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Maternal low-protein diet in rat pregnancy programs blood pressure through sex-specific mechanisms

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McMullen, Sarah, and Simon C. Langley-Evans. Maternal low-protein diet in rat pregnancy programs blood pressure through sex-specific mechanisms. Am J Physiol Regul Integr Comp Physiol 288: R85–R90, 2005. First published September 16, 2004; doi:10.1152/ajpregu.00435.2004.—Animal models support human epidemiological studies in demonstrating a relationship between impaired fetal growth and risk of adult hypertension. Undernutrition during pregnancy exerts programming effects on the developing kidney, and modulation of angiotensin receptor (ATR) expression has been observed persisting into adult life. Fetal overexposure to glucocorticoids is thought to be central to the nutritional programming of blood pressure and may act through an interaction with ATR expression. Pregnant female Wistar rats were fed a control (n = 6) or a maternal low-protein diet (MLP; n = 17) throughout pregnancy. The glucocorticoid dependency of MLP effects was tested using metyrapone, an inhibitor of corticosterone synthesis. MLP-fed rats were injected twice daily with metyrapone, metyrapone plus corticosterone, or vehicle over days 1–14 of pregnancy. At delivery, all animals were fed standard laboratory chow. MLP-exposed offspring 4 wk of age exhibited increased systolic blood pressure compared with controls (P < 0.05), which proved to be glucocorticoid dependent in males only. AT1R mRNA expression was independent of in utero dietary treatment. AT-R mRNA expression was downregulated in MLP-exposed females only (P < 0.05) and in a glucocorticoid-independent manner. Male offspring exhibited glucocorticoid-dependent hypertension with no modulation of renal ATR mRNA expression. In contrast, female offspring exhibited glucocorticoid-independent hypertension associated with reduced expression of renal AT1R mRNA. These data do not support the hypothesis that an interaction between glucocorticoid and ATR mRNA expression underlies the nutritional programming of blood pressure but instead suggest two independent mechanisms acting in a sex-specific manner.

glucocorticoids; angiotensin II receptors; gender

A LARGE BODY OF EPIDEMIOLOGICAL EVIDENCE indicates that risk of disease in later life is related to factors that impair fetal growth. Low birth weight and disproportion have been linked to increased risk of hypertension (5), coronary heart disease (12), and renal disease (18). On the basis of this evidence, it has been proposed that maternal undernutrition during pregnancy can program a predisposition to disease in later life (4). This hypothesis is strongly supported by animal studies that have shown that a range of nutritional manipulations in pregnancy can elevate blood pressure in the offspring (16, 29, 42, 54). It is suggested that undernutrition during pregnancy has a permanent programming effect on the developing tissues, including alterations to cell number and type and subsequent modulation of gene expression (23).

Feeding a maternal low-protein diet (MLP) to rats during pregnancy has consistently been shown to program an elevation in blood pressure in the resulting offspring (13, 24, 29). Studies of the MLP-exposed offspring suggest that the renin-angiotensin system plays a key role in the elevation of their blood pressure, in particular via modulation of angiotensin receptor (ATR) expression (35, 44, 48). There are three types of ATR expressed in the kidney of the rat, which exert opposing effects in the homeostasis of blood pressure (50). The type 1 receptors (AT1A-R and AT1B-R) mediate increases in blood pressure by promoting sodium reabsorption and vasoconstriction (1). In contrast, the type 2 receptor (AT2-R) mediates the release of bradykinins, prostaglandins, and nitric oxide, ultimately leading to vasodilation and decreased peripheral resistance (2, 37, 51). We previously demonstrated enhanced pressor responses to intravenous administration of angiotensin II (ANG II) in association with decreased expression of AT2-R in the kidneys of MLP offspring (35). In a similar model, increased expression of the AT1-R protein has been observed (44). Importantly, treatment with the AT1-R antagonist losartan within the first month of postnatal life permanently overcomes the hypertensive effect of intrauterine undernutrition (48). These observations suggest that upregulation of the renin-angiotensin system, specifically through an alteration in the balance of receptor expression, may directly increase blood pressure in rats exposed to an MLP diet in utero.

Glucocorticoids are important regulators of the renin-angiotensin system (15, 21), and fetal overexposure to glucocorticoids of maternal origin is thought to be central to the nutritional programming of blood pressure (8, 22). Administration of the synthetic glucocorticoid dexamethasone during pregnancy has been shown to induce hypertension in the offspring in rats (6, 31, 41) and sheep (9). Pharmacological adrenalectomy of MLP-fed rat dams prevents their offspring from developing hypertension (21). The developing fetal tissues are protected from the high levels of maternal glucocorticoids by the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD2), which converts naturally occurring glucocorticoids to inactive forms (10). The expression of this enzyme has been shown to
be nutritionally sensitive in both rats (7, 27) and sheep (36, 53), and downregulation of 11βHSD2 may result in overexposure of the fetal tissues to maternal glucocorticoids during critical periods of development. This study therefore investigates the hypothesis that an interaction between these two hormonal systems may provide a mechanism by which maternal under-nutrition programs blood pressure.

METHODS

All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Animals of the American Physiological Society.

Animals. Twenty-two virgin female Wistar rats (Harlan, Belton, UK) were mated at weights between 200 and 250 g. Upon confirmation of mating by the appearance of a semen plug on the cage floor, the rats were allocated to be fed isoenergetic diets containing 18% protein (control, 180 g of casein/kg; n = 6) or 9% protein (MLP, 90 g of casein/kg; n = 16) as previously described (21). The 11β-hydroxylase inhibitor metyrapone was used to inhibit the synthesis of corticosterone by the maternal and fetal adrenal glands (3, 21, 28) and thus test the glucocorticoid dependency of MLP effects. The MLP-fed rats received twice-daily injections of metyrapone (5 mg/kg body wt; n = 5), metyrapone plus corticosterone (15 mg/kg body wt; n = 5), or injection vehicle (saline; n = 6) over days 1–14 of pregnancy as previously reported (21, 28). Control rats were administered vehicle injections only, because metyrapone has been shown to have no effect on the blood pressure of their offspring (28), and thus the use of 12 additional litters was not warranted and could be inferred from an ethical perspective. Metyrapone was administered at a dose previously shown to have no adverse effect on reproductive outcome and to reduce maternal corticosterone concentrations by 90% (3, 21, 28). The dose of corticosterone administered was chosen on the basis of previous studies (13, 17).

The pregnant rats remained on the semisynthetic diets until they gave birth at 22 days’ gestation. All animals were then transferred to a standard laboratory chow diet (B&K Universal, Hull, UK), and the litters were culled to a maximum of eight pups to minimize variation in suckling nutrition. The offspring of the four treatment groups therefore differed only in prenatal nutritional and glucocorticoid exposures. The excess pups culled at birth were used to provide total and mean birth weights were also similar between groups (Table 2). Offspring body weight at 4 wk of age was significantly reduced after exposure to metyrapone in utero and was restored to normal by corticosterone administration. Systolic blood pressure was significantly increased in both male and female animals exposed to MLP diet in utero (Fig. 1, A and B; P < 0.05). This effect was not sex dependent and persisted after adjustment for body weight and litter of origin.

RESULTS

Maternal feed intake and weight gain during pregnancy were not affected by either diet or drug treatment. Litter size and total and mean birth weights were also similar between groups (Table 2). Offspring body weight at 4 wk of age was significantly reduced after exposure to metyrapone in utero and was restored to normal by corticosterone administration.

Table 1. Primer and probe sequences

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Primer and Probe Sequence</th>
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<tbody>
<tr>
<td>AT1aR</td>
<td>Forward 5'-TCA CAG TGT GCC GGT TTC AT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TGG TAA GCG CCA GCC CTA T-3'</td>
</tr>
<tr>
<td></td>
<td>Probe 5'-TGA GTG TCG GAA TTC GAC GCT GCC-3'</td>
</tr>
<tr>
<td>AT1bR</td>
<td>Forward 5'-TCA TGG AGC AGC ATG TCT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GAT GGT GAT GGT GAT CAT GCC-3'</td>
</tr>
<tr>
<td></td>
<td>Probe 5'-TGA AGT CGG TGC GCC GCC-3'</td>
</tr>
<tr>
<td>AT2R</td>
<td>Forward 5'-AAT TAC CCG TGA AGT CCT GAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AGA AGA TGG ACG GAC GC-3'</td>
</tr>
<tr>
<td></td>
<td>Probe 5'-AGC TGC TTT GCC AGT CAT TAC-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward 5'-TTG GTA CGA CCG AGG AGA AAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GGT GTA CGA CCG AGG AGA AAC-3'</td>
</tr>
</tbody>
</table>

AT1aR, angiotensin receptor 1A; AT1bR, angiotensin receptor 1B; AT2R, angiotensin receptor 2.
MLP-exposed male blood pressure elevated by 16 mmHg, MLP-exposed female blood pressure elevated by 15 mmHg. The MLP-induced increase in blood pressure in male offspring was prevented by treatment with metyrapone and restored by administration of corticosterone. In females, the effect of the MLP diet appeared to be independent of glucocorticoid treatment. The pulse rates of male offspring were not affected by diet or glucocorticoid experience (Fig. 1C). In female offspring, pulse rates were significantly elevated in the MLP-fed group (Fig. 1D; P < 0.05). This effect was prevented by metyrapone treatment but was not restored by corticosterone administration.

At 4 wk of age, the expression of AT1AR and AT1BR mRNA was again not affected by MLP in utero. However, an effect of glucocorticoid exposure on AT1AR was observed in both males and females. In males, AT1AR mRNA expression was significantly higher in the corticosterone-treated group than in any other group (Fig. 2B; P < 0.001). In females, AT1AR mRNA expression was significantly lower in the MLP metyrapone group than in any other group (Fig. 2C; P < 0.05). The expression of AT2R mRNA at 4 wk was significantly reduced by MLP exposure in utero in female offspring only (Fig. 2, B and C; P < 0.05). Maternal metyrapone treatment did not prevent this effect, but corticosterone significantly increased expression to control levels.

In kidneys from unsexed neonatal animals, expression of AT1AR and AT1BR mRNA was not significantly influenced by dietary or glucocorticoid experience in utero (Fig. 2A). There was a tendency for expression of AT2R mRNA to follow the same pattern across all four groups (Fig. 2A) as observed in 4-wk-old offspring, but this failed to reach statistical significance (P = 0.067).

### DISCUSSION

Evidence suggests that upregulation of the renin-angiotensin system, particularly through changes in ATR expression, may play a role in the fetal programming of blood pressure (35, 44, 47). Glucocorticoids are thought to play a central role in the fetal programming of adult disease and are potent regulators of the renin-angiotensin system (15, 22, 23). We therefore hypothesized that an interaction between glucocorticoids and ATR expression may provide a mechanism by which blood pressure is programmed in utero and have investigated this

### Table 2. Maternal weight gain and feed intake during pregnancy, litter size, and mean birth weight of offspring

<table>
<thead>
<tr>
<th></th>
<th>Control Saline</th>
<th>MLP Saline</th>
<th>MLP Met</th>
<th>MLP Cort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal wt, g</td>
<td>201.5±5.5</td>
<td>199.8±6.1</td>
<td>210.3±6.5</td>
<td>212.2±6.5</td>
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<tr>
<td>Maternal wt gain, g</td>
<td>110.7±7.4</td>
<td>106.8±5.1</td>
<td>106.5±7.9</td>
<td>112.2±7.8</td>
</tr>
<tr>
<td>Maternal FI, g/day</td>
<td>20.6±0.8</td>
<td>20.0±0.6</td>
<td>21.5±0.5</td>
<td>21.3±0.9</td>
</tr>
<tr>
<td>Litter size</td>
<td>10.8±1.0</td>
<td>11.0±1.1</td>
<td>12.5±0.7</td>
<td>13.3±1.0</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>5.5±0.1</td>
<td>5.1±0.2</td>
<td>5.5±0.2</td>
<td>5.6±0.2</td>
</tr>
<tr>
<td>Body weight at 4 wk, g</td>
<td>88.2±2.0</td>
<td>86.6±2.0</td>
<td>78.4±2.2</td>
<td>88.3±2.2</td>
</tr>
</tbody>
</table>

Data are means ± SE. Control saline, n = 6; MLP saline, n = 6; MLP met, n = 6; MLP cort, n = 5. aAdjusted for litter size. MLP, maternal low-protein diet; met, metyrapone; cort, corticosterone; FI, feed intake. a > b; P < 0.05.
sex-specific mechanisms in the programming of hypertension. In independent of in utero glucocorticoid experience, indicating development of high blood pressure in male offspring only. In coid-specific effects of the drug. Metyrapone group was intended to elucidate the glucocorticosterone action. Broad disturbances of maternal endocrine function due to effects only on maternal glucocorticoid synthesis. Metyrapone, however, is not specific to glucocorticoids in its action. Administration of metyrapone between days 1 and 14 of pregnancy, before activation of the fetal adrenal gland, ensures that corticosterone-reversible effects are attributable to effects only on maternal glucocorticoid synthesis. Metyrapone, however, is not specific to glucocorticoids in its action. Broad disturbances of maternal endocrine function follow the administration of this agent. The inclusion of the rejecting animal model was intended to elucidate the glucocorticoid-specific effects of the drug.

In the current study, metyrapone treatment prevented the development of high blood pressure in male offspring only. In female offspring, the programmed hypertension appeared to be independent of in utero glucocorticoid experience, indicating sex-specific mechanisms in the programming of hypertension. Although the majority of studies published show increased blood pressure in both males and females exposed to in utero dietary restriction (27, 54), gender differences in programmed responses have previously been reported. Male offspring of rat dams subjected to a 30% global nutrient restriction during pregnancy developed hypertension at an earlier age than did their female counterparts (42). Similarly, guinea pig fetuses subject to global nutrient restriction manifested hypertension only in the male offspring (20) In addition, a modest protein restriction (8.5% protein diet) during pregnancy was shown to program hypertension in males but not in females (55), whereas in the same model, severe protein restriction (5% protein diet) programmed hypertension in both sexes (56). The mechanisms behind such sex-specific effects remain unknown, although it has been postulated that sex steroids may offer female offspring protection against the rise in blood pressure (42). In contrast to the present study, the elevation in blood pressure in the MLP model was previously shown to be glucocorticoid dependent in both males and females at the older age of 7 wk (21). The onset of programmed hypertension was shown to be delayed in females (42), and we speculate that the glucocorticoid-dependent contributor to hypertension did not become apparent at 4 wk of age but may have been demonstrated at later time points had they been assessed. Despite this, it seems that at 4 wk, the female offspring are hypertensive through an alternative glucocorticoid-independent mechanism. The subsequent onset of glucocorticoid-dependent hypertension in females, which we suggest on the basis of previous evidence (21, 42) may occur at a later time point, would necessitate a loss of this glucocorticoid-independent component. Further ontogeny studies are required to ascertain whether this effect is indeed transient.

An intact renin-angiotensin system is essential for normal renal development and function during both fetal and adult life (32, 38, 40). The kidney is an important target organ for ANG II, with regard to which it plays a critical role in the regulation of blood flow, glomerular filtration rate, sodium absorption, and thus blood pressure (45, 49). We previously reported (35) a significant reduction in the renal expression of AT2R mRNA in MLP-exposed offspring at 4 wk of age. This is consistent with the findings of the present study, in which a decrease in AT2R mRNA expression was again observed. The original study was not designed to examine gender differences. However, the current study indicates that AT2R mRNA expression is downregulated in female offspring only. AT2R acts to oppose the classic effects of ANG II by promoting decreased peripheral resistance and vasodilation (2, 50, 51). Knockout mice lacking AT2R have increased blood pressure (19), and transgenic mice overexpressing AT2R exhibit a reduced pressor response to ANG II (48). Selective inhibition of AT2R in rats by injection of antisense oligodeoxynucleotides into the renal interstitial space significantly decreased cGMP and bradykinin concentrations, increased pressor responses to ANG II, and increased systolic blood pressure (37). Thus downregulation of AT2R may make the animal more sensitive to the effects of ANG II and contribute to the programmed hypertension. Indeed, increased pressor responses to ANG II have been observed in female offspring of the same model (35).

Our working hypothesis was that such downregulation may be mediated by glucocorticoids. However, the decrease in AT2R mRNA expression was not prevented by metyrapone treatment, and AT2R was in fact upregulated in response to

Fig. 2. Effect of in utero dietary and glucocorticoid experience on expression of angiotensin receptor 1A (AT1A), AT1B, and AT2R in kidneys of off-spring at birth (A; n = 13, 14, 15, 20) and at age 4 wk in males (B; n = 9, 10, 11, 12) and females (C; n = 12, 9, 10, 10). Values are means ± SE. a > b; P = 0.067. c > d; P < 0.001. e > f; P < 0.05. ab denotes a nonsignificant intermediate value.
maternal corticosterone treatment. This is consistent with work with the pregnant ewe in which an increase in AT2R expression in response to prenatal dexamethasone treatment was shown (39). This indicates that, although glucocorticoid sensitive, the reduction in AT2R mRNA in female rats exposed to a MLP diet in utero is not glucocorticoid dependent. On the basis of these data, it must be speculated that nutritional and steroid-based (39, 46) models of fetal programming cannot be treated as equivalent. The corticosterone-induced increase in AT2R mRNA expression was not associated with a change in blood pressure. This inconsistency highlights the need for further study to clarify whether the temporal association between decreased AT2R expression, increased sensitivity to ANG II, and increased blood pressure signifies a direct, causal, actual relationship, and pharmacological studies using ATR antagonists are underway to test this relationship.

The mRNA expression of AT1A and AT1B was not altered by MLP exposure. This is in agreement with our previous study in which we examined the expression of AT1A mRNA in offspring at age 4 wk (34). Similarly, in neonatal tissue, AT1R mRNA expression was unaffected by dietary experience. Interestingly, expression of AT1AR mRNA at 4 wk was affected by glucocorticoid experience in utero. Expression was downregulated in response to metyrapone in females and upregulated in response to corticosterone in males, suggesting a persistent upregulatory effect of glucocorticoids on the mRNA expression of AT1AR. This is consistent with receptor characterization studies in the rat demonstrating glucocorticoid response elements in the AT1AR promoter and a dexamethasone-induced increase in AT1AR transcription in vascular smooth muscle cells (15), as well as in cardiac fibroblasts and cardiomyocytes in culture (34). Although this fits with the hypothesis that glucocorticoid exposure in utero may increase sensitivity to ANG II, the absence of an effect of the MLP diet alone suggests that any increased glucocorticoid exposure experienced by fetuses exposed to a MLP diet is not sufficient to perturb renal AT1R mRNA expression, at least to the level of sensitivity of our real-time RT-PCR methodology. Increased levels of AT1R protein have been demonstrated in kidneys of offspring exposed to a MLP diet (44), and the possibility of programmed changes in posttranslational events requires further investigation.

This study is the first to examine the mRNA expression of ATR in the kidneys of MLP-exposed offspring at birth, and, although not reaching statistical significance (P = 0.067), it is of note that the same pattern of AT2R mRNA expression was observed across all four groups as observed in the female kidneys at age 4 wk. This indicates that the expression of AT2R mRNA is perturbed from an early stage of nephrogenesis and thus is not secondary to the hypertensive phenotype. ANG II has been shown to be critical for normal renal development and functioning of the fetal kidney (14, 32), and AT2R is the predominant ATR during fetal life (50). Downregulation of AT2R-mediated ANG II effects at this critical time may account for impaired renal development in this model as demonstrated by reduced nephron number (30, 33) and glomerular filtration rate (26, 43). Further studies are planned to clarify these neonatal data, including the effect of gender, and to elucidate the ontogeny of the differences between groups.

The results of this and our previous study indicate that, at 4 wk of age, male offspring have developed glucocorticoid-dependent hypertension with no involvement of altered renal ATR mRNA expression. In contrast, female offspring exhibit glucocorticoid-independent hypertension associated with a similarly glucocorticoid-independent decrease in renal AT2R mRNA expression, indicating a possible hypersensitivity to the hypertensive effects of ANG II. The absence of interactive effects of prenatal dietary and glucocorticoid experience on the renal mRNA expression of ATR indicates that an alternative mechanism must lie behind the glucocorticoid-dependent hypertension, perhaps involving changes in ATR expression in nonrenal central or peripheral circulation or altered sensitivity to other vasoactive compounds (11).

In summary, the results of this study do not support the hypothesis that an interaction between glucocorticoid and renal ATR mRNA expression provides a mechanism for the nutritional programming of blood pressure. Instead, the data suggest two independent mechanisms acting in a sex-specific manner.

ACKNOWLEDGMENTS

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GRANTS

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