Glucocorticoid exposure in late gestation in the rat permanently programs gender-specific differences in adult cardiovascular and metabolic physiology

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O’Regan, D., C. J. Kenyon, J. R. Seckl, and M. C. Holmes. Glucocorticoid exposure in late gestation in the rat permanently programs gender-specific differences in adult cardiovascular and metabolic physiology. Am J Physiol Endocrinol Metab 287: E863–E870, 2004.—Glucocorticoid overexposure in utero may underlie the association between low birth weight and subsequent development of common cardiovascular and metabolic pathologies. Previously, we have shown that prenatal dexamethasone (DEX) exposure in rat reduces birth weight and programs the hypothalamic-pituitary axis and fasting and postprandial hyperglycemia in adult males and hypertension in adult males and females. This study aimed to determine (1) whether there were gender differences in prenatal DEX-programmed offspring, and (2) whether the renin-angiotensin system (RAS) plays a role in the programming of hypertension. Rats exposed to DEX in utero (100 μg·kg⁻¹·day⁻¹ from embryonic days 14–21) were of lower birth weight (by 12%, P < 0.01) and displayed full catch-up growth within the first month of postnatal life. DEX-treated male offspring in adulthood selectively displayed elevated plasma adrenocorticotropic hormone (by 221%) and corticosterone (by 188%, P < 0.05), postprandial insulin-glucose ratios (by 100%, P < 0.05), and hepatic expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (by 38%, P < 0.05). Conversely, DEX-programmed females were hypertensive (by 11%, P < 0.05), with elevated hepatic angiotensinogen mRNA expression (by 9%, P < 0.05), plasma angiotensinogen (by 61%, P < 0.05), and renin activity (by 88%, P < 0.05). These findings demonstrate that prenatal glucocorticoids program adulthood cardiovascular and metabolic physiology in a gender-specific pattern, and that an activated RAS may in part underlie the hypertension associated with prenatal DEX programming.

glucocorticoids; birth weight; hypothalamic-pituitary axis; glucose homeostasis; blood pressure; renin-angiotensin system

Epidemiological studies in many human populations suggest that the common metabolic and cardiovascular disorders of adult life are influenced by intrauterine factors (7, 19). In particular, low birth weight (assumed to be a marker for an adverse intrauterine environment) is associated with a higher frequency of hypertension (5), type 2 diabetes (42), and death from ischemic heart disease in adulthood (7, 19, 42). The phenomenon of prenatal “programming” has been advanced to explain these findings whereby a factor, acting during a discrete developmental “window,” alters the maturation of specific organs, permanently altering their function (6, 10). The systems affected are determined by their particular vulnerability at the time of exposure (54). Although the precise mechanisms underpinning prenatal programming remain elusive, we hypothesized that excessive fetal exposure to glucocorticoids might be important on the basis of the documented effects of antenatal glucocorticoid administration to reduce birth weight and alter the trajectory of maturation of specific fetal organs (24). Glucocorticoid excess in adults or children causes many of the features of the “small baby syndrome.” In rats, we and others have shown that prenatal exposure to the synthetic glucocorticoid dexamethasone (DEX) or to endogenous maternal glucocorticoids by inhibition of the fetoplacental glucocorticoid “barrier enzyme” 11β-hydroxysteroid dehydrogenase type 2 reduces birth weight and produces permanently impaired glucose tolerance and hypertension in the adult offspring. These metabolic effects are associated with increased hypothalamic-pituitary-adrenal (HPA) axis activity and abnormal behavior reminiscent of anxiety (10, 29, 35–37, 58, 64, 65). The programming of glucose-insulin dyshomeostasis only occurs when glucocorticoids are administered in the final week of gestation (47), whereas the programming of hypertension is less time constrained (10, 35). Similar findings have been reported with DEX in sheep (10, 20, 28, 35) and guinea pig (30, 52), although the particular windows of vulnerability appear species specific.

In terms of mechanism, although prenatal DEX-programmed glucose intolerance is associated with permanently increased hepatic expression of a key gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PCK) (47), the basis of DEX programming of hypertension remains unknown, although some data in sheep have implicated the renin-angiotensin-aldosterone system (RAS) (21, 46). Importantly, in adult rats, glucocorticoids regulate all of the principle components of the RAS, including renin secretion (17), angiotensinogen synthesis (16), ANG-converting enzyme activity (43), ANG II (56), and mineralocorticoid receptor expression (55). Furthermore, in the fetal rat, hepatic angiotensinogen mRNA levels are regulated by glucocorticoids (26).

An additional complexity relates to gender. In several paradigms, antenatal manipulations that permanently alter adult HPA axis function are sex specific (31, 39, 61, 62), and the importance of gender in determining programmed outcomes has recently been suggested in both humans (1) and rodents (32, 49). Moreover, there is no reason a priori to assume that if similar programmed phenotypes are obtained that identical mechanisms underlie them. Indeed, early- and late-gestation DEX and 11β-hydroxysteroid dehydrogenase inhibition all produce HPA axis hyperactivity but involve distinct central...
mechanisms (63–65). However, little work has been done on the effects of antenatal steroids and peripheral programming phenotypes. DEX-programmed hypertension occurs in both sexes but may reflect distinct processes in each gender, whereas hyperglycemia/hyperinsulinemia has been shown only in male rats. In this study, we aimed to determine 1) whether there are gender differences in prenatal DEX-programmed offspring in terms of glucose intolerance and hypertension and 2) whether the prenatal programming of adult blood pressure by glucocorticoids is mediated by alterations in the RAS.

**MATERIALS AND METHODS**

**Animals.** Adult Wistar rats (200–250 g; Harlan, Bicester, UK) were maintained under conditions of controlled lighting (lights on 0700 to 1900; 24-h clock) and temperature (22°C) and allowed ad libitum access to food (standard rat chow; 61.9% carbohydrate, 18.8% protein, 3.4% oil, 0.6% NaCl; SDS, Wiltham, UK) and tap water. All experiments were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986.

**Prenatal treatments.** Virgin female rats were housed with adult male rats. Pregnancy was confirmed by the presence of a vaginal plug, checked every morning. Pregnant females were housed singly and randomly assigned to one of two treatment groups (n = 9–10/group). DEX (100 μg·kg⁻¹·day⁻¹, dissolved in 4% ethanol-0.9% saline, 200 μg·ml⁻¹) was added to the drinking water of the pregnant females from 200 days of age up to day 15 of gestation to induce DEX-programmed hypertension and 20-μg aliquots were separated on a 1.2% agarose gel containing 2% formaldehyde. RNA was blotted onto a nylon membrane (Zeta-PROBE GT; Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) and prehybridized for 2 h at 55°C in 10× standard saline phosphate buffer, 20% SDS, and 1 mg of denatured salmon testis DNA. Hybridization was carried out overnight at 55°C in an identical solution containing rat angiotensinogen cDNA, labeled with [32P]dCTP using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). After hybridization, the membrane was rinsed with 1× SSC, 0.1% SDS, followed by two washes in 0.3× SSC, 0.2% SDS, at 55°C. Membranes were rehybridized with U1 cDNA in the same manner to control for RNA loading and transfer. The level of hybridized probe was quantified using a Fugi FLA2000 phosphorimage analyzer. Values shown are normalized to U1 and expressed in arbitrary units.

**Real-time PCR.** Total RNA from hypothalamic and renal tissues was prepared using the TRIzol method. Five hundred nanograms were reverse transcribed (Reverse Transcription System; Promega, Southamption, UK) by incubation at 42°C for 50 min, followed by enzyme inactivation for 5 min at 99°C. Samples of cDNA were placed on ice, diluted 1:4 with nuclease-free water, and stored in aliquots at −20°C.

**TaqMan real-time PCR primers and probes (Table 1) were designed using the Primer Express Software (PE Applied Biosystems, Foster City, CA). A primer/probe mix for angiotensinogen was created, consisting of 25 μM forward primer, 25 μM reverse primer, 5 μM probe, and nuclease-free water. Equal volumes of this mix and a cyclophilin primer/probe mix (internal control) were added to TaqMan PCR core reagent, forming a master mix. Each sample was assayed in triplicate. Serial 1:10 dilutions of stock cDNA were added to the master mix to create a standard curve. No-template negative controls were constructed by substitution of nuclease-free water for cDNA. Negative RT controls were further added to the cDNA plate. One-in-four dilution of the stock cDNA acted as quality control. Plates were analyzed on a TaqMan ABI Prism 7700 Sequence Detector. Cycling parameters were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Data acquisition was processed with Sequence Detector 1.6.3 software.

**In situ hybridization for hepatic glucocorticoid receptor.** Ten-micrometer sections of liver from females aged 6 mo were thawed-mounted on gelatin/poly-l-lysine-coated slides and kept at −80°C. In situ hybridization, using [35S]-uridine 5′-triphosphate-labeled cRNA antisense probes for glucocorticoid receptor (GR), was performed as previously described (35). After hybridization and high-stringency washes, slides were dehydrated, dipped in NTB-2 in situ (Eastman, Kodak, Rochester, NY), and exposed for 3 wk. The mRNA expression was quantified by counting silver grains over individual hepatocytes under bright-field conditions using a computerized image analysis system.

**Oral glucose tolerance test.** Offspring underwent an oral glucose tolerance test at 6 mo. Animals were fasted overnight, and testing commenced between 0830 and 0900 the following morning. An oral glucose load of 2 g/kg was given by gavage, and blood samples were collected at 0, 30, and 120 min. Glucose was determined by the enzymatic (hexokinase) method using a kit (Sigma, Poole, UK). Inter- and intra-assay variations were <2%. Insulin was measured by ELISA (UltraSensitive Rat Insulin ELISA kit; Crystal Chem, Chicago, IL). Inter- and intra-assay variations were <10%.

**PEPCK enzyme activity.** At weaning (postnatal day 21), fed animals were killed by decapitation between 0900 and 1000, and livers were removed immediately. PEPCK activity was measured as previously described (47).
system (Carl Zeiss, Welwyn Garden City, UK). The analysis was carried out blind to the prenatal treatment. For each animal, 20 hepatocytes were assessed. Results were calculated as mean number of grains per hepatocyte after background counted over nonspecific areas was subtracted.

Statistical analyses. All data are expressed as means ± SE. Data were compared using one-way or multiple ANOVA followed by a least significant difference post hoc multiple comparisons test or unpaired Student's t-tests, where appropriate. Values were considered significant when P < 0.05. For Pearson correlation analyses, to standardize results for the effects of gender, individual values for each variable were subtracted from the mean of that gender and then divided by the standard deviation of that gender.

RESULTS

Gestational weight gain, birth phenotype, and catch-up growth. DEX administration throughout the final week of gestation resulted in a significant reduction in maternal weight gain (P < 0.05; Table 2). There were no differences in the length of gestation, litter size, ratio of male to female pups born, or pup viability. As previously reported, DEX treatment caused a significant reduction in the birth weight of offspring, to the same degree in both male and female pups (P < 0.05; Table 2). At weaning (postnatal day 21) and throughout adulthood, body weights of male and female offspring treated with DEX or vehicle were similar, i.e., full catch-up growth had occurred (Table 3).

HPA activity in adult offspring. In male offspring, prenatal DEX exposure resulted in significant increases in basal morning (0800) plasma ACTH and corticosterone concentrations (P < 0.05), whereas levels at the evening diurnal peak (2000) were similar to controls (Fig. 1). These hormonal profiles were not reflected in female offspring treated with DEX, with no differences observed at either diurnal nadir or peak (Fig. 1). Two-way ANOVA for treatment (vehicle/DEX) and gender showed a significant effect of gender: F(1,32) = 13.72 (P = 0.0007) for ACTH, and F(1,32) = 49.22 (P = 0.0001) for corticosterone and nonsignificant effects of treatment or interaction of the two parameters. Overall, female offspring displayed significantly higher basal and peak ACTH and corticosterone concentrations than males (P < 0.05).

Prenatal DEX, glucose homeostasis, and PEPCK activity. Male offspring of dams treated with DEX throughout the last week of pregnancy had permanent reactive hyperglycemia and hyperinsulinemia 120 min after an oral glucose load (P < 0.05 compared with vehicle control at that time point; Fig. 2, A–C). In contrast, glucose homeostasis in female offspring treated with DEX in utero did not differ from controls at any time point pre- or postoral glucose load (Fig. 2, A–C). Fasting glucose and insulin concentrations did not differ between treatment groups or sexes. Three-way ANOVA of the glucose-insulin ratio data as a whole for treatment, gender, and time showed a significant effect of gender [F(1,75) = 77.99, P < 0.0001] and time [F(1,75) = 21.54, P < 0.0001] and a significant interaction between treatment and time (P = 0.026), gender and time (P = 0.003), and treatment, gender, and time (P = 0.0093). This analysis confirmed that the effects of prenatal treatment on glucose homeostasis are gender specific. Consistent with previous observations (47), prenatally DEX-exposed male offspring also had significantly elevated hepatic PEPCK activity (P < 0.05), whereas no differences in enzyme activity were observed in females treated with vehicle or DEX in utero (Fig. 2D). Two-way ANOVA showed no significant effect of gender or treatment alone but a significant interaction between the two [F(1,20) = 68.86, P = 0.0017].

Systolic blood pressure. As shown in Fig. 3, only female offspring in the prenatal DEX group showed elevated systolic blood pressure above vehicle controls when measured at 6 mo (P < 0.05). In this cohort of male DEX-treated animals, systolic blood pressure was not elevated. Two-way ANOVA for treatment and gender showed a significant effect of treatment [F(1,44) = 8.564, P = 0.0054], no effect of gender, and a tendency for an interaction [F(1,44) = 3.616, P = 0.063].

Angiotensinogen expression and renin activity. Prenatal DEX-treated female offspring displayed a significant increase

Table 1. Taqman real-time PCR primers and probes

<table>
<thead>
<tr>
<th>Sequence (5’–3’)</th>
<th>Position</th>
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<td>Forward primer</td>
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<td>818–840</td>
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<td>Reverse primer</td>
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<tr>
<td>Probe</td>
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<td>Cyclophilin</td>
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<td>Forward primer</td>
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<tr>
<td>Reverse primer</td>
<td>aagttttctgccctgttggaact</td>
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<tr>
<td>Probe</td>
<td>caagggctegcactcagcctg</td>
<td>73–93</td>
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See materials and methods for details.

Table 2. Maternal weight, gestation length, litter size, male-to-female ratio, and birth weight

<table>
<thead>
<tr>
<th>Maternal Wt, g</th>
<th>Gestation Length, days</th>
<th>Litter Size, no.</th>
<th>Male-to-Female Ratio</th>
<th>Birth Wt, g</th>
</tr>
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<td>Before</td>
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<td>Gain</td>
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<td>Female</td>
</tr>
<tr>
<td>Vehicle</td>
<td>372±14 (n=8)</td>
<td>466±9</td>
<td>94±5</td>
<td>22±0</td>
</tr>
<tr>
<td>DEX</td>
<td>373±6 (n=9)</td>
<td>428±2.5*</td>
<td>55±4*</td>
<td>22±0</td>
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</table>

Results are means ± SE. Values in parentheses are nos. of rats/group. Maternal weight (wt) before treatment, maternal wt gain during the last week of pregnancy (dass 14–21), gestation length, litter size, male-to-female ratio, and group, male, and female offspring birth wt. DEX, dexamethasone. *P < 0.05 and †P < 0.01 compared with vehicle group.
Correlation analysis of blood pressure and plasma parameters. Analyses of the effects of DEX programming on blood pressure and the renin-ANG system are complicated by marked sex differences in blood pressure and plasma angiotensinogen. However, when male and female values were pooled after standardization, DEX offspring had statistically higher blood pressures ($P < 0.001$) and plasma renin activity (PRA) values ($P = 0.005$) than vehicle offspring but similar plasma angiotensinogen values ($P < 0.34$). Moreover, standardized blood pressure and PRA values ($r = 0.58, P = 0.002$), but not blood pressure and angiotensinogen ($r = 0.26, P = 0.2$), were correlated. The lack of an overall influence, albeit indirect, of angiotensinogen on blood pressure was surprising, given that pooled male and female standardized PRA and angiotensinogen values were correlated ($r = 0.5, P = 0.009$). One possible explanation is that the role of angiotensinogen in determining renin activity and blood pressure differs between males and females. This is supported by separate analysis of male and female data. In females, plasma angiotensinogen correlated with PRA ($r = 0.75, P = 0.005$) and blood pressure ($r = 0.64, P = 0.024$) and was significantly increased in DEX offspring compared with vehicle (Fig. 3). Although PRA correlated with blood pressure in males ($r = 0.67, P = 0.009$), plasma angiotensinogen values did not correlate with either blood pressure or PRA and were not increased in DEX-programmed rats.

**Effect of prenatal DEX on hepatic GR expression in female offspring.** Consistent with previous findings in DEX-treated male offspring (47), GR mRNA was significantly increased in the liver of DEX-treated female offspring (vehicle $158 \pm 51$ grains/cell area, DEX $317 \pm 44$ grains/cell area; $n = 5$ group, Student’s $t$-test, $P < 0.05$).

**DISCUSSION**

We have shown that, although DEX administration in the last week of gestation reduces birth weight to a similar degree in male and female rat littermate offspring, adult cardiovascular and metabolic physiology is programmed in a sex-specific
manner. In male offspring, prenatal glucocorticoid exposure programs elevate hepatic PEPCK activity and produce post-glucose hyperglycemia and hyperinsulinemia in adulthood. In contrast, prenatal DEX programs elevate hepatic angiotensinogen mRNA expression, elevate plasma angiotensinogen and renin activity, and produce hypertension in female offspring.

Birth weight reduction and postnatal catch-up growth were observed in both males and females in this study, whereas the adult “programmed” phenotypes differed considerably. This reinforces the current notion that birth weight is a marker of exposure to an adverse (late) gestational environment, rather than a specific cause of pathophysiology in the offspring. Below we discuss particular aspects of the adult phenotype.

**HPA axis.** As with some other antenatal challenges (stress, alcohol, and so forth), antenatal DEX exerts an effect on the adult HPA axis. In males, DEX increased morning basal ACTH and corticosterone levels, findings in agreement with earlier studies, which further showed decreased levels of the glucocorticoid and mineralocorticoid receptors in the hippocampus, suggesting decreased HPA axis feedback sensitivity (35, 65). In females, no differences in corticosterone or ACTH levels were observed between DEX- and vehicle-treated controls. However, basal values in both groups were 10- to 15-fold higher than male values, which, undoubtedly, would obscure any subtle programming effect. There are two likely reasons

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Fig. 2. Plasma glucose (A), insulin (B), and insulin-to-glucose ratios (C) in male and female adult offspring exposed to vehicle or DEX during the final week of pregnancy (embryonic days 14–21). After an overnight fast, offspring underwent an oral glucose tolerance test. Plasma glucose and insulin were measured at 0, 30, and 120 min after an oral glucose load (2 g/kg). D: activity of hepatic phosphoenolpyruvate carboxykinase (PEPCK), assayed at weaning (postnatal day 21), in fed male and female offspring treated in utero with vehicle or DEX during the 3rd wk of gestation. Results are means ± SE; n = 8/group. *P < 0.05 compared with male vehicle group.

Fig. 3. Systolic blood pressure (BP) measured by tail cuff plethysmography in adult male and female offspring exposed to vehicle or DEX throughout the final week of intrauterine life (embryonic days 14–21). Results are means ± SE; n = 8/group. *P < 0.05 compared with female vehicle group.

Fig. 4. Hepatic expression of angiotensinogen mRNA in adult male (A) and female (B) offspring treated with vehicle or DEX during the last week of gestation (embryonic days 14–21). Data are from Northern blots, graphically represented in arbitrary units (AU), of angiotensinogen mRNA in adult rat liver. Values shown are normalized to U1 used as loading control. Results are means ± SE; n = 6/group. *P < 0.05 compared with vehicle group.
why female values were so much higher than males. First, it is well known that circulating corticosterone levels and adrenal gland size in female rodents are greater than in males (3). Second, females are known to be much more reactive than males to any stressor (45). In the present study, female rats showed obvious stress levels of both corticosterone and ACTH despite precautions taken to obtain “basal” plasma hormone measurements (handled for 2 wk before blood sampling, housed individually to prevent disturbance stress, and blood sampling completed within 2 min of disturbing cage) identical to those for male rats. Other antenatal programming paradigms have been shown to affect the HPA axis in female rats to a variable extent (2, 50, 62). Although DEX did not appear to program the HPA axis in females in the present study, it is significant that expression of hepatic GR mRNA is increased by DEX as it is in males (47).

Glucose-insulin homeostasis. In agreement with previous observations (47, 48), DEX-treated male offspring were hyperglycemic after an oral glucose load, with features suggestive of insulin resistance (higher 120-min insulin-to-glucose ratio). This effect was not seen in female littersmates, and it is significant that both DEX- and vehicle-treated control males were markedly more insulin resistant than females. Because corticosterone is known to inhibit glucose-stimulated insulin release from the pancreas (8, 14), it is tempting to speculate that raised plasma corticosterone in females accounts for gender-specific differences in glucose tolerance tests. Similarly, gender-specific programming of insulin resistance has been observed in offspring of protein-restricted dams (48, 57), again with evidence that males are more insulin resistant than females. This parallel between the two antenatal programming models is perhaps not surprising given that glucocorticoid-mediated activation of the sympathetic nervous system (12, 13) also contributes to the elevation of plasma renin activity (60) leading to hypertension (33). Similarly, mutations in the angiotensinogen gene are associated with hypertension in humans (18, 41, 53). It has been suggested that angiotensinogen is not merely a passive substrate reservoir but also stabilizes plasma renin concentrations (by decreasing its metabolic clearance) (15). Thus the higher hepatic angiotensinogen mRNA levels and consequent elevation in plasma angiotensinogen concentrations causes PRA in females to be increased, which in turn may drive hypertension.

Whether programmed activation of the sympathetic nervous system (12, 13) also contributes to the elevation of plasma renin is not known. In contrast, prenatally DEX-treated males showed reduced hepatic angiotensinogen mRNA levels, unal-

<table>
<thead>
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<th>Plasma Parameter</th>
<th>Male</th>
<th>DEX</th>
<th>Female</th>
<th>DEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renin activity, ng·h⁻¹·ml⁻¹</td>
<td>1.205±0.129</td>
<td>1.494±0.169</td>
<td>792.8±86</td>
<td>1.493±28.8*</td>
</tr>
<tr>
<td>Angiotensinogen, pg/ml</td>
<td>1,686±243</td>
<td>1,801±61</td>
<td>482±89</td>
<td>773±103*</td>
</tr>
<tr>
<td>Estradiol, pg/ml</td>
<td>18±4</td>
<td>20±2</td>
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</table>

Results are means ± SE; n = 6–8/group. Measurements were made in ad libitum-fed 6-mo-old male and female rat offspring treated in utero during the last wk of pregnancy (embryonic days 14–21) with vehicle or DEX. PRA, plasma renin activity. *P < 0.05 compared with vehicle group.
tered plasma angiotensinogen and PRA, and no effect on blood pressure.

The observation in the present study that elevated systolic blood pressure was confined to female offspring was surprising. Prior investigations have demonstrated variable responses to in utero programming. We and others (10, 35, 58) have noted increased blood pressure in both sexes, whereas others found males to be more responsive than females (49). The precise reasons for this variation are unknown, but species, timing and duration of programming stimuli, the age of offspring, and techniques used to measure blood pressure are important (10, 28, 32, 38, 58).

In conclusion, we demonstrate that prenatal DEX administration in the final week of gestation results in reduced birth weight and subsequent gender-specific abnormalities in cardiovascular and metabolic physiology. Alterations within the RAS may, in part, underlie the hypertension associated with prenatal glucocorticoid treatment. Recent human data in preterm babies have suggested that brief prenatal glucocorticoid treatment, commonly used in obstetric practice, is associated with increased adolescent blood pressure (22). Our experimental observations, in conjunction with others’, highlight the necessity for meticulous follow-up studies to ascertain whether antenatal glucocorticoid exposure in humans produces these adverse effects and to further determine whether they are gender specific.

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GRANTS

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