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Intergenerational consequences of fetal programming by in utero exposure to glucocorticoids in rats

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Drake, Amanda J., Brian R. Walker, and Jonathan R. Seckl. Intergenerational consequences of fetal programming by in utero exposure to glucocorticoids in rats. Am J Physiol Regul Integr Comp Physiol 288: R34–R38, 2005. First published June 3, 2004; doi: 10.1152/ajpregu.00106.2004.—Epidemiological studies linking low birth weight and subsequent cardiometabolic disease have given rise to the hypothesis that events in fetal life permanently program subsequent cardiovascular risk. The effects of fetal programming may not be limited to the first-generation offspring. We have explored intergenerational effects in the dexamethasone-programmed rat, a model in which fetal exposure to excess glucocorticoid results in low birth weight with subsequent adult hyperinsulinemia and hyperglycemia underpinned by increased activity of the key hepatic gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK). We found that the male offspring of female rats that had been exposed prenatally to dexamethasone, but were not manipulated in their own pregnancy, also had reduced birth weight (5.66 ± 0.06 vs. 6.12 ± 0.06 g, P < 0.001), glucose intolerance, and elevated hepatic PEPCK activity (5.7 ± 0.6 vs. 3.3 ± 0.2 nmol·min⁻¹·mg protein⁻¹, P < 0.001). These effects resolved in a third generation. Similar intergenerational programming was observed in offspring of male rats exposed prenatally to dexamethasone mated with control females. The persistence of such programming effects through several generations, transmitted by either maternal or paternal lines, indicates the potential importance of epigenetic factors in the intergenerational inheritance of the “programming phenotype” and provides a basis for the inherited association between low birth weight and cardiovascular risk factors.

Epidemiological studies in diverse populations have demonstrated an association between low birth weight and the subsequent development of hypertension, insulin resistance, Type 2 diabetes, and cardiovascular disease (2). This association appears to be independent of classical adult lifestyle risk factors (3). In explanation, it has been proposed that a stimulus or insult acting during critical periods of growth and development permanently alters tissue structure and function, a phenomenon termed “fetal programming.” Indeed, evidence from both human and animal studies (2, 25) suggests that adult pathophysiology can be induced by manipulating the fetal environment. Intriguingly, there is evidence that this phenomenon is not limited to the first-generation (F1) offspring and that programming effects may persist in subsequent generations (8). Epidemiological studies in humans suggest intergenerational effects on birth weight, cardiovascular risk factors, and type 2 diabetes (8). Similarly, transgenerational effects on birth weight, glucose tolerance, blood pressure, and the hypothalamic-pituitary-adrenal axis have been reported in animal models (8).

One major hypothesis to explain fetal programming invokes overexposure of the fetus to glucocorticoids (10). Steroid hormones exert long-term organizational effects and regulate organ development and maturation (1, 13); indeed, glucocorticoids are exploited therapeutically in the perinatal period to alter the rate of maturation of organs such as the lung (34). Glucocorticoid treatment during pregnancy reduces birth weight in animals and in humans (24, 28). Furthermore, cortisol levels are increased in human fetuses with intrauterine growth retardation or in pregnancies complicated by preeclampsia, which may indicate a role for endogenous cortisol in fetal growth retardation (12).

The fetus is normally protected from high circulating levels of glucocorticoids in the mother by placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD 2), which catalyzes the conversion of active glucocorticoids (cortisol in humans, corticosterone in rats) to their inactive 11-keto metabolites (cortisone and 11-dehydrocorticosterone). We have shown that rats exposed to dexamethasone (Dex; a poor substrate for 11β-HSD 2) during the last third of pregnancy, or to 11β-HSD inhibitors, are of low birth weight and develop hypertension and glucose intolerance in adulthood (5, 20, 21, 25). The offspring glucose intolerance is thought to be mediated, at least in part, by permanent elevation in the activity of hepatic phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in gluconeogenesis (25). The majority of studies in this model have been conducted on male animals (25), although effects on blood pressure have been shown in both sexes (5). We have used the prenatal Dex model of programming in rats to test for intergenerational effects on birth weight, hepatic PEPCK activity, and glucose tolerance and to investigate whether these intergenerational effects are transmitted through the maternal and/or paternal line.

MATERIALS AND METHODS

Animals. Female Wistar rats (200–250 g; Charles River UK, Margate, UK) were maintained under controlled lighting (lights on 7:00 AM to 7:00 PM) and temperature (22°C) and allowed free access to food and water. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
to food and water (standard rat chow, Special Diets Services, Witham, Essex, UK). After 2 wk of acclimatization, rats were timed mated. A single virgin female was housed with a male in a breeding cage until an expelled vaginal plug was noted (designated E0, day zero of pregnancy); females were then housed singly throughout pregnancy until delivery, which occurred on days 20–22. Pregnant females were injected subcutaneously with a solution of 100 μg/kg Dex in 0.9% saline containing 4% ethanol (Dex mothers) or with an equivalent volume of vehicle (Veh mothers) at the same time each morning between days 15 and 21 of pregnancy inclusive. Litters were termed F1 Dex and F1 Veh (n = 8 F1 Dex litters, 7 F1 Veh litters). F1 females weighing approximately 230–250 g were timed-mated with nonsibling F1 males from the same prenatal treatment group. Females were caged separately during pregnancy and not manipulated in any way. Pups were termed second generation (F2) Dex-mother/Dex-father (F2 Dex/Dex) and F2 Veh-mother/Veh-father (F2 Veh/Veh) (n = 9 litters for both groups). Mature F2 Dex/Dex and F2 Veh/Veh females (230–250 g) were timed-mated subsequently with unrelated males from the same group to give 10 third-generation (F3) Dex/Dex litters and 9 F3 Veh/Veh litters.

Further experiments were performed to dissect paternal and maternal effects on the F2 phenotype. F1 animals were generated as described above. Dex or Veh F1 females weighing 230–250 g were mated with Dex or Veh nonsibling F1 males to produce litters with all combinations, including 8 Dex-mother/Dex-father (F2 Dex/Dex), 8 Veh-mother/Veh-father (F2 Veh/Veh), 5 Dex-mother/Veh-father (F2 Dex/Veh), and 6 Veh-mother/Dex-father (F2 Veh/Dex). Females were caged separately and not manipulated in any way during pregnancy. In all cases, offspring were weighed at birth and culled to eight pups per litter. Offspring were then weighed at weaning (21 days) and monthly thereafter. Experimental cohorts included males selected randomly from as many litters as possible.

All animal procedures were carried out under the terms of the UK Animals (Scientific Procedures) Act 1986.

Enzyme activity. At 3–12 wk of age, nonfasted male animals (n = 8–10) from each group were culled by cervical dislocation between days 21–22 of age in the F2 offspring. Animals were fasted overnight; at 9:00 AM the following morning, 2 g/kg glucose solution (0.5 g/ml) was administered by gavage, and tail-nick blood samples were collected at 0, 30, and 120 min. Plasma was stored at −20°C. Glucose was determined by the enzymatic (hexokinase) method (kit supplied by Sigma Chemical, Poole, UK) and plasma insulin by ELISA (Crystal Chem, Chicago, IL).

Statistics. Data are expressed as means ± SE. Groups were compared using unpaired Student’s t-tests, with two-way (PEPCK) or repeated-measures ANOVA (glucose tolerance tests, longitudinal body weight), as appropriate.

RESULTS

F1 offspring. Treatment of pregnant rats with Dex reduced birth weight in both males and females in the F1 offspring without altering gestation length or litter size (Table 1). F1 Dex

**Table 1. Gestation length, litter size, and birth weight for F1, F2, and F3 offspring**

<table>
<thead>
<tr>
<th>Gestation Length</th>
<th>Litter Size</th>
<th>Birth Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 Dex (n = 74)</td>
<td>20.8 ± 0.3</td>
<td>11.5 ± 0.8</td>
</tr>
<tr>
<td>F1 Veh (n = 81)</td>
<td>20.9 ± 0.1</td>
<td>12.7 ± 0.7</td>
</tr>
<tr>
<td>F2 Dex/Dex (n = 101)</td>
<td>22.8 ± 0.1*</td>
<td>11.6 ± 0.9</td>
</tr>
<tr>
<td>F2 Veh/Veh (n = 108)</td>
<td>22.0 ± 0.2</td>
<td>12.0 ± 0.6</td>
</tr>
<tr>
<td>F3 Dex/Dex (n = 108)</td>
<td>21.6 ± 0.2</td>
<td>10.7 ± 0.7</td>
</tr>
<tr>
<td>F3 Veh/Veh (n = 107)</td>
<td>21.8 ± 0.2</td>
<td>12.0 ± 0.5</td>
</tr>
</tbody>
</table>

Data are means ± SE. Birth weights shown are for both males and females. F1, F2, and F3, first, second, and third generation; Dex, dexamethasone; Veh, vehicle. *P < 0.05 compared with control for each cohort.

Glucose tolerance tests. Glucose tolerance testing was performed in male animals at 6 mo of age in each generation and also at 4 mo of age in the F2 offspring. Animals were fasted overnight; at 9:00 AM the following morning, 2 g/kg glucose solution (0.5 g/ml) was administered by gavage, and tail-nick blood samples were collected at 0, 30, and 120 min. Plasma was stored at −20°C. Glucose was determined by the enzymatic (hexokinase) method (kit supplied by Sigma Chemical, Poole, UK) and plasma insulin by ELISA (Crystal Chem, Chicago, IL).

**Fig. 2. Hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity in F1 and F2 males.** A: PEPCK activity in F1 males (n = 34–50). B: PEPCK activity in F2 males (n = 24–38). Veh, vehicle; Dex, dexamethasone.
males showed catch-up growth so that they weighed the same as F1 Veh controls from weaning at 21 days (Fig. 1). Hepatic PEPCK activity tended to be higher in livers of 12-wk-old male F1 Dex offspring than in Veh controls ($P = 0.10$; Fig. 2); although this difference did not reach statistical significance, mean values were consistent with previous studies (25). Glucose tolerance testing at 6 mo of age revealed no differences in plasma glucose or insulin between F1 Dex and F1 Veh males (Fig. 3).

**F2 offspring.** Breeding of F1 animals, without further manipulation, produced F2 offspring. In F2 Dex/Dex offspring, birth weight was lower in males and females (Table 1), and in males, hepatic PEPCK activity at 5 wk was higher (Fig. 2) than in F2 Veh/Veh offspring, although storage of the liver homogenate at $-80^\circ$C before analysis reduced PEPCK activity in both F2 Dex/Dex and F2 Veh/Veh animals. F2 Dex/Dex males showed catch-up growth, and body weights were not different from Veh/Veh controls from weaning onward (Fig. 1). In glucose tolerance tests at 4 mo of age, F2 Dex/Dex males had higher plasma glucose than F2 Veh/Veh (Fig. 3), although there were no differences in plasma insulin (Fig. 3). At 6 mo, F2 Dex/Dex males additionally had higher basal insulin levels (F2 Dex/Dex 1.08 ± 0.05 vs. F2 Veh/Veh 0.92 ± 0.04 ng/ml, $P = 0.04$).

**F3 offspring.** The Dex-programmed phenotype did not persist into a third generation. There were no differences between F3 offspring groups in birth weight (Table 1), litter number, or gestation length (Table 1); postnatal growth patterns (not shown); hepatic PEPCK activity at 12 wk of age (F3 Dex/Dex 9.97 ± 1.14 vs. F3 Veh/Veh 10.61 ± 0.61 nmol·min$^{-1}$·mg protein$^{-1}$, $P = 0.62$); or glucose tolerance (Fig. 3).

**Parental effect on phenotype.** In a second cohort of animals prepared in the same way, birth weights of F2 Dex/Dex were again significantly lower (Table 2), and in males, hepatic PEPCK activity was higher at 3 wk of age (Fig. 4) than in F2 Veh/Veh offspring. Among the F2 offspring of F1 fathers treated with Veh, maternal Dex exposure in utero reduced birth weight (Table 2) and elevated PEPCK (Fig. 4). However, differences in F2 animals were not entirely explained by maternal prenatal Dex exposure because paternal Dex had similar effects. Thus, among the F2 offspring of F1 mothers treated with Veh, paternal Dex exposure in utero also signifi-

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![Fig. 3. Oral glucose tolerance tests results for F1, F2, and third-generation (F3) males. Plasma glucose (A) and insulin (B) in F1 males at 6 mo of age ($n = 10$ per group). Plasma glucose (C) and insulin (D) in F2 males at 4 mo of age ($n = 10$ per group). Plasma glucose (E) and insulin (F) in F3 males at 6 mo of age ($n = 8$ per group). Data are means ± SE and were analyzed by repeated-measures ANOVA. *$P < 0.05$.](http://ajpregu.physiology.org/)
However, maternal characteristics alone do not explain all aspects of the intergenerational programming described in this study. Both maternal and paternal Dex exposure were associated independently with intergenerational effects on offspring birth weight and PEPCK. The effects were not additive, suggesting that they may be mediated by a common pathway. Human epidemiological studies have revealed both maternal and paternal effects on offspring birth weight and type 2 diabetes (8). Extensive studies in the Pima Indian community, which has a high prevalence of maternal diabetes, have shown that low birth weight is associated with the subsequent development of type 2 diabetes only if paternal diabetes is also present (18). It has been proposed that such effects reflect the influence of genetic factors (9, 14); however, this interpretation may not be complete if our findings can be extrapolated to humans. One potential mechanism invokes “epigenetic” modification, which may affect the phenotype without changing the DNA sequence. Such epigenetic modification of genes has been proposed to underpin the association of lower offspring birth weight with paternal diabetes in Pima populations (18, 19) and in the parental differences in transmission of the variable number tandem repeat minisatellite of the insulin gene (INS-VNTR), itself associated with type 2 diabetes (15). Although the epigenetic silencing of alleles according to the parent of origin, or parental imprinting, is erased through meiosis and reestablished in the offspring, recent evidence suggests that some epigenetic modifications may not be completely erased during gametogenesis and embryogenesis, potentially resulting in the intergenerational inheritance of the epigenetic state (23, 26, 30). Recent studies suggest that environmental factors, including nutrition and glucocorticoids, may influence gene expression and potentially affect both fetal growth and later disease risk (32, 35–37). If such epigenetic modifications were not erased during gametogenesis and embryogenesis, this could lead to the transgenerational inheritance of “programmed effects” (27). Furthermore, an interaction between programmed alterations in maternal physiology and epigenetic modifications may explain some of the differences between the inter-

Table 2. Birth weights, gestation length, and litter size for F2 offspring with contrasting maternal and paternal prenatal exposure to Dex

<table>
<thead>
<tr>
<th>Litter</th>
<th>F2 Veh/Veh</th>
<th>F2 Dex/Dex</th>
<th>F2 Veh/Dex</th>
<th>F2 Dex/Veh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veh</td>
<td>22.3±0.2</td>
<td>22.0±0.2</td>
<td>21.5±0.7</td>
<td>22.4±0.2</td>
</tr>
<tr>
<td>Dex</td>
<td>15.8±0.4</td>
<td>14.8±0.8</td>
<td>13.2±2.2</td>
<td>13.6±2.2</td>
</tr>
<tr>
<td>Father</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veh</td>
<td>6.15±0.05</td>
<td>5.92±0.05†</td>
<td>5.71±0.09*</td>
<td>5.96±0.08†</td>
</tr>
<tr>
<td>Dex</td>
<td>0.05*†</td>
<td>0.05*†</td>
<td>0.05*†</td>
<td>0.05*†</td>
</tr>
</tbody>
</table>

Data are means ± SE. Analysis by ANOVA revealed no differences between the groups for gestation length or litter size. Birth weights shown are for both males and females and were recorded for 126 F2 Veh/Veh offspring, 116 F2 Dex/Dex offspring, 71 F2 Veh/Dex offspring, and 62 F2 Dex/Veh offspring. *P < 0.05 compared with F2 Veh/Veh offspring; †P < 0.05 compared with F2 Veh/Dex offspring.

DISCUSSION

These data show that in utero exposure to Dex is associated with intergenerational effects in the rat. In agreement with previous reports, exposure to Dex in utero during the last week of pregnancy reduced birth weight (25) and was associated with a permanent increase in hepatic PEPCK activity in F1 male offspring (25). We have extended these studies to show that, without further intervention, second-generation male offspring of Dex-programmed rats also have reduced birth weight, elevated hepatic PEPCK activity, and glucose dyshomeostasis. The effects had resolved by the third generation. In addition, we have demonstrated independent effects of maternal and paternal early life experience on offspring birth weight and PEPCK.

F2 Dex/Dex males demonstrated a significant increase in PEPCK activity and showed evidence of glucose dyshomeostasis by 4 mo of age. Previous experiments in F1 males using this model have demonstrated the presence of glucose dyshomeostasis after 6 mo of age, which may be secondary to the permanent increase in hepatic PEPCK activity (25). The lack of any detectable effect on glucose tolerance in the F1 Dex animals described here may reflect the nonsignificant increase in PEPCK in the F1 Dex males and perhaps indicates a milder programming phenotype in this cohort. Although the differences in birth weight and glucose/insulin responses are relatively small, such changes are consistent with those found in our previous and others’ programming experiments (16, 21, 25) and indeed reflect the human epidemiology (2).

The concept of perpetuation of programming effects in subsequent generations is commonly attributed to maternal characteristics such as maternal size (7, 33) or exposure of the fetus to a hyperinsulinemic environment (4, 6, 11, 17, 22, 29, 31). However, maternal characteristics alone do not explain all of the differences.
generational effects of maternal and paternal antenatal Dex exposure on offspring birth weight and PEPCK.

In conclusion, we have shown an effect of both maternal and paternal early life experience on offspring characteristics in this animal model of programming. The mechanisms may include inherited epigenetic effects. Environmental factors, including glucocorticoids, may alter the expression of genes in the placenta, the fetus, or both, with effects on fetal growth and later disease risk. This intergenerational programming may masquerade as a “genetic” explanation for the association between birth weight and inherited risk of cardiovascular disease.

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