Developmental Programming of Renal Glucocorticoid Sensitivity and the Renin-Angiotensin System

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Abstract—Fetal glucocorticoid excess leads to subsequent adult hypertension, but the mechanisms involved in this developmental programming remain largely unknown. In this study we tested the hypothesis that programmed hypertension in rats is linked to altered renal expression of the glucocorticoid receptor, mineralocorticoid receptor, and 11β-hydroxysteroid dehydrogenase type 2 and components of the intrarenal and adipose renin-angiotensin system. The interactive effects of a postnatal diet enriched in omega-3 fatty acids, which prevents emergence of the hypertensive phenotype, were also examined. Maternal dexamethasone (0.75 μg/mL of drinking water from day 13 to term) markedly increased renal expression of the glucocorticoid receptor in 6-month–old offspring, and this was associated with hypomethylation of the glucocorticoid receptor promoter; renal MR was unaffected. In contrast, maternal dexamethasone reduced renal 11β-hydroxysteroid dehydrogenase type 2 in offspring, but this effect was prevented by a high omega-3 diet. Consistent with these effects, renal Na/K-ATPase-α1 was elevated in offspring of dexamethasone-treated mothers, but only in those raised on the standard diet. Maternal dexamethasone also programmed increased expression of renal and adipose angiotensin-converting enzyme and renal renin, but among these changes, only that of renal angiotensin-converting enzyme was prevented by the omega-3 diet. Our data support the hypothesis that programmed hypertension is mediated, in part, by increased renal glucocorticoid sensitivity, with consequent stimulatory effects on Na/K-ATPase-α1 and intrarenal renin-angiotensin system components. Partial prevention of programmed changes in renal gene expression by postnatal dietary omega-3 fatty acids provides insight into how this intervention prevents hypertension induced by fetal glucocorticoid excess. (Hypertension. 2007;50:579-584.)

Key Words: prenatal programming ▪ hypertension ▪ glucocorticoids ▪ 11β-hydroxysteroid dehydrogenase type 2 ▪ renin-angiotensin system ▪ kidney ▪ omega-3 fatty acids

Developmental programming is now recognized as a key determinant of the adult phenotype, most notably in relation to regulation of blood pressure, insulin sensitivity, and adiposity.1 Thus, fetal insults, such as undernutrition or glucocorticoid excess, can lead to adult hypertension and insulin resistance, but the mechanisms underlying such programming outcomes remain largely unknown.2,3 Moreover, the severity of the programmed phenotype can be either reduced or exacerbated by the postnatal environment. Specifically, adult hypertension programmed by fetal undernutrition is worsened by a hypercaloric diet in postnatal life,4 whereas we recently reported that hypertension programmed by maternal dexamethasone treatment is prevented when offspring are raised on a diet enriched with omega-3 (n-3) fatty acids.5 This rescue of the programmed phenotype is consistent with the well-recognized, beneficial effects of dietary n-3 fatty acids in relation to human hypertension.6 Interestingly, hyperleptinemia was also a feature of the programmed phenotype in our model (despite no change in adiposity) and was similarly prevented by a high n-3 diet.5 Given the link between elevated leptin and hypertension,7 programmed hypertension likely develops, in part, as a consequence of hyperleptinemia. However, in addition, the hypertensive phenotype is potentially driven by several other factors, the effects of which must also be reversed or at least compensated for by a high n-3 diet.

Various models point to a central role for the kidney in the etiology of programmed hypertension.2,3 For example, nephron deficit is a consistent feature of hypertension programmed by fetal glucocorticoid excess,8–11 maternal low protein diet,12–14 and uterine ligation.12 Other renal regulators of blood pressure are also implicated, with increased glucocorticoid receptor (GR) and reduced 11β-hydroxysteroid dehydrogenase (11β-HSD2) expression observed in a rat maternal low-protein diet programming model.15 These changes in renal GR and 11β-HSD2 would be expected to elevate blood pressure via increased renal glucocorticoid sensitivity and activation of the mineralocorticoid receptor (MR) by glucocorticoids16 and consequent upregulation of genes promoting sodium retention, including Na/K-ATPase-α1 and -β1. Disturbances in the renin-angiotensin system (RAS) may also underlie hypertension programmed...
during fetal life by both nutritional insult\textsuperscript{14,17–19} and glucocorticoid excess.\textsuperscript{11,17,20,21} The latter upregulates adult plasma renin and angiotensinogen (AGT) levels\textsuperscript{21} and renal expression of angiotensin II receptors,\textsuperscript{11,17} but whether programming of other RAS components occurs in renal or adipose tissues is unknown.

Therefore, the present study tested the hypothesis that hypertension programmed by maternal dexamethasone treatment is mediated via increased renal glucocorticoid/mineralocorticoid sensitivity and upregulation of the intrarenal and adipose RAS. We also examined whether any programmed changes were prevented by a postnatal n-3 fatty acid diet. Specifically, renal expression of mRNAs encoding 11β-HSD2, GR, MR, and Na/K-ATPase-α1 and -β1, as well as all of the major components of the renal and adipose RAS (ie, renin, AGT, angiotensin-converting enzyme [ACE], and angiotensin II receptors [AT1aR, AT1bR, AT2R]) were measured. Among the programmed changes observed were altered renal expression of the GR and 11β-HSD2 genes, the promoter regions of which have been shown previously to be susceptible to methylation changes.\textsuperscript{22,23} Therefore, we also tested the hypothesis that maternal dexamethasone treatment alters the methylation status of these genes.

**Methods**

**Diets and Animals**

Two isocaloric, semipure diets were used in this study, each formulated with identical ratios of protein, carbohydrate, fat, and salt, but with markedly different n-3 fatty acid contents as reported previously.\textsuperscript{5} The semipure diets were manufactured by Specialty Feeds and were sterilized by gamma irradiation. Nulliparous albino Wistar rats aged between 8 and 10 weeks were obtained from the Western Australia Animal Ethics Committee. The rats consumed acidified water and food ad libitum. All of the mothers on normal rat chow. Within 24 hours of birth, all of the rats consumed acidified water and food ad libitum. All of the procedures involving animals were approved by the University of Western Australia Animal Ethics Committee.

Rats were mated overnight, and the day on which spermatozoa were present in a vaginal smear was designated day 1 of pregnancy. Dexamethasone acetate (Sigma) was administered in the drinking water (0.75 μg/mL) from day 13 of pregnancy until birth in half of the mothers on normal rat chow. Within 24 hours of birth, all of the pups from control (con) and dexamethasone-treated (dex) mothers were cross-fostered to a mother on either a standard diet (con) or high n-3 diet (high n-3). Cross-fostering resulted in 4 treatment groups (con/standard, con/high n-3, dex/standard, and dex/high n-3), and pups remained with their foster mothers until weaning, at which point male and female offspring were caged separately and remained on their allocated diets (standard or high n-3). Blood pressure data from the animals used in this study have been reported previously; maternal dexamethasone programmed offspring hypertension by 2 months of age in males and 6 months in females, but these effects were prevented by high n-3 diets.\textsuperscript{5}

**Tissue Collections**

At 6 months of age, 1 male and 1 female rat were randomly chosen from each litter, fasted overnight, and anesthetized with halothane (in oxygen/NO: 0.2:0.8). Portions of left kidney and retroperitoneal fat were collected, snap frozen in liquid nitrogen, and stored at −80°C until analysis of gene and protein expression.

**Quantitative RT-PCR Analysis**

Total RNA was extracted from kidney samples using TRI Reagent (Molecular Research Center) and from retroperitoneal fat using a QIAGEN RNeasy Lipid Tissue mini kit. Extracted RNA was treated using the Ambion DNA-free kit to remove contaminating genomic DNA. RNA (1 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer’s instructions. The cDNA was purified using the UltraClean PCR cleanup kit (MoBio Laboratories Inc). The primers for amplification of rat GR, MR, 11β-HSD2, Na/K-ATPase-α1 and -β1, and components of the RAS (renin, AGT, ACE, AT1Ra, AT1Rb, and AT2R) were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and ribosomal L19 was used as an internal control.\textsuperscript{25} For each gene the primer sequences are shown in the online supplemental data along with reaction conditions and product sizes (please see http://hyper.ahajournals.org). Quantitative PCR was performed using the Ro-torgene 3000 system (Corbett Research) as described previously.\textsuperscript{5}

**DNA Methylation-Sensitive PCR of GR and 11β-HSD2 Promoters**

To determine methylation status of the renal GR and 11β-HSD2 gene promoters, genomic DNA was subjected to methylation-sensitive PCR as described previously.\textsuperscript{23} Genomic DNA was extracted from renal tissue with TRI Reagent and subjected to digestion by AciI (New England Biolab). For 11β-HSD2, the primers amplified a region of the promoter that contains 21 AciI sites.\textsuperscript{22} For the GR, the primers targeted the exon 1 promoter, which is the predominant GR promoter in the kidney and comprises 17 AciI sites.\textsuperscript{23,26} The rat hexokinase I gene was used as an internal control, because its promoter region contains no CpGs and no AciI recognition sites. Quantitative PCR was performed in a manner similar to that for RT-PCR, and in each case melt curve analysis showed a single PCR product, the identity of which was confirmed by sequence analysis (data not shown). Details of primers and PCR conditions for GR, 11β-HSD2, and hexokinase I are presented in the online supplemental data available at http://hyper.ahajournals.org.

**Western Blot Analysis**

Renal samples were homogenized in radioimmunoprecipitation assay buffer and 100 μg of cleared lysate supernatant were resolved by SDS-PAGE. Proteins were transferred to Hybond C+ Super membrane (Amersham). Membranes were exposed to primary antibody overnight (1:500 dilution for 11β-HSD2, 1:250 for Na/K-ATPase α1, and 1:5000 for β-actin). The 11β-HSD2 antibody (RAH23) was a kind gift from Dr Zygmunt Krozowski (Baker Heart Research Institute, Melbourne, Australia), the Na/K-ATPase-α1 antibody was purchased from Santa Cruz Biotechnology, and the β-actin antibody was purchased from Chemicon (Temecula, California).

**Figure 1.** Relative expression of GR mRNA in kidneys of 6-month-old male offspring of control (Con) and dexametha-
sone-treated (Dex) mothers. Female offspring showed a similar pattern of expression. Offspring were raised on either a standard diet (Std) or a diet enriched with omega-3 fatty acids (Hn3). Values are the mean±SEM (n=7 to 8 per group). Data were analyzed by ANOVA (variance attributed to sex, prenatal treatment, and postnatal diet) followed by posthoc LSD tests. Values without common notation differ significantly (P<0.05; LSD test).
was from Sigma. Membranes were incubated with a horseradish peroxidase-conjugated secondary antibody and immunoreactive bands visualized by chemiluminescence. Immunoreactive bands for 11β-HSD2 (40 kDa) and Na/K ATPase-α1 (100 kDa) were standardized against corresponding β-actin bands.

Statistical Analysis
All of the data are expressed as mean±SEM, with each litter representing n=1. In all of the cases, n=6 to 8 for each experimental group. All of the variables were analyzed by ANOVAs (1-, 2-, or 3-way as appropriate to account for variation because of sex, maternal treatment, and diet) followed by posthoc least-significant difference (LSD) tests. When significant interaction terms were found in these ANOVAs, analyses of subsets of data were made (see Results for specific applications).

Results

Renal Expression of GR, MR, 11β-HSD2, and Na/K-ATPases
Maternal dexamethasone treatment increased renal GR mRNA expression in both male and female offspring at 6 months of age (P<0.001; Figure 1), and this effect was not altered by postnatal consumption of a high n-3 diet. The renal mRNA expression of MR was similar among all of the experimental groups in both sexes (data not shown).

Maternal dexamethasone treatment reduced renal 11β-HSD2 mRNA expression in adult male and female offspring consuming a standard diet (P<0.001; Figure 2A), but not in those consuming the high n-3 fatty acid diet. Indeed, renal 11β-HSD2 expression in male offspring that had been exposed to dexamethasone in utero but then raised on a high n-3 fatty acid diet was even higher than in control offspring that consumed the standard diet (Figure 2A). Female control offspring on the high n-3 diet also exhibited an increase in 11β-HSD2 mRNA. Consistent with these changes, the abundance of 11β-HSD2 protein was lower in kidneys of dexamethasone-exposed female offspring consuming a standard diet (P<0.05), but this effect was not apparent in dexamethasone-exposed females on a high n-3 diet. A similar pattern was observed in males, although in this instance renal 11β-HSD2 protein levels in dexamethasone-exposed offspring on a high n-3 diet were intermediate between those in the control and dexamethasone-exposed groups raised on the standard diet and not significantly different from either (Figure 2B).

Consistent with observations on 11β-HSD2 expression, renal mRNA expression of Na/K-ATPase-α1 was increased in both male and female offspring of dexamethasone-treated mothers (P<0.001; Figure 3A), but this effect was eliminated by dietary n-3 fatty acids. These alterations in mRNA expression were reflected in protein abundance of Na/K-ATPase-α1 in male offspring, with increased levels in dexamethasone-exposed offspring consuming the standard diet (P<0.05; see Figure 3B) but not in those consuming a high n-3 fatty acid diet.
n-3 diet. In female offspring, however, the abundance of Na/K-ATPase-α1 protein did not parallel mRNA changes, with variation not attributable to either prenatal dexamethasone or postnatal diet. The expression of Na/K-ATPase-β1 mRNA was similar among all of the groups (data not shown).

Renal GR and 11β-HSD2 Promoter Methylation Status

GR exon 10 promoter methylation status was markedly decreased in male and female offspring exposed to dexamethasone in utero (P<0.001; Figure 4), and this effect was not altered by postnatal diet. In contrast, the methylation status of the 11β-HSD2 promoter did not vary because of prenatal treatment, postnatal diet, or sex (data not shown).

Renal and Adipose Expression of RAS Components

Renal ACE mRNA expression in adult male and female offspring exposed to dexamethasone in utero was elevated (P<0.05) only in those animals raised on a standard diet (Table). In adipose tissue (ie, retroperitoneal fat), ACE expression was also elevated in both sexes as a result of in utero dexamethasone exposure (P<0.001; Table), but this effect was not altered by n-3 fatty acids. Renal renin mRNA expression was increased in all of the offspring of mothers treated with dexamethasone regardless of their postnatal diet (P<0.001; Table), whereas adipose expression of renin was not affected by either maternal dexamethasone treatment or postnatal diet (Table). Expression of renal and adipose AGT and renal AT1aR, AT1bR, and AT2R did not differ among groups (data not shown).

Discussion

This study explored changes in renal glucocorticoid sensitivity and the renal and adipose RAS associated with programmed hypertension in adult male and female offspring of mothers treated with dexamethasone during pregnancy. The major findings were that programming of adult hypertension was associated with increased mRNA expression of adiposal ACE and renal GR, ACE, renin, and Na/K-ATPase-α1 but decreased renal expression of 11β-HSD2. In contrast, a postnatal diet rich in n-3 fatty acids prevented the programmed changes in renal 11β-HSD2, ACE, and Na/K-ATPase-α1, thus providing insight as to how postnatal dietary n-3 fatty acids prevent programmed hypertension.

Previous studies indicate that GR expression is particularly susceptible to developmental programming, with fetal glucocorticoid excess shown to upregulate GR in adult rat liver27 and adipose tissue.28 The present study, however, is the first to show programming of the adult renal GR after fetal glucocorticoid excess. Maternal protein restriction similarly programs increased GR expression in both the kidney23 and liver.23 In contrast, fetal glucocorticoid excess programs reduced expression of the GR in hippocampus29 and skeletal muscle,24 highlighting the complex organization of the 5’-end of the GR gene, which includes multiple and tissue-specific promoters.26 In this regard we also show that upregulation of renal GR is associated with decreased methylation of the exon 1 promoter of the GR gene, comparable to the effects of maternal protein restriction on the hepatic GR of adult offspring.23 Similarly, variations in maternal care in early postnatal life determine hippocampal GR expression via effects on methylation of the exon 1 GR promoter.30

Our data also show that fetal glucocorticoid excess programs reduced expression of renal 11β-HSD2, a change not attributable to altered methylation of the 11β-HSD2 promoter as assessed by methylation-sensitive PCR. Renal 11β-HSD2 normally serves to prevent illicit access of glucocorticoids to the renal MR, thereby maintaining MR specificity for aldosterone, and, accordingly, humans with a deficiency in 11β-HSD2 exhibit hypertension.16 A programmed reduction in renal 11β-HSD2 would be expected to increase the ability of glucocorticoids to activate both GR and MR, resulting in increased transcription of both and a consequent increase in blood pressure. This increase in glucocorticoid sensitivity may be exacerbated by hyperactivation of the hypothalamic-
pituitary-adrenal axis, because several previous studies show that prenatal glucocorticoids elevate adult plasma corticosterone,\textsuperscript{21,29} apparently driven by increased hypothalamic corticotropin releasing hormone expression.\textsuperscript{31}

Consistent with the proposal that increased renal glucocorticoid sensitivity underlies programmed hypertension, expression of the renal glucocorticoid-responsive genes Na/K-ATPase-\(\alpha_1\), ACE, and renin were all elevated in offspring of dexamethasone-treated mothers. These programmed changes would be expected to increase renal sodium retention and thereby elevate plasma volume and, thus, blood pressure. A similar mechanism seems to underlie hypertension in a rat maternal low-protein programming model in which adult offspring have higher renal expression of both Na/K-ATPase-\(\alpha_1\) and -\(\beta_1\).\textsuperscript{15} Interestingly, only the Na/K-ATPase-\(\alpha_1\) isoform was upregulated in the present study, and although this effect was evident in both sexes at the mRNA level, protein expression of Na/K-ATPase-\(\alpha_1\) was increased only in kidneys of male offspring. It is unclear why programming effects were observed for Na/K-ATPase-\(\alpha_1\) mRNA but not protein in females, although it is possible that turnover of Na/K-ATPase-\(\alpha_1\) protein may vary across the estrous cycle. Further studies are required to clarify this mRNA/protein discrepancy. Regardless of the mechanism underlying this sex difference, the more potent effect of programming on Na/K-ATPase-\(\alpha_1\) protein expression in males may account, in part, for the earlier emergence of programmed hypertension in males in this model.\textsuperscript{5}

The finding that renal RAS components (ie, ACE and renin) and adipose ACE were upregulated in adult offspring of dexamethasone-treated mothers supports the recent proposal that increased RAS activity is central to programmed hypertension.\textsuperscript{2} Previously, O’Regan et al\textsuperscript{21} reported higher plasma renin activity and AGT in conjunction with hypertension in 6-month–old female, but not male, rats exposed to dexamethasone in utero. No major sex differences were evident in our model with respect to either RAS components (present study) or hypertension,\textsuperscript{5} consistent with several previous reports,\textsuperscript{32,33} whereas others have shown that fetal glucocorticoid excess programs hypertension only in male offspring.\textsuperscript{5} Such discrepancies likely reflect differences in treatment protocols and, thus, the timing and level of fetal glucocorticoid exposure. In any event, the increases in expression of ACE (renal and adipose) and renin (renal) observed in the present study provide the first evidence for programming of these RAS components, regardless of the type of fetal insult (undernutrition or glucocorticoid excess). On the other hand, renal expression of AT1aR, AT1bR, or AT2R was unaffected in our model, unlike a recent report showing that all 3 receptor types were elevated in 4-week–old offspring of corticosterone-treated mothers.\textsuperscript{11} Similarly, renal AT1aR mRNA was increased in 4-week–old offspring in maternal low-protein models.\textsuperscript{18,19}

The capacity of postnatal dietary n-3 fatty acids to prevent developmental programming of hypertension\textsuperscript{5} raised the question as to whether this dietary manipulation reverses programmed gene expression and/or alters other regulatory pathways that counter programmed hypertension via compensatory mechanisms. The present findings suggest that both reversal and compensation are involved, because dexamethasone programming of adult renal 11\(\beta\)-HSD2, Na/K-ATPase-\(\alpha_1\), and ACE expression was prevented by the high n-3 diet, whereas other programmed changes were unaffected by diet. Most notably, the programmed increase in renal GR expression was still evident in animals raised on a high n-3 diet, although these animals were normotensive. Nevertheless, it would be premature to conclude from these data that increased renal GR expression is not involved in programmed hypertension. Rather, the positive effects of the high n-3 diet on other aspects of blood pressure regulation, such as vascular reactivity and prevention of hyperleptinemia, may compensate for GR-mediated increases in blood pressure.

The mechanism by which n-3 fatty acids increased renal 11\(\beta\)-HSD2 expression may relate to their well-documented, anti-inflammatory actions.\textsuperscript{34} Proinflammatory cytokines such as tumor-necrosis factor-\(\alpha\) and interleukin (IL)-1\(\beta\) have been shown to reduce 11\(\beta\)-HSD2 expression in several sites, including kidney epithelial cells\textsuperscript{35} and placenta.\textsuperscript{36} Moreover, we have preliminary data showing that plasma levels of interleukin-1\(\beta\) and interleukin-6 are elevated in offspring of dexamethasone-treated mothers raised on a standard diet but not in those raised on the high n-3 diet (C.S. Wyrwoll, P.J. Mark, and B.J. Waddell, unpublished observations, 2007), consistent with suppression of 11\(\beta\)-HSD2 by these proinflammatory cytokines. Postnatal dietary n-3 fatty acids also prevented the programmed increases in renal Na/K-ATPase-\(\alpha_1\) and ACE mRNA expression, likely to be downstream effects of the restored 11\(\beta\)-HSD2 and an associated fall in local corticosterone. In addition, n-3 fatty acids could reduce ACE expression via activation of the peroxisome-proliferator activated receptors (PPARs), transcription factors known to influence renal regulation of blood pressure.\textsuperscript{37} Indeed, isomer-proliferator activated receptors \(\alpha\) and \(\gamma\) agonists were shown recently to suppress vascular ACE expression in streptozotocin-induced diabetic rats.\textsuperscript{38}

**Perspectives**

This study shows that adult hypertension programmed by maternal dexamethasone treatment is linked to marked changes in renal expression of the GR, 11\(\beta\)-HSD2, and components of the RAS. Some but not all of these “programmed” changes were no longer observed when hypertension was prevented by a postnatal n-3 fatty acid diet. Thus, our data suggest that the beneficial effects of a postnatal n-3 fatty acid diet on blood pressure in this model reflect a mix of reversal and compensatory changes in gene expression and highlight the extent of developmental plasticity that exists beyond the fetal period.

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**Disclosures**

None.
References


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