four experimental groups were included in the study: 13 control fetuses remained intact and were not bled (Intact-Control); 9 fetuses remained intact and were made anemic (Intact-Anemic); 6 fetuses were adrenalectomized and made anemic (Adx-Anemic); 11 fetuses were adrenalectomized and were not bled (Adx-Control). Fetuses were often chosen to be controls if catheters were not fully functional. Sample sizes vary among groups and various measured parameters based upon continued functionality of catheters, and whether hearts were utilized for protein analysis or cardiomyocyte isolation. Fetuses assigned to an anemia group had 75 ± 12 ml of blood removed over one hour daily for 5 days. Numbers vary in the sample size for physiological measurements because catheter failures dictated that not all values could be collected for every fetus at all the time points. In addition, heart mass tissue collection for protein analysis could not be performed on hearts removed and perfused for cardiomyocyte dissociation Intact-Control and Adx-Control fetuses (see below).

Tissue Collection

Ewes were euthanized with an overdose of a commercially available sodium pentobarbital solution (~65 mg/kg). Fetuses were immediately exteriorized and the fetal hearts were arrested in diastole with an intravenous solution of saturated potassium chloride. Fetuses were weighed and their hearts were removed. For fetuses selected for myocardial protein analysis (5–9 in each group), hearts were dissected into anatomical components and each component weighed. A thin strip of tissue isolated from the mid-ventricular wall 1 cm below

the atrio-ventricular groove was placed in buffered 2% formaldehyde for fixation. The remaining tissue was then cut into small pieces and immediately frozen in liquid nitrogen. The time from organ harvest until freezing did not exceed 45 seconds. A subset of Intact-Control and Adx-Control fetuses were selected for cardiomyocyte morphometry; their hearts were instead dissociated using collagenase and protease and then fixed, as previously described (Jonker 2007).

Cortisol Assay

Circulating cortisol levels were assessed using a commercially available radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA).

Quantitative Immunoblot Analysis

Immunoblots were performed as described previously to quantify protein expression (Olson 2006a). Cardiac samples were homogenized and then sonicated in a buffer containing soybean trypsin inhibitor, leupeptin and PMSF in 50 mM Tris, 10mM EDTA, 150 mM NaCl and 0.1% mercaptoethanol. Cellular debris was removed by centrifugation and samples were quantified spectrophotometrically. Twenty micrograms of protein was separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 hour in 5% nonfat milk, and then incubated with primary antibodies overnight at 5°C. Bound antibody was detected by incubation with infrared-labeled secondary antibodies (IRDye 800 or IRDye 700 700DX, Li-Cor Biotechnology, Lincoln, NE). Blots were read and quantified on a Li-Cor Odyssey Imagine System (Li-Cor Biotechnology). Equal protein loading was assessed by Ponceau S staining. Protein loading controls were not utilized due to uncertainty about constitutive expression of typically used proteins (GAPDH, beta actin) in our experimental conditions.

Primary antibodies included antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) specific to: total ERK1/2 at a dilution of 1:1000 (sc-93); phosphorylated ERK1/2 at a dilution of 1:1000 (sc-7383); total JNK1/2/3 at a dilution of 1:1000 (sc-1648); phosphorylated JNK1/2/3 at a dilution of 1:1000 (sc-6254); total p38 at a dilution of 1:1000 (sc-9212); MKP-1 at a dilution of 1:1000 (sc-1199). Two antibodies from Cell Signaling Technology (Beverly, MA) were used, specific to: phosphorylated p38 at a dilution of 1:250 (9122); and proliferating cell nuclear antigen (PCNA) at 1:1000 (2586).

Cardiomyocyte morphometry

The long-axis (length) and maximal cross-sectional diameter (width) dimensions of cardiomyocytes were measured as previously described (Jonker 2007). Fixed myocytes were prepared in a wet mount with methylene blue, and selected for measurement according to a random, non-repeating and unbiased method employing a counting frame. Myocytes were photographed at 40x on a light microscope (Zeiss Axiophot, Bartels and Stout, Bellevue, WA), and photomicrographs immediately analyzed by calibrated software (Image Pro Plus, MediaCybernetics, Bethesda, MD). At least 50 cells of each type (mononucleated or binucleated) were measured per ventricle per fetus. Separately, at least 300 myocytes from each ventricle of each fetus were counted to determine the number of nuclei per cardiomyocyte.

Cell cycle activity

The anti-Ki-67 antibody MIB-1 (DAKO, Carpinteria, CA) was used to immunohistologically detect cell cycle activity in dissociated cardiomyocytes as previously described (Jonker 2007). Detection of antibody binding was carried out using an avidin-biotin system (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and staining with

diaminobenzidine. At least 500 myocytes were counted per fetal ventricle for cell cycle activity analysis.

Cardiac Histology

Fixed myocardial samples were embedded in paraffin, sliced into 5 μm sections, and stained with Masson's Trichrome. Photomicrographs were taken at 400x magnification from randomly selected locations of each sample. An unbiased estimate of structural volume fractions was calculated based on random point counting techniques described previously (Jonker 2010). A grid was overlaid on each photomicrograph and the structures at each grid intersection were counted into appropriate categories. Volume fractions of tissue components were then determined.

Statistical Analysis

Circulating cortisol levels were initially compared using a Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Daily oxygen content values for Anemic groups were tested by one-way analysis of variance (ANOVA) followed by a test for linear trend. Blood chemistry and hemodynamic parameters at baseline and final day for all groups were compared with Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Body and organ weights, cardiomyocyte dimensions, myocardial morphometry, and immunoblot comparisons were carried out by 2-way ANOVA followed by Bonferroni post-test. A P value of less than 0.05 was accepted as significant for all analyses. Data are presented as mean $\pm$ standard error.

RESULTS

Thirty-nine fetuses in four groups were studied (Intact-Control, Intact-Anemic, Adx-Control and Adx-Anemic) to determine the effects of anemia and adrenalectomy on heart growth. Blood chemistry parameters were measured in all fetuses with patent catheters on baseline and final experimental day (Table 1). All fetal groups had similar arterial pH, PCO₂, PO₂, hematocrit, total hemoglobin and oxygen contents at baseline. Daily isovolemic hemorrhage resulted in a gradual decrease in arterial oxygen content in both Intact and Adx fetuses (Figure 1). Final day hematocrits, total hemoglobins and blood oxygen contents of anemic groups were reduced significantly ($p < 0.05$) compared to their baseline values and compared to Intact-Control and Adx-Control fetuses (Table 1). Because no differences in cortisol were found at baseline between Intact-Control and Intact-Anemic or between Adx-Control and Adx-Anemic fetuses, these fetuses were pooled to make an Intact baseline group and an Adx group. These baseline values were compared with final day values for each experimental group, and with each other, by Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Baseline cortisol values were similar between adrenal intact fetuses, though significantly greater on the final day of study in Intact-Anemic compared to Intact-Control animals. Cortisol was undetectable in Adx fetuses.

Hemodynamic parameters were measured in all fetuses with patent catheters (Table 1). Mean arterial pressures and heart rates were similar among all groups and were unaffected by Adx or anemia. Neither Adx nor anemia had an effect fetal body weight (Table 2). Absolute ventricular free-walls plus septum (V+S) weight was significantly reduced in the Adx-Control group compared to the Intact-Control group ($P < 0.05$) (Table 2). The presence of anemia removed the effect on V+S weight produced by Adx. Left ventricular (LV) weight was reduced in Adx-Control compared to Intact-Control fetuses ($P < 0.01$). The presence of anemia removed the effect on LV weight produced by Adx. Right ventricular (RV), septum, right atrial (RA) and left atrial (LA) weights were similar between groups. Notably, the ratio of V+S to body weight was unchanged by Adx or anemia, suggesting differences in absolute

weights of heart chamber walls related to differences in overall fetal body weight for those animals included in the measurements.

Expression levels of total and phosphorylated components of the MAP kinase pathway were measured by immunoblot (Figure 2) and reported as relative expression to Intact-Control (Table 3). No significant differences in levels were detected among groups, except for p-ERK protein. Anemia was a significant source of variation for p-ERK in both ventricles by analysis of variance ($P < 0.02$), with the Intact-Anemic group found to have higher RV p-ERK levels than the Intact-Control group by post-test (Table 3).

Myocardial volume fractions (the percent of the myocardium occupied by the constituent tissue types) were not affected by anemia or adrenalectomy (Table 4). In addition, no differences in cardiomyocyte dimensions were found between groups (Table 5).

Cardiomyocyte cell cycle activity was assessed by PCNA (Figures 2, 3) and Ki67 (Figure 4) protein expression. Anemia and adrenalectomy were significant sources of variation by analysis of variance. The Adx-Anemic group had higher PCNA levels than the Adx-Control group (LV: $P < 0.05$, RV: $P < 0.05$) and Intact-Anemic group (LV: $P < 0.05$; RV: $P < 0.001$) by post-test. To further assess the effect of Adx on cell cycle activity, Ki67 immunostaining of cardiomyocyte nuclei was performed in the Intact-Control and Adx-Control groups, with no statistically significant differences identified.

DISCUSSION

The fetal cardiac response to anemia occurs rapidly with a marked increase in myocardial mass (Davis 1991; Davis 1999; Mascio 2005; Olson 2006a). Using the same bleeding protocol outlined in the present study, we previously found that cardiac enlargement was present after 7 days (Jonker 2010), but not 4 days (Jonker, unpublished observation). To examine potential signaling mechanisms and whether endogenous cortisol plays a role in this hypertrophic response, animals were studied at 5 days, a time point prior to significant changes in myocardial mass due to anemia. Interestingly, activation of myocyte proliferation, as measured by PCNA expression, was only modestly increased by anemia at 5 days. Adrenalectomy significantly enhanced myocyte proliferation, suggesting an additive effect of the two interventions on cell cycle activity, although cardiomyocyte dimensions were not influenced by subacute anemia or adrenalectomy. Finally, exploration of the MAP kinase signaling found that chronic anemia, but not adrenalectomy, increased myocardial ERK phosphorylation during subacute fetal anemia (prior to gross cardiac enlargement), suggesting an involvement of the MAP kinases in the cardiac growth response to anemia that is not modified by the absence of adrenal hormones.

MAPK Pathway

The MAP kinase pathway consists of a sequence of kinase reactions that result in phosphorylation and activation of the terminal kinases ERK, JNK and p38 (Bogoyevitch 2000). Initiation of the pathways may result from stress (cardiomyocyte stretch), G-protein coupled receptors, receptor tyrosine kinases and receptor serine/threonine kinases (Heineke 2006). Activated MAP kinases translocate to the nucleus where they activate a number of transcription factors that then play a key role in the response of the cell to physiological and pathological stimulation. The specific roles of MAP kinases in regulating physiological and pathological cardiac growth remain unclear, in part related to conflicting results of *in vitro* and *in vivo* studies (Choukroun 1998; Liao 2001; Yamazaki 1993; Zou 1996). Studies involving transgenic and knockout mice have provided novel insights into the functions of MAP kinases in the heart. Overexpression of active MAP kinase kinase 1 (MEK1), which activates ERK (Bueno 2000) results in concentric, compensated cardiac hypertrophy. In

contrast to the role of ERK, *in vivo* activation of p38 and JNK result in mice developing a dilated cardiomyopathy with death at a young age (Liao 2001; Petrich 2002; Petrich 2003). Of significance, targeted inhibition of JNK and p38 results in cardiac enlargement, suggesting that these proteins negatively regulate cardiac hypertrophy (Braz 2003).

There are limited studies on the contributions of MAP kinases in regulating cardiomyocyte or heart growth in the fetus. Sundgren and colleagues found that activation of ERK and PI3K by IGF-1, and ERK alone by angiotensin II, stimulates fetal sheep cardiomyocyte proliferation *in vitro* (Sundgren 2003a; Sundgren 2003b). The ERK cascade was also required for the *in vitro* hypertrophic effects of phenylephrine. Thus, upstream signal-transduction pathways appear important in modulating the effects of ERK activation on fetal cardiomyocyte responses. Our group has examined changes in the cardiac levels of MAP kinases in response to increased load in the late gestation fetal heart *in vivo* (Olson 2006b). In these studies, pressure overload induced by constrictive bands on the proximal aorta or pulmonary artery resulted in significant increases in activated p38 but not ERK or JNK. In 136 dGA but not 108 dGA fetuses made anemic using a protocol similar to that in the current study, increased RV mass was associated with significantly decreased levels of activated ERK (Olson 2008). No changes in p38 or JNK were seen at either age in response to chronic anemia. We acknowledge, however, that such *in vivo* studies do not allow for determination of rapid and perhaps transient changes in MAPK signaling in response to various stimuli and that time course studies have not been performed.

In the current study, designed to examine the role of adrenal hormones on the adaptive response of the fetal heart to chronic anemia, we found levels of p38 and JNK to be similar among groups. In contrast to our previous study, we found that chronic anemia resulted in increased levels of activated ERK in both the LV and RV, independent of the presence or absence of cortisol. Reasons for the differences in results between the two studies are unknown, though may reflect differences in the duration of anemia (8 days versus 5 days). Differences in tissue oxygen delivery or requirements may have also been present, although hemoglobin and blood oxygen content were similar among the anemic fetuses in both studies.

Importantly, and in contrast to the findings of Olson and colleagues (Olson 2006a), the hearts studied at the 5-day time point in the current study did not have an increase in mass. Thus, augmented ERK phosphorylation (activation) during the subacute anemia stage, prior to gross cardiac enlargement, may activate genes needed for the subsequent development of cardiac hypertrophy. After cardiac adaptation (growth) occurs, the stimuli driving cardiomyocyte growth and ERK phosphorylation may then diminish. Levels of MKP-1, an important member of the dual-specificity phosphatase family that regulates inactivation of nuclear p38, JNK and ERK, were similar among the 4 groups of fetuses (Table 3), suggesting activity of this phosphatase did not contribute to the changes observed in phosphorylated ERK. Taken together, these findings suggest a biphasic signaling response to bleeding that mirrors the subacute and chronic phases of fetal anemia. Supporting this interpretation is the observation that cardiac VEGF and metabolic genes that are stimulated during subacute fetal hemorrhage return towards normal values in the chronic phase of anemia when expansion of the coronary tree and gross cardiac hypertrophy are manifest (Davis 1999; Mascio 2005).

Adrenalectomy

Cortisol is a glucocorticoid produced by the adrenal cortex that plays an integral role in the maturation of several fetal organs, including the respiratory, renal and cardiovascular systems. Excess or absence of glucocorticoids can disrupt normal growth (Fowden 1996; Jobe 1998; Lajic 1998; Trahair 1987). Jonker et al. previously reported in a group of slightly

older gestational age fetal sheep that circulating cortisol levels increased from approximately 5 ng/ml at 129 d gestation to 45 ng/ml at 138 d gestation (Jonker 2010). The development of chronic anemia had no significant effect on this maturational response. We failed to identify a maturational increase in cortisol levels, likely related to the slightly younger fetuses and shorter length of study compared to the Jonker study. However, we did find that anemia resulted in a small but significant increase in cortisol compared to age matched, Intact-Controls. However, heart weight, heart-to-body weight ratio, myocardial volume fractions, and cardiomyocyte dimensions were unchanged following adrenalectomy, suggesting that at this gestational age adrenal hormones may not be critical for normal cardiac growth. In fetal sheep, adrenalectomy reduces the catecholaminergic response to acute hypoxemia (Simonetta 1996), but does not alter the hemodynamic response to hemorrhage (Ray 1988).

The specific role of cortisol on cardiomyocyte growth and maturation is unclear. Altered steroid levels due to congenital adrenal hyperplasia and its treatment have been implicated in cardiomyopathy postnatally (Al Jarallah 2004; Donaldson 1994; Scire 2007). Infusion of high levels of cortisol into late gestation fetal sheep caused cardiomyocyte enlargement without a change in maturation (Lumbers 2005). In contrast, infusion of non-pressor levels of cortisol to the fetus at the same age stimulates cardiomyocyte cell cycle activity (Giraud 2006). Although we originally hypothesized that cortisol release during the stress on anemia contributes to cardiomyocyte proliferation, we found that PCNA, a marker of proliferation, significantly increased only in Adx-Anemic animals. This finding suggests that *in vivo*, adrenal hormones may function to inhibit myocardial cell cycle activation rather than mediating the pro-growth effects of anemia on the fetal heart. Phosphorylation of ERK, which is known to promote fetal cardiomyocyte proliferation, and has been shown by others to be inhibited by cortisol *in vitro* (Gonzalez 2010), was unaffected by adrenalectomy. The phosphoinositol-3 kinase (PI3K) cascade has been implicated as a necessary co-signal for fetal cardiomyocyte proliferation *in vitro* (Sundgren 2003a), and it is possible that the increased proliferative activity following adrenalectomy acted this pathway. It is clear that the *in vivo* role of cortisol in the immature heart is far from settled.

In addition to cortisol, the fetal adrenal glands produce aldosterone, epinephrine and norepinephrine, which may influence heart growth. Adrenalectomy of the near-term fetus reduces the circulating level of aldosterone without affecting the fluid balance of the fetus because of the primary role of the placenta in electrolyte control (Benson 1995). Although it has not been well studied, aldosterone is capable of stimulating cardiomyocyte hypertrophy *in vitro* (Okoshi 2004). Catecholamines also play a role in cardiomyocyte growth, although their role in fetal cardiac growth is unknown. This study does not differentiate between the roles of these hormones, and cortisol, in the fetal cardiac growth response to adrenalectomy.

The mechanism responsible for stimulating cardiac growth pathways during fetal anemia remains elusive. Candidates include a very sensitive response to altered diastolic stress, molecules involved in regulation of oxygen delivery to the myocardium, or signaling resulting from increased coronary vascular shear. Experiments to address some of these possibilities may be quite difficult; for instance, accurately measuring diastolic stress in the fetal heart is very challenging. However, dissecting the mechanisms driving physiological adaptation in the fetal heart will ultimately lead to better understanding and improved tools designed to address congenital and adult cardiovascular disease.

PERSPECTIVE

In the subacute phase of fetal anemia, prior to gross cardiac enlargement, the ERK arm of the MAP kinase signaling pathway is activated. This activation is not modulated by the absence of the fetal adrenals, and adrenalectomy has no effect on the cellular composition of

the fetal heart. Adrenal hormones do not play an integral role in anemia-induced activation of myocardial growth pathways. In fact, adrenal hormones may have an inhibitory role on myocardial proliferation during anemia. The key mechanisms modulating cardiac growth during fetal anemia remain to be determined.

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Figure 1.

Arterial oxygen content in animals bled daily for 5 days. Arterial oxygen content decreased gradually in Intact-Anemic (open circles, -0.4562 ml/dL/day, $R^2=0.6816$, $P<0.0001$) and Adx-Anemic (closed circles, -0.5717 mg/dL/day, $R^2=0.5421$, $P<0.0001$) fetuses.

Figure 2.

Representative Western Blots of p38, phosphorylated p38, ERK1/2, phosphorylated ERK 1/2, JNK1/2/3, phosphorylated JNK1/2/3, MKP-1 and PCNA performed on myocardial protein isolated from left ventricular free wall of Intact-Controls (I/C), Intact-Anemic (I/An), Adrenalectomy-Control (Ad/C) and Adrenalectomy-Anemic (Ad/An) fetuses.

Figure 3. PCNA levels in fetal myocardial extracts. A) left ventricle, and B) right ventricle. Anemia increased cardiac PCNA expression only in Adx fetuses.

Figure 4. Percentage of Ki-67 positive cells in mononucleated cardiomyocytes from Intact-Control and Adx-Control fetuses. A) left ventricle, and B) right ventricle.

Table 1

Fetal blood chemistry and hemodynamic parameters

	Intact-Control		Intact-Anemic		Adx-Control		Adx-Anemic	
	baseline	final day	baseline	final day	baseline	final day	baseline	final day
pH	7.34 ± 0.01 (2)	7.34 (1)	7.35 ± 0.01 (9)	7.35 ± 0.01 (9)	7.34 ± 0.01 (6)	7.33 ± 0.01 (8)	7.34 ± 0.02 (6)	7.33 ± 0.02 (6)
PCO ₂ (mmHg)	57 ± 1 (2)	61 (1)	55 ± 2 (9)	57 ± 2 (8)	56 ± 2 (6)	58 ± 2 (8)	55 ± 2 (6)	59 ± 2 (6)
PO ₂ (mmHg)	18 ± 2 (2)	14 (1)	17 ± 1 (9)	14 ± 1 (9)	17 ± 2 (6)	14 ± 1 (8)	17 ± 2 (6)	14 ± 2 (6)
Hematocrit (%)	34 ± 0 (2)	33 (1)	35 ± 1 (9)	21 ± 1 [*] (9)	38 ± 2 (4)	40 ± 5 (2)	37 ± 1 (6)	20 ± 2 (6) [*]
Total Hemoglobin (g/dL)	11.3 ± 0.0 (2)	11.2 (1)	11.2 ± 0.5 (9)	6.1 ± 0.3 [*] (9)	12.7 ± 0.7 (4)	12.1 ± 1.0 (3)	11.9 ± 0.4 (6)	5.6 ± 0.4 (6) [*]
O ₂ Content (g/dL)	7.8 ± 0.7 (2)	5.7 (1)	7.9 ± 0.6 (9)	3.3 ± 0.2 [*] (9)	6.9 ± 0.5 (4)	6.6 ± 0.6 (3)	8.2 ± 1.3 (6)	2.6 ± 0.5 (6) [*]
Cortisol (nMol/L)	19 (1)	23 ± 6 (4)	43 ± 7 (6)	72 ± 19 [†] (8)	ND (3)	ND (6)	ND (4)	ND (4)
Mean Arterial Pressure (mmHg)	43 ± 1 (2)	44 ± 1 (2)	45 ± 1 (9)	42 ± 1 (9)	46 ± 2 (3)	45 ± 2 (2)	46 ± 1 (6)	44 ± 2 (6)
Heart Rate (bpm)	155 ± 2 (2)	134 ± 8 (2)	159 ± 6 (9)	162 ± 7 (9)	190 ± 18 (3)	151 ± 26 (2)	167 ± 6 (6)	185 ± 11 (6)

^{*} P < 0.05 compared to baseline value of same group and final day of Intact-Control.[†] P < 0.05 compared to final day of Intact-Control. Values in parenthesis represent sample size for determined parameter. Adx, adrenalectomized; ND, not detected.

Table 2

Fetal body and heart weights

	Intact-Control	Intact-Anemic	Adx-Control	Adx-Anemic
Body (kg)	4.0 ± 0.2 (13)	3.9 ± 0.1 (9)	4.0 ± 0.2 (11)	3.8 ± 0.1 (6)
Heart (g)				
Left Atrium	2.1 ± 0.2 (6)	1.5 ± 0.1 (9)	1.4 ± 0.1 (5)	1.6 ± 0.1 (6)
Right Atrium	2.1 ± 0.2 (6)	1.7 ± 0.2 (9)	1.6 ± 0.2 (5)	1.9 ± 0.2 (6)
Left Ventricle ^{1,2}	8.7 ± 0.6 (6)	7.7 ± 0.3 (9)	6.6 ± 0.3 (5) ³	7.6 ± 0.5 (6)
Right Ventricle	7.7 ± 0.7 (6)	7.1 ± 0.4 (9)	5.8 ± 0.2 (5)	7.1 ± 0.4 (6)
Septum	5.7 ± 0.4 (6)	5.0 ± 0.2 (9)	4.8 ± 0.2 (5)	5.1 ± 0.4 (6)
Ventricles + Septum ^{1,2}	22.1 ± 1.3 (6)	19.8 ± 0.9 (9)	17.2 ± 0.4 (5) ³	19.8 ± 1.3 (6)
Ventricles + Septum/Body (g/kg)	5.1 ± 0.1 (6)	5.1 ± 0.1 (9)	4.9 ± 0.1 (5)	5.2 ± 0.3 (6)

¹ Adrenalectomy is a significant source of variation by 2-way analysis of variance.² There is significant interaction between adrenalectomy and anemia by 2-way analysis of variance.³ Different from Intact-Control. Values in parenthesis represent sample size.

Table 3

Immunoblot analysis of cardiac MAPK expression

	Left Ventricle				Right Ventricle			
	Intact-Control (6)	Intact-Anemic (9)	Adx-Control (5)	Adx-Anemic (6)	Intact-Control (6)	Intact-Anemic (9)	Adx-Control (5)	Adx-Anemic (6)
P38	100 ± 10	94 ± 10	102 ± 12	114 ± 6	100 ± 12	106 ± 6	98 ± 4	116 ± 10
p-P38	100 ± 6	87 ± 6	85 ± 6	92 ± 6	100 ± 13	126 ± 17	132 ± 15	128 ± 21
ERK	100 ± 10	98 ± 11	90 ± 8	88 ± 8	100 ± 9	98 ± 7	87 ± 5	91 ± 8
p-ERK*	100 ± 16	164 ± 28	108 ± 20	159 ± 20	100 ± 16	185 ± 27	110 ± 17	169 ± 24
JNK	100 ± 6	104 ± 5	102 ± 8	102 ± 8	100 ± 10	102 ± 7	115 ± 8	89 ± 12
p-JNK	100 ± 12	99 ± 9	103 ± 17	103 ± 9	100 ± 7	128 ± 12	121 ± 9	134 ± 14
MKP-1	100 ± 17	129 ± 31	91 ± 22	150 ± 30	100 ± 11	83 ± 16	127 ± 21	119 ± 15

Values expressed as % of Intact-Control value. Values in parenthesis represent sample size.

* Anemia is a significant source of variation by 2-way analysis of variance.

Table 4

Volume fractions of fetal heart tissue

	Intact-Control	Intact-Anemic	Adx-Control	Adx-Anemic
Left Ventricle				
Cardiomyocyte (%)	96.3 ± 0.7 (7)	96.5 ± 0.8 (6)	95.1 ± 1.3 (6)	95.3 ± 1.4 (6)
Other (%)	3.7 ± 0.7 (7)	3.5 ± 0.8 (6)	4.9 ± 1.3 (6)	4.7 ± 1.4 (6)
Right Ventricle				
Cardiomyocyte (%)	97.0 ± 0.2 (7)	97.0 ± 0.3 (6)	97.2 ± 0.4 (6)	96.8 ± 0.7 (6)
Other (%)	3.0 ± 0.2 (7)	3.0 ± 0.3 (6)	2.6 ± 0.5 (6)	3.0 ± 0.8 (6)

Values in parenthesis represent sample size.

Table 5

Fetal cardiomyocyte profile

	Left Ventricle		Right Ventricle	
	Intact-Control	Adx-Control	Intact-Control	Adx-Control
Binucleation (%)	48 ± 5 (7)	56 ± 4 (6)	52 ± 5 (7)	55 ± 5 (6)
Cell Cycle Activity (%)	3.3 ± 0.5 (7)	6.1 ± 0.9 (6)	4.9 ± 0.8 (7)	8.7 ± 2.0 (6)
Mononucleated Length	67.1 ± 0.8 (7)	67.8 ± 1.2 (6)	69.8 ± 0.7 (7)	69.3 ± 1.1 (6)
Mononucleated Width	11.2 ± 0.2 (7)	11.4 ± 0.1 (6)	13.3 ± 0.3 (7)	13.4 ± 0.4 (6)
Binucleated Length	87.6 ± 1.7 (7)	88.8 ± 1.2 (6)	92.3 ± 1.8 (7)	92.8 ± 2.2 (6)
Binucleated Width	12.9 ± 0.2 (7)	13.3 ± 0.2 (6)	15.2 ± 0.4 (7)	15.8 ± 0.4 (6)

Values in parenthesis represent sample size.