Prenatal betamethasone exposure results in pituitary-adrenal hyporesponsiveness in adult sheep

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Sloboda DM, Moss TJ, Li S, Doherty D, Nitsos I, Challis JR, Newnham JP. Prenatal betamethasone exposure results in pituitary-adrenal hyporesponsiveness in adult sheep. Am J Physiol Endocrinol Metab 292: E61–E70, 2007. First published August 1, 2006; doi:10.1152/ajpendo.00270.2006.—Fetal exposure to synthetic glucocorticoids in sheep results in increased fetal hypothalamic-pituitary-adrenal (HPA) axis activity persisting to one year of age. We aimed to determine the effects of single or repeated maternal or fetal betamethasone injections on offspring HPA activity at 2 and 3 yr of age and whether changes in adrenal mediators of steroidogenesis contribute to changes in pituitary-adrenal function. Pregnant ewes or their fetuses received either repeated intramuscular saline or betamethasone injections (0.5 mg/kg) at 104, 111, 118, and 124 days of gestation (dG) or a single betamethasone injection at 104 dG followed by saline at 111, 118, and 124 dG. Offspring were catheterized at 2 and 3 yr of age and given corticotrophin-releasing hormone + arginine vasopressin challenges. Adrenal tissue was collected for quantitative RT-PCR mRNA determination at 3.5 yr of age. In 2-yr-old offspring, maternal betamethasone injections did not alter basal ACTH or cortisol levels, but repeated injections elevated ACTH responses. At 3 yr of age, basal ACTH was elevated, and both basal and stimulated cortisol levels were suppressed by repeated maternal injections. Basal and stimulated cortisol-to-ACTH ratios and basal cortisol-to-cytochrome P-450c17 mRNA ratios were suppressed by repeated injections. Repeated fetal betamethasone injections attenuated basal ACTH and cortisol levels in offspring at 2 but not 3 yr of age. Plasma changes were not associated with altered adrenal P450c17, ACTH receptor, β-hydroxysestosterone dehydrogenase, or glucocorticoid receptor mRNA levels. These data suggest that maternal, but not fetal, betamethasone administration results in adrenal suppression in adulthood.

hypothalamic-pituitary-adrenal; glucocorticoid programming; adrenal; steroidogenic enzymes

A number of models of developmental programming exist that suggest that fetal adaptations to intrauterine influences can result in adverse health outcomes: one such model is fetal glucocorticoid exposure. A number of circumstances during intrauterine development can lead to fetal exposure to elevated circulating glucocorticoids. Maternal stress, resulting in an elevation of maternal glucocorticoid levels, has been associated with alterations in postnatal endocrine function in offspring as well as alterations in temperament and cognition (2, 3, 9, 16, 24, 41). Overexposure to either endogenous or exogenous glucocorticoids during fetal life “programs” a number of organ systems and increases the predisposition to several disease states in later life (8, 28, 42).

In the ovine fetus, low-dose dexamethasone infusion altered the basal set point of the hypothalamic-pituitary-adrenal (HPA) axis and enhanced fetal HPA axis responses to acute stress (7). In sheep, we have shown previously that maternal administration of synthetic glucocorticoids at levels sufficient to induce fetal lung maturation (12) reduced prenatal (36) and postnatal weight to 3 mo of age (22). Offspring demonstrated elevated insulin responses to a glucose load at 1 yr of age (22) and increased hepatic capacity for glucose production at 3 yr of age (35). We have also shown that maternal betamethasone injections significantly increased basal plasma ACTH, cortisol, and cortisol-binding capacity (CBC) in the fetus late in gestation (36) and increased HPA responsiveness at 1 yr of age (34). It is unknown, however, whether these HPA effects persist to adulthood. It is also unknown whether changes in HPA activity are mediated through betamethasone-induced changes in adrenal steroidogenesis.

We hypothesized in the present study that exogenous glucocorticoid (betamethasone) administration in the latter third of pregnancy would alter long-term HPA responsiveness in adult offspring. Furthermore, based on our previous work (26, 34), we reasoned that there would be differential effects of maternal and fetal administration on HPA responses. Direct fetal injection under ultrasound guidance is of interest as it may provide a possible clinical alternative to maternal intramuscular injections. We have shown previously that direct fetal injection in sheep does not carry the growth-restricting effects seen after maternal injections (26). Our pharmacokinetic studies have shown that the duration of fetal exposure to betamethasone is shorter following fetal injections (21). Furthermore, since the fetal sheep HPA axis undergoes progressive maturation over the last one-third of pregnancy (4), we reasoned that it is likely that single and repeated betamethasone injections during this developmental window would produce different outcomes. We therefore also investigated the effects of single vs. repeated betamethasone injections.

To address these issues, we have treated ewes or fetuses with betamethasone as a single intramuscular injection or with four repeated intramuscular injections at 1-wk intervals during the latter one-third of pregnancy. We allowed the ewes to deliver spontaneously and measured the responsiveness of the HPA axis, as reflected in plasma ACTH and cortisol concentrations.
after a challenge with corticotrophin-releasing hormone (CRH) + arginine vasopressin (AVP), in offspring at 2 and 3 yr of postnatal age. To investigate molecular changes associated with potential alterations in adrenal function, we determined mRNA levels of the adrenocorticotrophin receptor (ACTHr), the glucocorticoid receptor (GR), cytochrome P-450 17α-hydroxylase (P450c17), and 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) in the adrenals of these same animals at 3.5 yr of age.

MATERIALS AND METHODS

All experimental procedures were approved by the Animal Experimentation Ethics Committee of The University of Western Australia.

Prenatal and Postnatal Procedures

Prenatal interventions, pregnancy outcomes, and postnatal care of animals have been described in detail previously (13, 34, 36). Pregnant ewes bearing singleton fetuses were allocated randomly to receive either maternal or fetal injections of saline and/or betamethasone. All ewes were injected intramuscularly with 150 mg of medroxyprogesterone acetate (Depo Provera; Upjohn) at ~100 days gestation (dG) to reduce pregnancy losses due to betamethasone treatment, as described previously (22, 25, 34). Saline-treated groups were injected with normal saline at 104, 111, 118, and 125 dG; single-dose groups were injected with betamethasone at 104 dG and saline at 111, 118, and 125 dG; repeated-dose groups were injected with betamethasone at 104, 111, 118, and 125 dG. Maternal betamethasone (Celestone Chronodose; Schering Plough) injections were given intramuscularly in a dose of 0.5 mg/kg maternal body wt; saline injections were of a comparable volume (5–6 ml). Fetal intramuscular injections of betamethasone (0.5 mg/kg estimated fetal body wt) were given under ultrasound guidance using an established technique, as described previously (26).

Management of offspring from birth to 2 yr of age has been described previously (22, 34). Briefly, ewes delivered their lambs spontaneously in a field environment. At ~2 mo of age, lambs were immunized, their tails were cropped, and males were castrated. Weaning occurred at 3 mo of age. Offspring (of mixed sex) were grouped according to prenatal treatments as follows: maternal saline injection, M5 (male = 3, female = 2) or fetal saline injection, F5 (male = 1, female = 3); single maternal betamethasone injection, M1 (male = 3, female = 2) or single fetal betamethasone injection, F1 (male = 4, female = 3); four maternal betamethasone injections, M4 (male = 2, female = 2) or four fetal betamethasone injections, F4 (male = 2, female = 2). We have published previously the results of HPA experimentation on these offspring at 6 mo and 1 yr of age (34) and metabolic experimentation at 6 mo and 1 yr (22) and 2 and 3 yr (35). Before HPA experimentation at 2 and 3 yr of age, estrous cycles of female offspring were synchronized with intravaginal progesterone sponges (30 mg flugestone acetate; ChronoGest30) to eliminate any effect of differing estrous cycles. At ~3.5 yr of age, all offspring were weighed and painlessly killed (pentobarbitone overdose, Valabarb, Jurox) for the collection of tissues. Adrenals were recovered as quickly as possible, weighed, and snap-frozen in liquid N2 and stored at ~80°C until RNA extraction and gene analyses. Other organs were removed and weighed and were the subject of other investigations (23, 35).

CRH + AVP Challenges at 2 and 3 yr of Postnatal Age

Offspring underwent CRH + AVP challenges as described previously (34). Briefly, offspring underwent aseptic surgery to implant femoral arterial and venous catheters at 2 yr of age and carotid and jugular catheters at 3 yr (halothane anesthesia, 1–2% in O2, following induction with im ketamine and xylazine). Because these offspring underwent experimentation at 6 mo and 1 and 2 yr of postnatal age using femoral vessels (22, 34), these vessels were not used again at 3 yr of age. Offspring were allowed at least 3 days to recover from surgery before undergoing CRH + AVP challenges. Food was withdrawn 12 h before challenges, but the animals were allowed free access to water. Basal arterial blood samples (5 ml) were drawn at 30 (~30) and 15 (~15) min and immediately before (0) the administration of an intravenous bolus of ovine CRH (0.5 µg/kg body wt) and AVP (0.1 µg/kg body wt; Bachem) followed by a 10-ml saline flush. Arterial blood samples (5 ml) were collected at 5, 10, 20, 30, 60, 90, 120, and 180 min after CRH + AVP administration and centrifuged for 10 min at 4°C, and plasma was collected and stored at ~80°C. All challenges were administered between 0800 and 0900 to minimize the impact of circadian variability on measurements of plasma ACTH and cortisol. Catheters were removed after completion of experiments.

Measurement of Plasma ACTH, Cortisol, and Dehydroepiandrosterone Levels

Plasma immunoreactive ACTH concentrations were measured using a commercial RIA kit (Diasorin, Minneapolis, MN) previously validated for use in sheep (34). The intra-assay coefficient of variation was 5%, and the interassay coefficient of variation was 6%. The mean assay sensitivity was 15 pg/ml. The ACTH antibody cross-reacted <0.01% with α-melanocyte-stimulating hormone, β-melanocyte-stimulating hormone, β-endorphin, and β-lipotropin. Plasma cortisol concentrations were quantified using a commercial RIA kit (Diasorin) previously used in sheep (38). The cortisol antibody cross-reacted 100% with cortisol and <0.04% with corticosterone, aldosterone, cortisone, and progesterone. The intra-assay coefficient of variation was 6%. The interassay coefficient of variation was 11%. Basal plasma dehydroepiandrosterone (DHEA) levels were measured at only 3 yr of age using a commercial RIA kit (Diagnostic Systems Laboratories, Webster, TX). The antibody cross-reacted 100% with DHEA, 0.02% with DHEA sulfate, and 0.5% with androstenedione, 11-deoxycorticosterone, and androstosterone. All samples were run in a single assay, and the intra-assay coefficient of variation was <3%.

Molecular Analyses

RNA extraction and RT. Total adrenal RNA was extracted using the RNeasy Midi kit (QIAGEN, Clifton Hill, Victoria, Australia). Possible genomic DNA contamination was removed from each sample using a DNase treatment (Ambion, Austin, TX). Briefly, samples were incubated in 10× DNase I buffer and recombinant DNase I for 25 min at 37°C. Samples were eluted through microcolumns and then incubated with DNase Inactivation Reagent, centrifuged at 10,000 g, and stored at ~80°C. Total RNA (1 µg) was reverse transcribed in a 10-µl reaction containing 5× Moloney murine leukemia virus (MMLV) RT reaction buffer, 10 mM dNTP, 10 mM random hexamers, and 200 U/µl MMLV (H-RT) RT (Promega, Madison, WI). The RT reactions were carried out in a Peltier Thermal Cycler (MJ Research) at 22°C for 10 min, 55°C for 50 min, and 70°C for 15 min. Each sample of cDNA was purified using the QIAGEN PCR Purification Kit (QIAGEN) and stored at ~20°C.

Quantitative PCR assays of relative concentrations of adrenal ACTHr, GR, P450c17, and 11βHSD2 mRNA. For the quantification of adrenal ACTHr, GR, P450c17, and 11βHSD2 mRNA levels and the endogenous reference 18S ribosomal RNA (18S), a quantitative PCR assay was performed (Rotor-Gene 3000/Corbett Research, Sydney, Australia). For quantitative PCR, all primers were either designed using Primer 3.0 (for ACTHr accession no. AF116874; for 18S by 10.220.32.247 on June 27, 2017 http://ajpendo.physiology.org/ Downloaded from
Table 1. Primer sequences used in quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5'→3'</th>
<th>Product Size, bp</th>
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<td>ACTH Receptor</td>
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<td></td>
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<tr>
<td>Forward</td>
<td>ACATGGTTAATCCTGAGCC</td>
<td>152</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGATTTGATGATGATGCTGCA</td>
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</tr>
<tr>
<td>P450c17</td>
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<td></td>
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<tr>
<td>Forward</td>
<td>TGATGATGAGCCACCACTAG</td>
<td>298</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGAAGAGAGGCGTCGAGCTGAC</td>
<td></td>
</tr>
<tr>
<td>GR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>ACTGCCCCCAATGTAACAGA</td>
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</tr>
<tr>
<td>Reverse</td>
<td>GCCGCAATTCACAGCCTTAAC</td>
<td></td>
</tr>
<tr>
<td>11βHSD2</td>
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<td></td>
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<tr>
<td>Forward</td>
<td>ACGAGGGAGATCGCGGTTC</td>
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<tr>
<td>Reverse</td>
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<tr>
<td>Internal control 18S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>ATCCGGGAGATTGCAAATTTC</td>
<td>92</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTGTAGAAGAAGGGGGCACT</td>
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</table>

P450c17, cytochrome P-450 17α-hydroxylase; GR, glucocorticoid receptor; 11βHSD2, 11β-hydroxysteroid dehydrogenase type 2.

11βHSD2, and internal control 18S sets are presented in Table 1. PCR reactions were carried out in 25-μl volumes consisting of 10× IMMUNO Buffer (Bioline, Randolph, MA) 3 mM MgCl2 (Bioline), 10 mM dNTPs (Roche, Indianapolis, IN), 0.5 μM forward primer, 0.5 μM reverse primer, SYBR Green (1:2,000; Fisher Biotech), and 5 U/μL IMMOLASE DNA polymerase (Bioline). cDNA was amplified using the following cycling conditions: 95°C for 0 s, 61°C for 15 s, and 72°C for 10 s for 45 cycles. Melting-curve analysis demonstrated a single PCR product for adrenal ACTHr, P450c17, GR, and 11βHSD2, and internal control 18S, and this was confirmed by gel electrophoresis as the presence of a single band at the appropriate molecular weight (data not shown). All samples for each gene of interest were run in duplicate in a single assay. Where it was apparent that RNA was degraded, this sample was removed from the analysis. Intra-assay coefficients of variation were <5%.

A comparative cycle of threshold fluorescence (C<T>) method was used to assess relative gene expression levels, where a C<T> value reflects the cycle number at which DNA amplification is first detected. Equal amplification efficiencies were confirmed between the gene of interest and the internal control. One control sample within each assay for each gene was used as a calibrator. Thus comparative C<T> calculations for the expression of adrenal ACTHr, P450c17, GR, and 11βHSD2 were all relative to a calibrator. First, 18S C<T> values were subtracted from adrenal ACTHr, P450c17, GR, and 11βHSD2 mRNA values for each sample to give a ΔC<T> value. ΔΔC<T> values were achieved by subtracting the calibrator ΔC<T> value from each sample ΔC<T> value. The expression of adrenal ACTHr, P450c17, GR, and 11βHSD2 relative to the calibrator was evaluated using the expression 2<ΔΔC<T>>. This method of analysis has been previously published using sheep RNA (5, 20).

Statistical Analysis

Basal values of ACTH and cortisol were calculated from the means of three measurements collected before administration of CRH + AVP (−30, −15, and 0 min). ACTH and cortisol responses to the CRH + AVP challenge were summarized using areas under the ACTH or cortisol response curves (AUC), which were calculated using the trapezoidal rule. Maximal ACTH and cortisol levels were also determined. Group results are reported as means and SE. Analyses of basal, maximal, and the overall ACTH and cortisol responses (using AUC summaries) were performed using linear regression models with betamethasone treatments as fixed effects and individual sheep modeled as random effects. AUC summaries were log transformed (base e) to achieve data normality. Simultaneous adjustments for basal ACTH, cortisol levels, and birth weight were considered in all analyses. Time effects (year 2 vs. year 3) on the ACTH and cortisol responses to CRH + AVP challenge were evaluated with the introduction of an additional fixed factor. Although the effect of sex on the ACTH and cortisol basal and stimulated responses was not evaluated specifically because of small sample sizes, sex was controlled for in all statistical analyses. All mRNA analyses were performed using one-factor ANOVA. Molecular data that were not normally distributed were log transformed (base e) to achieve data normality. Linear regression analyses were performed to determine the relationship between birth weight and outcome variables such as cortisol responsiveness. Data analysis was carried out using SPSS (Chicago, IL), SAS (Cary, NC), or Sigma Stat (Jandel Scientific) statistical software. P values <0.05 were considered statistically significant.

RESULTS

Maternal Betamethasone Administration

Effects of maternal betamethasone injections on basal ACTH, cortisol, and DHEA levels. Basal ACTH and cortisol levels in adult offspring after maternal betamethasone administration are shown in Fig. 1. No significant effect of prenatal betamethasone was observed in basal ACTH and cortisol or cortisol-to-ACTH ratios at 2 yr of age (Fig. 1, A–C). Plasma ACTH levels in M4 offspring were significantly higher than those observed in saline controls (MS) at 3 yr of age (P = 0.034; Fig. 1D). At 3 yr, basal cortisol levels were significantly decreased in M4 offspring compared with MS controls (P < 0.05; Fig. 1E). Consistent with this, basal cortisol-to-ACTH ratios were significantly different between treatment groups at 3 yr of age (P = 0.045); M4 animals demonstrated the lowest cortisol-to-ACTH ratio (Fig. 1F). Basal plasma DHEA levels at 3 yr of age were elevated in M4 offspring compared with MS although differences were not statistically significant (Table 2). The basal DHEA-to-cortisol ratio, however, was significantly higher in the M4 offspring compared with both MS (P = 0.025) and M1 (P = 0.017) offspring (Table 2).

Effects of maternal betamethasone administration on adult ACTH and cortisol responses to CRH + AVP challenge. ACTH and cortisol responses to CRH + AVP challenge after maternal betamethasone administration are shown in Fig. 2. In all offspring, ACTH responsiveness to the CRH + AVP challenge was evident within 5–10 min postbolus and within 5–30 min postbolus for cortisol responses (Fig. 2). A significant treatment effect on ACTH responsiveness in offspring was observed at 2 yr of age (P < 0.05; Fig. 2A); M4 animals demonstrated the highest ACTH response to CRH + AVP challenge (Fig. 2A). Consistent with this, ACTH AUC was significantly higher in M4 compared with MS offspring (P < 0.05; Fig. 2A, inset). At 2 yr of age, maternal betamethasone administration did not affect either maximal or overall cortisol responsiveness (Fig. 2B). At 3 yr of age, ACTH responses to CRH + AVP were similar between groups (Fig. 2C). However, maternal betamethasone injection resulted in modestly reduced cortisol responses to challenge (P = 0.07; Fig. 2D), and cortisol AUC values were significantly lower in M4 offspring compared with MS offspring (P = 0.007; Fig. 2D, inset). Stimulated cortisol-to-ACTH ratios (represented by cortisol AUC-to-ACTH AUC ratio) were lowest in the M4...
offspring (Fig. 3) at both 2 and 3 yr of age, although these differences did not attain statistical significance (P < 0.20 at 2 yr and P < 0.10 at 3 yr).

Effects of maternal betamethasone administration on adult (3.5 yr of age) adrenal ACTHr, GR, P450c17, and 11βHSD2 relative mRNA concentrations. Maternal betamethasone administration did not significantly affect relative adrenal mRNA expression levels of ACTHr, GR, P450c17, and 11βHSD2 (data not shown). Differences in P450c17 mRNA levels approached significance (MS: 0.473 ± 0.25; M1: 1.057 ± 0.14; M4: 1.32 ± 0.29; P = 0.10). Basal cortisol-to-P450c17 mRNA ratios were significantly decreased in M4 offspring (P = 0.031; Fig. 4A), and stimulated cortisol-to-P450c17 levels were lowest in the M4 offspring but differences did not attain statistical significance (P = 0.13; Fig. 4B). Neither the ratio of basal DHEA-to-P450c17 mRNA nor the ratio of ACTH-to-ACTHr

Table 2. Basal DHEA levels and DHEA-to-cortisol ratios at 3 yr of postnatal age following prenatal maternal or fetal betamethasone administration

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>DHEA, pg/ml</th>
<th>DHEA-to-Cortisol Ratio</th>
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<tr>
<td>MS</td>
<td>184.6 ± 21.0</td>
<td>16.57 ± 3.6</td>
</tr>
<tr>
<td>M1</td>
<td>169.8 ± 27.0</td>
<td>11.05 ± 2.77*</td>
</tr>
<tr>
<td>M4</td>
<td>210.0 ± 15.0</td>
<td>34.93 ± 4.36†</td>
</tr>
<tr>
<td>FS</td>
<td>168.2 ± 12.1</td>
<td>6.21 ± 0.94</td>
</tr>
<tr>
<td>F1</td>
<td>250.3 ± 40.6</td>
<td>30.86 ± 16.59</td>
</tr>
<tr>
<td>F4</td>
<td>207.0 ± 48.0</td>
<td>22.66 ± 11.58</td>
</tr>
</tbody>
</table>

Values are presented as means ± SE. MS, maternal saline; M1, maternal single betamethasone injection; M4, maternal 4 betamethasone injections; FS, fetal saline; F1, fetal single betamethasone injection; F4, fetal 4 betamethasone injections. *P = 0.017 vs. M4; †P = 0.025 vs. MS.
mRNA was affected by maternal betamethasone treatment (data not shown).

**Fetal Betamethasone Administration**

**Effects of fetal betamethasone injections on basal ACTH, cortisol, and DHEA levels.** Basal ACTH and cortisol levels in offspring after fetal betamethasone administration are shown in Fig. 5. Basal ACTH levels at 2 yr of age were significantly lower in F4 offspring (Fig. 5A). Basal cortisol levels were highest in F1 offspring and were significantly different from F4 offspring (Fig. 5B). Basal cortisol-to-ACTH ratios at 2 yr of age were not different between groups (Fig. 5C). Basal ACTH and cortisol levels and cortisol-to-ACTH ratios were not different between groups at 3 yr of postnatal age (Fig. 5, D, E, and F). Basal plasma DHEA levels were highest in the F1 and F4 offspring compared with FS at 3 yr of age, but differences were not statistically significant (Table 2). Basal DHEA-to-cortisol ratios were not statistically different between groups (Table 2).

**Effects of fetal betamethasone administration on ACTH and cortisol responses to CRH + AVP challenge.** ACTH responsiveness was significantly different in offspring treated with fetal betamethasone at 2 yr of age; F1 and F4 offspring demonstrating the lowest responses to CRH + AVP challenge ($P < 0.05$; Fig. 6A). As such, ACTH AUC values were significantly lower in F1 and F4 offspring compared with saline controls ($P < 0.05$; Fig. 6A, inset). Maximal cortisol levels were different between groups ($P = 0.040$); F1 offspring had higher responses than both F4 ($P = 0.015$) and FS ($P = 0.008$; Fig. 6B) offspring. Cortisol AUC was significantly higher in F1 offspring vs. F4 ($P = 0.010$; Fig. 4; B), but not FS. Consistent with these observations, stimulated cortisol-to-ACTH ratios were significantly elevated in F1 offspring at 2 yr of age ($P = 0.045$ vs. FS; Fig. 7). ACTH and cortisol responses to challenge were similar between groups at 3 yr of age (Fig. 6, C and D) and stimulated cortisol-to-ACTH ratios at 3 yr of age were unaltered (Fig. 7).

**Effects of fetal betamethasone administration on adult (3.5 years of age) adrenal ACTH-r, GR, P450c17 and 11ßHSD2 relative mRNA concentrations.** Fetal betamethasone administration did not affect adrenal ACTHr, GR, P450c17, or 11ßHSD2 relative mRNA levels in offspring at 3.5 yr of age (data not shown). The ratio of basal or stimulated cortisol to P450c17 mRNA was not affected by prenatal treatment, nor were basal DHEA-to-P450c17 ratios (data not shown).

**DISCUSSION**

In the present study, we hypothesized that prenatal betamethasone exposure would result in long-term changes in adult HPA function, possibly because of the resetting of the fetal HPA axis. In our previous studies, we found that weekly prenatal betamethasone injections in sheep at 104, 111, 118, and 124 dG resulted in a $23\%$ decrease in fetal weight and increased levels of ACTH, cortisol, and CBC in cord plasma (36). We have also reported that maternal betamethasone injection in the offspring of the present study resulted in fetal growth restriction and decreased postnatal weight to 3 mo of postnatal age (22) but not thereafter (23). We have demonstrated that changes in fetal HPA axis activity were associated with elevated cortisol responses to a CRH + AVP challenge at 1 yr of postnatal age with normal ACTH responses, suggesting heightened adrenal sensitivity (34). In the present study, re-evaluation of HPA axis function in these same offspring has...
demonstrated that, at 2 yr of age, offspring exposed previously to maternally administered betamethasone had increased ACTH responses without changes in cortisol levels, suggesting an increased pituitary drive required to maintain cortisol levels similar to controls. This was reflected in a modest reduction in stimulated cortisol-to-ACTH ratios. Our new data indicate that, at 2 yr of age, offspring treated with repeated maternal injections of betamethasone demonstrated reduced adrenal ACTH sensitivity. This reduction in adrenal sensitivity was even greater at 3 yr of age. Basal ACTH levels were ~2-fold greater in M4 offspring and were associated with an ~2.5-fold decrease in basal cortisol levels and significant reductions in basal and stimulated cortisol-to-ACTH ratios. Furthermore, overall cortisol responsiveness to a CRH + AVP challenge was significantly reduced, as indicated by reduced cortisol AUC response values.

We have reported previously that maternal betamethasone administration in the latter one-third of pregnancy increases HPA activity in fetuses (36) and postnatal 1-yr-old sheep (34). These data combined with our current data suggest that the long-term effects of HPA programming evolve with postnatal age. Banjanin and colleagues (1) have also suggested that effects of HPA programming are not constant throughout postnatal life. Consistent with our observations, Liu et al. (18) have shown that prenatal dexamethasone exposure resulted in reduced basal and activated HPA function in young (~65 days) male guinea pig offspring and was associated with increases in hippocampal mineralocorticoid receptor expression. They also demonstrated that male offspring had significantly higher testosterone levels (18). In a follow-up study investigating older offspring (150 days), Banjanin et al. (1) reported no effect of prenatal dexamethasone treatment on basal or stimulated HPA function and no difference in testosterone levels. These studies combined with our own (34, 36) highlight the importance of investigating the effects of developmental programming serially throughout postnatal life rather than at a single cross-sectional time point.

Other models of developmental programming resulting in exposure of the fetus to elevated levels of glucocorticoids have demonstrated HPA programming in offspring. Increases in...
Basal cortisol concentrations were observed in the early postnatal period in rat pups (15, 39), lambs (33), guinea pigs (14), and goat kids (32) that were born from mothers stressed during pregnancy. Our current data extend those of previous reports later into adult life and suggest that the well-documented "programming" of elevated HPA activity may not be sustained indefinitely. Rather, we have shown the development of reduced adrenal sensitivity with age at least in offspring that were exposed repeatedly to maternal betamethasone injections. It appears, however, that a single dose of maternal betamethasone does not have significant long-term effects on adult pituitary-adrenal activity.

We observed increased ACTH levels at 2 and 3 yr of age in betamethasone-exposed offspring. It is unknown in the present study whether this apparent increased pituitary drive at 2 and 3 yr of age in maternal-exposed offspring is associated with changes in pituitary corticotroph function or gene expression levels. Studies in pigs have shown that prenatal exposure to elevated maternal glucocorticoids (via maternal ACTH injection) resulted in changes in hypothalamic and pituitary gene expression levels (11). Porcine offspring demonstrated increased CRH in the hypothalamus and increased proopiomelanocortin mRNA levels in the pituitary at 30 and 60 days of age. Exposed offspring also demonstrated an increased cortex-to-medulla ratio, suggesting alterations in adrenal cortical growth (11). Future studies investigating hypothalamic, pituitary, and even hippocampal mechanisms of HPA axis activity in our cohort are warranted.

Our data suggest that prenatal betamethasone does not have long-term effects on adrenal ACTHr, P450c17, 11βHSD2, or GR relative mRNA levels in adult offspring. Therefore, cortisol levels in M4 animals appear not to be limited by ACTHr or P450c17 levels or the result of increased intra-adrenal GR or 11βHSD2 levels. In fact, our observation that basal cortisol-

**Fig. 5.** Histograms representing basal ACTH and cortisol levels and basal cortisol-to-ACTH ratios in offspring at 2 (A–C) and 3 (D–F) yr of postnatal age after fetal betamethasone administration. Groups are as follows: fetal saline (FS), single fetal betamethasone injection (F1), and four fetal betamethasone injections (F4). Values are presented as means ± SE. *P < 0.05 vs. FS, †P < 0.05, F4 vs. F1.
to-P450c17 ratios were decreased suggests that P450c17 levels are likely abundant. The fact that DHEA-to-cortisol ratios were elevated in M4 offspring suggests that changes in other important steroidogenic enzymes in the cortisol biosynthetic pathway (such as 3β-hydroxysteroid dehydrogenase) may divert synthesis away from glucocorticoids (cortisol) to the Δ5 steroidogenic pathway (DHEA, androstenediol). Other enzymes may also participate in the regulation of adrenal cortisol levels. Recent data suggest that adrenal 5α-reductase may play a key role in the inactivation of glucocorticoids, mineralocorticoids, and progesterone in mice (19). Investigation into other steps in the steroidogenic pathway is therefore warranted.

We have shown previously that repeated maternal betamethasone injections resulted in a significant increase in cord plasma CBC and hepatic corticosteroid binding globulin (CBG) levels (37). Our current data describing reductions in basal cortisol and cortisol-to-ACTH ratios are not likely the result of changes in cortisol clearance due to alterations in either CBC or CBG because, as we have reported previously, hepatic CBG levels are not different in these offspring (35). It seems likely that our observed effects are due to changes at the level of the adrenal.

In this study, we did not differentiate between different adrenal cortical regions; therefore, it is possible that the long-term effects of maternal betamethasone administration may target the development of cortical fasciculata and reticularis differentially. This speculation can be supported by our observation that DHEA levels are elevated in M4 offspring. It is unknown in our present study whether sympathoadrenal activity was altered in betamethasone-exposed offspring. Low birth weight has been associated with significant changes in sympathetic-adrenomedullary system activity and behavior in school-aged children (6). It is possible that prenatal exposure to betamethasone alters long-term sympathoadrenal activity in offspring, and further studies are needed to investigate this possibility.

Low birth weight is associated with elevated HPA axis function in humans. Studies in humans have shown that cord blood levels of ACTH and cortisol are increased in association with reduced fetal growth (10, 17, 30, 31). We have reported previously that birth weight and postnatal weight at 3 mo of age were reduced in the M1 and M4 offspring of the current study (22). Our current and previous data on HPA function raise the possibility that low birth weight may be associated initially with increased cortisol levels and subsequently with reduced cortisol levels in adulthood. Phillips et al. (29) have shown that fasting plasma cortisol levels in men were inversely related to birth weight and that elevated cortisol levels were significantly associated with higher blood pressure, plasma glucose levels, fasting triglyceride levels, and insulin resistance. More recent follow-up of low-birth-weight men by Phillips and colleagues has shown conflicting findings. Ward et al. (40) have found no significant group differences between the ACTH and cortisol response to CRH or baseline ACTH and cortisol levels after dexamethasone in a cohort of high-birth-weight (>3.86 kg) and low-birth-weight (<3.18 kg) men. Interestingly, however, they found that low-birth-weight men had significantly lower ACTH and cortisol responses to CRH testing after dexamethasone and reductions in peak and increment responses that bordered on being significant (40). The
authors suggested that these data reflect an increased pituitary sensitivity to dexamethasone and resemble alterations in HPA activity associated with pathological syndromes demonstrating low basal cortisol levels such as chronic fatigue syndrome (CFS). Although there are no current data describing relationships between low birth weight, prenatal glucocorticoid exposure, and CFS, these data suggest that further investigations are warranted.

Developmental programming by glucocorticoids is influenced by the route of administration. We have demonstrated in the current study as well as previously that the long-term outcomes of injecting betamethasone in the mother or fetus are different. Maternal intramuscular betamethasone injection resulted in a dose-dependent decrease in birth weight, whereas fetal intramuscular injections of betamethasone did not (22, 27). Furthermore, we have demonstrated that maternal intramuscular injection of betamethasone significantly altered postnatal cortisol responsiveness in offspring at 1 yr of age, but direct fetal betamethasone injection did not (34). Fetal circulating levels of betamethasone after maternal injection differ from those observed after fetal injection. Cumulative exposure of the fetus to betamethasone was higher after fetal injection than after maternal injection, but the length of exposure was shorter after fetal injection (21). This observation, in effect, provides us with two models of glucocorticoid exposure with markedly different exposure times and the opportunity to evaluate long-term HPA axis activity accordingly.

We have confirmed in the present study our previous observations of the differential effects of maternal vs. fetal prenatal betamethasone administration on postnatal HPA responsiveness. At 2 yr of postnatal age, offspring treated prenatally with one fetal intramuscular injection of betamethasone had the lowest ACTH and the highest cortisol responses to a challenge of CRH + AVP. Interestingly, this response was no longer apparent at 3 yr of age. We have previously demonstrated this diminished ACTH response and a modestly heightened cortisol response in these same offspring at 1 yr of age (34), possibly reflecting increased adrenal sensitivity to ACTH. Why these animals do not show circulating changes in ACTH and cortisol at 3 yr is unknown. Because this study ended at 3.5 yr postnatal age, it is unknown whether the fetally treated offspring would have gone on to develop relative adrenal hyporesponsiveness as the maternally exposed offspring had by 3 yr. Our data do suggest, however, that both maternal and fetal administration of prenatal betamethasone have long-term effects on ACTH and cortisol responsiveness to CRH + AVP, albeit following different postnatal developmental time frames. The mechanisms regulating these differential effects are unknown, and the effects of maternal betamethasone injection on the development and function of the placenta are unclear. Further investigations into the effects of maternal betamethasone injection on placental growth are currently being completed.

In conclusion, the findings from our investigations of HPA activity in these animals (34, 36) suggest that repeated maternal betamethasone injection results in persistent long-term changes in HPA axis activity and function in offspring. It appears that effects on postnatal HPA function evolve from a heightened adrenal sensitivity early in life (34, 36), to a transitional phase where adrenal sensitivity begins to decrease (2 yr), to a final stage of decreased adrenal sensitivity (3 yr). It appears from our observations that altered adrenal cortisol output is not associated with significant changes in the expression of ACTHr, GR, P450c17, or 11βHSD2 but may be associated with changes in other steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase. The clinical implications of a reduced basal cortisol and blunted response to a stressor remain uncertain, although the widespread use of maternal glucocorticoids and the possibility of adrenal insufficiency in adult life necessitate further investigation.

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