Increased maternal cortisol in late-gestation ewes decreases fetal cardiac expression of 11β-HSD2 mRNA and the ratio of AT1 to AT2 receptor mRNA

Seth A. Reini,^2 Charles E. Wood,^2 Ellen Jensen,^2 and Maureen Keller-Wood^1

^1Department of Pharmacodynamics, College of Pharmacy and ^2Department of Physiology and Functional Genomics, College of Medicine, University of Florida, Gainesville, Florida

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Reini, Seth A., Charles E. Wood, Ellen Jensen, and Maureen Keller-Wood. Increased maternal cortisol in late gestation ewes decreases fetal cardiac expression of 11β-HSD2 mRNA and the ratio of AT1 to AT2 receptor mRNA. Am J Physiol Regul Integr Comp Physiol 291: R1708–R1716, 2006. First published May 10, 2006; doi:10.1152/ajpregu.00294.2006.—Moderately elevated maternal cortisol levels in late-gestation ewes decreases fetal cardiac expression of 11β-HSD2 mRNA and the ratio of AT1 to AT2 receptor mRNA. Am J Physiol Regul Integr Comp Physiol 291: R1708–R1716, 2006. First published May 10, 2006; doi:10.1152/ajpregu.00294.2006.—Moderately elevated maternal cortisol levels in late gestation cause enlargement of the fetal sheep heart. We have used quantitative real-time PCR to examine expression of candidate genes in fetal hearts from mothers in whom cortisol levels were increased (by infusion of 1 mg cortisol·kg⁻¹·day⁻¹) or decreased (by adrenalectomy and replacement to 0.5 mg cortisol·kg⁻¹·day⁻¹) from 115 to 130 days gestation. Control ewes were not treated with steroid. Expression of mineralocorticoid receptor (MR), glucocorticoid receptor (GR), 11β-hydroxysteroid dehydrogenases 1 and 2 (11β-HSD1 and -2), IGF I and II, IGF receptors 1 and 2 (IGF-1R and IGF-2R), endothelial nitric oxide synthase, VEGF, myotrophin, angiotensinogen, the angiotensin receptors 1 and 2 (AT1R and AT2R), and the angiotensin converting enzymes 1 and 2 were measured. MR mRNA abundance in fetal hearts was found to be similar in that in adult kidney and hippocampus. Although there were no significant changes in most genes, 11β-HSD2 and IGF-1R expression were significantly decreased in the high cortisol group and 11β-HSD2 expression negatively correlated to left ventricular wall thickness. There was also a significant change in the ratio of AT receptor expression, with increased AT2R and decreased AT1R in the high cortisol group. MR, GR, and 11β-HSD1 immunoreactivity was found in cardiomyocytes and cardiac blood vessels in 126–128 day fetal sheep; in contrast 11β-HSD2 staining was predominantly in blood vessels. These results indicate that cortisol could indeed act in the fetal heart to induce enlargement and suggest that the renin-angiotensin system may play a role.

myotrophin; insulin-like growth factor

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 (22, 23). The fetal consequences are similar to those observed with maternal hypovolemia (8), 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 (39, 54). Even modest chronic increases in maternal cortisol levels increase fetal heart growth while causing a reduction in overall fetal growth rates (24). 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 (7, 44, 48). In rats, short-term prenatal treatment resulted in programming effects, including increased postnatal plasma cortisol levels and blood pressure (29). In sheep, postnatal hypertension results after glucocorticoid treatment only when it is administered in early or mid-gestation and does not occur after synthetic glucocorticoid treatment in late gestation (15, 59). Acute glucocorticoid treatment in the late gestation ewe also does not appear to increase fetal heart weight (39). 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 (23). We hypothesized that cortisol acts on MRs or GRs in the fetal heart to induce genes involved in cardiac growth. In this study we 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: IGFs and their receptors, endothelial nitric oxide synthase (NOS-3), VEGF, myotrophin, angiotensin receptors (AT1R and AR2R), angiotensinogen, and angiotensin converting enzymes (ACEs).

MATERIALS AND METHODS

Experimental Design

RNA was extracted from the left ventricles taken from three groups of sheep fetuses from a previous study (23). These studies were approved by the University of Florida Institutional Animal Care and Use Committee. In that study, one group of ewes was treated with cortisol (1 mg·kg⁻¹·day⁻¹) between 115 and 130 days of gestation (high cortisol group), a second group of ewes was adrenalectomized and treated with cortisol (0.5 mg·kg⁻¹·day⁻¹) between 115 and 130...
were 7.0 plasma cortisol concentrations as previously reported (23) at 130 days of gestation. Although there was no overall effect of maternal of gestation and blood pressure was measured on -HSD1 and 2, The genes analyzed in this study were MR, GR, 11

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe Sequence</th>
</tr>
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<tr>
<td>IGF-II</td>
<td>CTGCCCTCTAGCCGCGTGCTTT</td>
<td>TGGCTTCAGAGGGTGTCAGATTGGG</td>
<td>TCAAGACATACCCCCTGGGCAAG</td>
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<td>AGAACAGGCTCGGCTTACTGACG</td>
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<td>GCGGCTACCTTTTGGAGGTCTTT</td>
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<td>TGGAGCTGGACGACACTT</td>
<td>CTTCTGCTTCTGCTTCATGTCAGGAC</td>
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<tr>
<td>Myotrophin</td>
<td>GGGAATGAGGCGACCATGTTGA</td>
<td>GGATCCTGCTCTGCTGATG</td>
<td>CTTGCTGATGAGAGATGCTGAG</td>
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<tr>
<td>11β-HSD1</td>
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<td>GGCTGCTGCTCTGCTGATG</td>
<td>ATGTGTCAATCACCCTCTGTATTCT</td>
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<td>GAGCTGCGCTGCTGCTGCTT</td>
<td>SYBR</td>
</tr>
<tr>
<td>ACE2</td>
<td>GAGGAGCCAGCCTCAGTATGGG</td>
<td>AGAAGATTTTTCATTGTGTCATGTCATC</td>
<td>SYBR</td>
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Nuclotide sequences are 5'-3'. 11β-HSD1, 11β-hydroxysteroid dehydrogenase.

days of gestation (low cortisol group), and a third group of normal ewes had no alterations of cortisol between 115 and 130 days of gestation (control group). The high cortisol treatment regimen produces circulating cortisol levels that are chronically elevated but are within the range of maternal cortisol levels measured with mild maternal stress. The treatment regimen 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 (23). 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 130 days 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 10-fold in the low cortisol group and were decreased by ~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. Furthermore, it is likely that the increase in plasma cortisol by 130 days in fetuses in the low cortisol group results 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 with 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 (23).

Real-time PCR

Total RNA was extracted (Trizol; Invitrogen, Carlsbad, CA) from 0.2 to 0.3 g of left ventricular free wall of fetal sheep in the control (n = 6), low cortisol (n = 4), and high cortisol groups (n = 5). All sheep were euthanized, and tissues were collected at ~130 (range, 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 DNA 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, AT1R and AT2R, and ACE1 and ACE2. For measurement of mRNA for MR and GR (26), IGF-I (37), NOS-3 (60), angiotensinogen (4), 11β-HSD2, AT1R and AT2R (10, 23), 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 (28, 35). Probes and primers used for IGF-1R, IGF-II, IGF-2R, ACE1 and -2, and 11β-HSD1 were designed using Primer Express 2.0 (Applied Biosystems, Table 1) based on ovine sequences in the NCBI database or previously published for other. For the IGF-1R probe/primer design, the ovis aries IGF-1R sequence (GenBank accession no. AY162434) was used; the amplified sequence corresponds to base pairs 319–380. The IGF-II ovine sequence (GenBank accession no. M97975) was used for IGF-II probe/primer design (base pairs 385–463) while the IGF-2R ovine sequence (GenBank accession no. 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 (GenBank accession no. AJ920032) between the base pairs 662–726, whereas ACE2 primers were designed using an ACE2 bovine sequence (GenBank accession no. BT021667) between the base pairs of 1245–1327. For both ACE1 and ACE2 SYBR Green (Bio-Rad, Hercules, CA) was used instead of probes. The primers and probe for 11β-HSD1 were designed using the ovine sequence published by Yang et al. (62) (base pairs 664–737 of published sequence). The VEGF probe and primers were designed from the sequence published by Cheung and Brace (6); 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 human RNA using primers designed from the published bovine sequence (GenBank accession no. 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. The sequence obtained was 94% homologous to the corre-

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

Table 2. Partial sequence of ovine myotrophin

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sponding bovine sequence (GenBank accession no. NM 203362.2; bp 321–674) and 90% homologous to the corresponding human sequence (GenBank accession no. 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). A 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 cycle number at which the threshold amplitude is achieved (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.

Radioimmunoassay

Fetal plasma angiotensin II levels were measured by radioimmunoassay after extraction of the peptide from plasma using previously described methods (40). 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 killed, 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-micrometer 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 peroxidase 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°C for 30 min.

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, Santa Cruz, CA) were used to localize MR, GR, 11β-HSD1 (47), and 11β-HSD2 (25) in the sections. Immunohistochemistry for MR was performed following the methods described by Gomez-Sanchez et al. (20) 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 1 h with 0.05 M Tris pH 7.6, 5% milk, 5% goat serum, and 0.2% SDS, followed by incubation with primary antibody in blocking solution for 1 h, and incubation with biotinylated secondary antibody (goat anti-rabbit; Zymed) for 1 h. As a tertiary agent, streptavidin-peroxidase (Zymed) was used. Metal-enhanced diaminobenzidine (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 Axiosplan 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 ANOVA using the ΔCt values. For graphical purposes, fold changes of the genes were calculated using the expression 2^ΔCt with respect to the control group (33). Comparisons of MR, GR 11β-HSD1, and 11β-HSD2 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 concentrations levels, fetal plasma cortisol concentrations levels, AT1R mRNA, AT2R mRNA, IGFB mRNA, IGFR mRNA, 11β-HSD1 mRNA, 11β-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

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 (Fig. 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).

Fig. 1. Expression of mRNA for mineralocorticoid receptor (MR), glucocorticoid receptor (GR), 11β-hydroxysteroid dehydrogenase-1 (11β-HSD1), and 11β-HSD2 in left ventricles from fetuses of the control (white bars), high (black bars), and low maternal cortisol (gray bars) groups. Fold changes of the genes were calculated using the expression 2^ΔCt with respect to the control group and are expressed as mean fold change ± SE. **P < 0.05 vs. control.
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 compared with the control and low cortisol groups (Fig. 1).

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 (Fig. 2).

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 compared with control and low maternal cortisol levels (Fig. 3).

Angiotensinogen, AT1R, AT2R, ACE1, and ACE2. We 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 (Fig. 4). However, we found that AT1R mRNA tended to decrease, whereas AT2R mRNA tended to be increased, in fetuses of ewes with increased cortisol compared with controls. There was a statistically significant increase in the ratio of AT2 to AT1 receptor expression in the high cortisol group (Fig. 4, top right).

Immunohistochemistry and Collagen Staining

MR, GR, and 11β-HSD1 staining was found in both cardiac blood vessels and myocytes in normal fetal hearts, whereas 11β-HSD2 showed very limited staining in myocytes and slightly more in blood vessels (Fig. 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 colocalize 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 compared with the angiotensin II levels in the control group, which appear to remain relatively constant between days 120 and 130 (Table 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 \( (r = 0.624, P < 0.02; \text{Fig. 6}) \). 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 130 days. There were also no correlations between left ventricular wall thickness and either MR.
(r = 0.113, P = 0.71) or GR expression (r = 0.065, P = 0.83) in the hearts. Negative relationships of left ventricular wall thickness with 11β-HSD1, AT1R, and IGF-1R that 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 = -0.0541, 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 ventricular wall 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, we found that both MR and GR are expressed in the fetal heart, as in adult myocytes in many species, including humans (34). We found that MR and 11β-HSD1 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. Our results further suggest that changes in the ratio of 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 11β-HSD1 and -2. Whereas 11β-HSD1 primarily converts cortisol into cortisone in most tissues (49), 11β-HSD2 converts cortisol into cortisone, which is inactive at MR and GR (38). Furthermore, action of 11β-HSD2 alters the 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 coex-
pressed with MR results in a “MR protective” effect that reduces basal MR activation by cortisol or corticosterone but permits aldosterone action at the MR receptors (38). 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 is 11β-HSD2 appreciably expressed in cardiomyocytes postnatally (3, 57).

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 (17, 19). Evidence for both MR- 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 (14, 16). In humans with severe heart failure, there is a reduction in severity of cardiac hypertrophy after blockade of the MR receptor (41). 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 (32). Interestingly, in 11β-HSD2 knockout mice, postnatal mortality is high, but surviving mice have enlarged hearts without evidence of cardiac fibrosis (27), suggesting that fibrosis only occurs in adult hearts, or when hearts have been subjected to ischemic damage. Overexpression of 11β-HSD2 in murine cardiomyocytes, however, results in cardiac hypertrophy, interstitial fibrosis, and heart failure (42). 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 11β-HSD2, 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 (18). 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 we cannot determine from these studies whether MR or GR is responsible for the observed effects, the relative levels of MR and 11β-HSDs 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 11β-HSD1 appears to predominate over 11β-HSD2 expression, particularly in the myocytes. In sheep as in other species, MR has greater affinity for cortisol than does GR (43), 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 (Fig. 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, our 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). We do not have any data regarding distribution of 11β-HSD2 in tissues from the treated fetuses, and so we cannot speculate on whether the decrease in 11β-HSD2 with maternal cortisol infusion altered myocyte or vascular expression of the protein.

Our 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, we reported that fetal blood pressure was not significantly elevated by the chronic maternal infusion of cortisol (23); fetal mean arterial pressure at 130 days 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 days 120 to 130 and left ventricle wall thickness. Studies in several animal models of hypertrophy, including

![Fig. 6. 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.](http://ajpregu.physiology.org/DownloadedFrom/)
plasma angiotensin per se stimulates the increase in wall saline-infused control group, it is not likely that the increase in response to the chronic cortisol infusion compared with the fetal plasma angiotensin II concentrations tend to increase in response to the chronic cortisol infusion compared with the fetal plasma angiotensin II concentrations tend to increase in fetal sheep stimulates left ventricular growth (50), in congenital heart enlargement. In adult hearts, angiotensin appears to cause cardiac hypertrophy, and pressure overload causes a ventricle-specific increase in myotrophin, as well as wall thickness. Treatment of cultures of neonatal myocytes with myotrophin increased the size of the myocytes and stimulated protein synthesis without increasing DNA synthesis. The absence of a change in myotrophin 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 (55), and increased IGF protein has been implicated in the increase in heart size in fetuses of undernourished ewes (11). 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 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 downregulation in skeletal muscle in fetal sheep (30, 31); glucocorticoids have also been shown to decrease IGF-2R in fetal rat osteoblasts in culture (45). 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 we can conclude that IGFs do not play any role in the enlargement.

Role of Growth-Related Genes: VEGF, NOS-3, Myotrophin, and IGFs

Several genes that might be expected to be related to growth were not found to be increased. We 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 (Fig. 2). Myotrophin was also not increased in the enlarged hearts (Fig. 2). Myotrophin has been suggested as a causative agent in cardiac hypertrophy in both humans and in rodents (1, 46, 53). In mice, overexpression 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 myocytes with myotrophin increased the size of the myocytes and stimulated protein synthesis without increasing DNA synthesis. The absence of a change in myotrophin in our study suggests, therefore, that the increased size of the fetal heart may result from hyperplasia rather than hypertrophy.

Role of the Renin-Angiotensin System

Our observation regarding the relative expression of AT2 to AT1 receptors in the fetal hearts suggests 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 (64); whereas infusion of angiotensin II in fetal sheep stimulates left ventricular growth (50), in cultures of ovine fetal cardiomyocytes, angiotensin II stimulates hyperplastic growth (56). Although our results indicate that fetal plasma angiotensin II concentrations tend to increase in response to the chronic cortisol infusion compared with 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 130 days (23). We 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 demonstrated that a more acute treatment with much larger doses of cortisol (~72 mg/day for 2–3 days) directly into the fetus late in gestation caused left ventricular hypertrophy and increased angiotensinogen gene expression in the heart (36). 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). Because dexamethasone treatment results in increased expression of angiotensinogen in cultured neonatal myocytes (13), 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 overexpressed in rat hearts (58) and induce cardiac arrhythmia, enlargement of the atria, and sudden death when overexpressed in mouse hearts (61). ACE2, however, is thought to be cardioprotective 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 (9). We observed no significant change in ACE1 or ACE2 expression between the cortisol groups, again suggesting local production of angiotensin II is not playing a major role in the enlargement.

In our studies, the magnitude of the increase in ventricular wall thickness was related to the relative expression of AT2 and AT1 receptors. Our finding that the AT2-to-AT1 receptor mRNA ratio increases also differs from that of Lumberts et al. (36) who found no significant change in expression in either of the receptors. In adult hearts, the hypertrophy caused by angiotensin II is thought to be mediated by AT1 receptors (64). It has been observed in human hearts that are failing that AT2 receptor expression increases or remains constant, whereas AT1 receptor expression decreases (51). The AT2 receptor is traditionally believed to have anti-AT1 receptor-mediated effects, whereas the AT1 receptor is known to be pro-growth (12). Other studies in adult hearts have indicated that an increase in the AT2-to-AT1 receptor ratio in the heart is associated with an antigrowth effect in response to cardiac hypertrophy (2). It is therefore possible that the rise in the AT2R-to-AT1R ratio is simply a chronic response that 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 (21), and in rats, there is no expression of AT2 receptors in myocytes at any age (52).

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 (5). 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 (51), 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 suggest 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. Our 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 range that will be observed changes in cardiac and vascular remodeling. Nephron Physiol 94: 47–50, 2003.

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