Programming Effects of Short Prenatal Exposure to Dexamethasone in Sheep

Miodrag Dodic, Tamara Abouantoun, Anne O’Connor, E. Marelyn Wintour, Karen M. Moritz

Abstract—Recent studies have linked fetal exposure to a suboptimal intrauterine environment with adult hypertension. The aims of the present study were to see whether prenatal dexamethasone administered intravenously to the ewe between 26 to 28 days of gestation (1) resulted in high blood pressure in male and female offspring and whether hypertension in males was modulated by testosterone status, and (2) altered gene expression for angiotensinogen and angiotensin type 1 (AT₁) receptors in the brain in late gestation and in the adult. Basal mean arterial pressure (MAP) at 2 years of age was significantly higher in wethers exposed to prenatal dexamethasone (group D; 106±5 mm Hg, n=9) compared with the control group (group S; 91±3 mm Hg, n=8; P<0.01). Infusion of testosterone for 3 weeks had no effect on MAP in either treatment group. At 130 days of gestation, dexamethasone administered between 26 to 28 days of gestation (group DF; n=8), resulted in an increased expression of angiotensinogen in hypothalamus (in arbitrary units: 2.5±0.3 versus 1.3±0.3 in the saline group [group SF], n=10; P<0.05). In addition, there was higher expression of the AT₁ receptors in medulla oblongata in group DF (2.6±0.6 versus 1.1±0.2 in group SF; P<0.01). This effect of prenatal dexamethasone treatment was still evident in females at 7 years of age (group DA; n=5; 2.6±0.5 versus 1.1±0.2 in group SA; n=6, P<0.05). In conclusion, brief prenatal exposure of the pregnant ewe to dexamethasone leads to hypertension in adult animals of both sexes. Most interestingly, the mechanism leading to programming of hypertension might be linked with the brain angiotensin system. (Hypertension. 2002;40:729-734.)

Key Words: brain ● glucocorticoids ● hypertension, experimental ● sheep

Epidemiological evidence suggests that babies born small for gestational age have an increased incidence of adult-onset diseases or dysfunction, including syndrome X (hypertension, non–insulin-dependent diabetes mellitus, and hyperlipidemia).1–3 It is hypothesized that a suboptimal intrauterine environment during a critical stage of development permanently alters, or “programs,” the development of fetal tissues. This may ensure the short-term survival of the fetus but also may bring adverse consequences in postnatal life.

Animal models using maternal undernutrition or restriction of specific dietary components (iron, protein), either throughout pregnancy or during parts of gestation, have confirmed that restriction of fetal growth leads to elevated blood pressure in the progeny of rats.4–6 A second type of animal model has examined the long-term/programming effects caused by prenatal glucocorticoid exposure. When adult rats were exposed to large doses of carbenoxolone (an 11β-hydroxysteroid dehydrogenase [HSD] inhibitor, which blocks placental inactivation of endogenous glucocorticoids) throughout gestation, offspring were of low birth weight and had high blood pressure.7–9 The synthetic glucocorticoid dexamethasone, which is poorly metabolized by placental 11β-HSD, given throughout rat pregnancy resulted in offspring with high blood pressure.10

In the sheep, we11 have shown that exposure to dexamethasone, for 2 days very early in gestation (at a mean age of 27 days of the 150-day gestation period) results in hypertensive female offspring by 3 to 4 months of age. This hypertension amplifies with age and is associated with an increased cardiac output.12 By 7 years of age, these animals had developed left ventricular hypertrophy with reduced cardiac functional reserve.13 In these studies, only female offspring were studied. However, in many models, the programming effects of the prenatal treatment are only seen in male offspring,14 or they were more pronounced in male offspring compared with female offspring.4 These studies proposed that programming, at least in some models, may be gender specific. For this reason, in the present study we examined the blood pressure in castrated male offspring, at ≈2 years of age, that had been exposed to maternal dexamethasone treatment (0.48 mg/hour for 48 hours) at 27 days of gestation. We hypothesized that this treatment would result in hypertension in male and female offspring from the flock. The male offspring were given chronic testosterone replacement to test the hypothesis that the hypertension may be exaggerated in the presence of testosterone.

To elucidate possible mechanisms involved in our model of hypertension, we examined the effect of prenatal dexam-
methasone treatment on gene expression in specific parts of the brain (medulla oblongata and hypothalamus) from a cohort of late gestation fetuses and cohort of 7-year-old hypertensive female offspring. Although several studies suggested the role of the kidney renin-angiotensin system in programming high blood pressure in offspring exposed to various prenatal perturbations, no study has yet looked for a possible alterations in the brain angiotensin system. We have chosen to examine gene expression for components of the angiotensin system in the brain, particularly because the central angiotensin system is known to be important in blood pressure regulation and fluid homeostasis.17

Methods

Animals
All experiments were approved by the animal ethics committee of the Howard Florey Institute in accordance with National Health and Medical Research Council guidelines. Pregnant Merino ewes (n=59), weighing between 45 to 55 kg, were infused intravenously with isotonic saline (0.19 mL/hour, n=32) or dexamethasone (0.48 mg/hour, n=27) for 48 hours as described previously.11 Maternal blood samples (10 mL) were taken for ions and glucose before and at completion of the infusion.

The first cohort of ewes was allowed to lamb. There were 17 male (group S, n=8; group D, n=9) and 20 female (group S, n=8; group D, n=12) animals. To avoid seasonal variations in sex hormone levels, female animals were oophorectomized at 1 year of age (performed under general anesthesia; see below), and males had castration and tail docking performed at 2 months of age.10 The animal ethics committee of the Howard Florey Institute requires all animals to be castrated and tail docked at weaning.) In addition, because it is our intention to study these animals for several more years, animals are kept on a farm for long periods between experiments. Carotid artery loops were constructed in both males and females at 1 year of age under general anesthesia. General anesthesia was induced with 5% thiopentone sodium (pentothal, Rhone Merieux) via the jugular vein, an endotracheal tube was inserted, and anesthesia was maintained on an isoflurane anesthetic (Isoflo inhaled, Merieux) via the jugular vein, an endotracheal tube was inserted, and anesthesia was maintained on an isoflurane anesthetic (Isoflo inhaled, Merieux) via the jugular vein, an endotracheal tube was inserted, and anesthesia was maintained on an isoflurane anesthetic (Isoflo inhaled, Merieux) via the jugular vein, an endotracheal tube was inserted, and anesthesia was maintained on an isoflurane anesthetic (Isoflo inhaled, Merieux) via the jugular vein, an endotracheal tube was inserted, and anesthesia was maintained on an isoflurane anesthetic (Isoflo inhaled, Merieux). A second cohort of ewes (group SF, n=5; group DF, n=4) were maintained until fetuses were 130 days of gestation, at which time they were killed (100 mg/kg pentobarbitone, Lethabarb, Arnolds), and fetal organs were weighed and collected. The brain was dissected (medulla oblongata and hypothalamus), and pieces were frozen in liquid nitrogen. All ewes killed at this stage carried twin fetuses. Thus, there were tissues from 10 saline and 8 dexamethasone fetuses.

The third cohort of ewes was also allowed to lamb. Oophorectomized offspring (group SA, n=6; group DA, n=5) have been studied extensively before being killed at 7 years of age for tissue collection.11,12,13

Both male and female animals from the first cohort had basal blood pressure measurements. Blood pressure response to testosterone was studied after basal blood pressure measurements only in males from the same cohort. Animals from the second and third cohorts were used for gene expression studies.

Gene Expression Studies
Total RNA was extracted from brain tissues (medulla oblongata and hypothalamus) by use of the phenol-chloroform method,22 and 1 μg of sample was reverse transcribed.23 Control reactions that did not include reverse transcriptase were included in a separate reverse transcription reaction with all total RNA samples. A comparative Ct (cycle of threshold fluorescence) method was used to determine relative mRNA expression levels of the angiotensin II type 1 and type 2 receptors (AT1 and AT2) and angiotensinogen in fetal brain tissues. 18S ribosomal RNA was used as an endogenous reference.

The expression of AT1 and angiotensinogen was studied also in medulla oblongata and hypothalamus of adult animals killed at 7 years of age. The real-time polymerase chain reaction (PCR) has been described in more detail elsewhere.12,24 Optimal concentrations of primers and probe have been determined in preliminary experiments and are previously shown.25 The ΔCt value of each sample was used to give a final value for each sample relative to the calibrator. Coefficients of variation (CVs) for 1 sample run 5 times in 1 assay were 16.1%, 23.5%, and 23%, respectively, for AT1, AT2, and angiotensinogen. We have used the mean ΔCt value of the saline group for any particular gene as the calibrator.

Blood Pressure and Heart Rate Measurement Protocol
Animals were allowed at least 7 days to acclimatize to the laboratory environment before mean arterial pressure (MAP) and heart rate (HR) were measured continuously for 3 days as previously described.11–13 Animals (both male and female) were between 16±1 and 24±4 months of age. The pressure was corrected to compensate for the height of transducer above the level of the heart. The analogue signal was digitally converted via a DT 301 Board Data Translation device (Marlboro), and blood pressure and HR data were collected at 100 Hz (HEM 3.1; Notocord, Kent Scientific Corp). HR was calculated by the software using the formula dp/dtMax and took into account the pick of the dp/dt curve during systole versus time.

Testosterone Implant
After 3 days of control MAP measurement, male animals, age 24±4 months (group S; n=8) and 21±4 months (group D; n=9), were implanted with testosterone (testosterone propionate pellets, 23.5 mg/pellet; Dover Laboratories) subcutaneously in the dorsal aspect of the outer ear. HR, blood pressure, and plasma testosterone concentrations were measured for 2 consecutive days after the testosterone implant (T1 and T2, period of increasing plasma testosterone levels) and again a week later for 2 consecutive days (T8 and T9, a plateau in plasma testosterone concentrations). Cannulae were then removed, and after 14 days, animals were recanulated and measurements made at T22 and T23 (period of declining plasma testosterone concentrations).

Testosterone Assay
Blood (10 mL) was chilled on withdrawal from the animal, and plasma was obtained after centrifugation. Plasma testosterone concentration was analyzed by use of a described immunoradiometric method adapted from O’Donnell and colleagues for use in sheep.

Ions and Glucose Assays
Plasma was analyzed for osmolality by freezing point depression using an Advanced Osmometer (Advanced Instruments). Concentrations of sodium, potassium, chloride, magnesium, phosphate, urea, creatinine, glucose, and total protein were measured by use of the synchronized CX5 clinical system (Beckman).

Statistical Analysis
An unpaired t test was used to test for differences in gene expression and blood pressure of the 2 treatment groups. When data were not normally distributed, the Mann-Whitney test was used and data were presented as median, 25%, and 75%; otherwise, data are presented as mean±SEM. Repeated-measures ANOVA was used to test for blood pressure differences during testosterone replacement. Analysis was performed using Sigmastat software. Statistical significance was set at P<0.05.
Basal Blood Pressure in Adult Offspring Exposed to Prenatal Dexamethasone Treatment

Blood pressure was examined in male lambs at 24±4 (group S, n=8) and 21±4 (group D, n=9) months of age. The body weights were not different (group S, 45±5 kg; group D, 41±5 kg). As shown in Figure 1, basal MAP was significantly higher in males from group D (106±5 mm Hg) compared with group S (91±3 mm Hg; P<0.01). HR was not different between the groups, being 86±3 bpm in group S and 85±3 bpm in group D (Figure 1).

Blood pressure was examined in female lambs at 16±1 (group S; n=8) and 17±1 (group D; n=12) months of age. Body weights were not different (group S, 38±1 kg; group D, 41±5 kg). As found previously, the basal MAP was significantly higher in females from group D (86±2 mm Hg) compared with group S (81±2 mm Hg; P<0.05) (Figure 1). HR was not significantly different between the groups, with values of 81±2 bpm in group S and 83±3 bpm in the group D. As shown in Figure 1, in each treatment group, it was found that the males had significantly higher MAP than did the females (P<0.05).

Plasma Testosterone Levels and Blood Pressure During Testosterone Replacement

As shown in Table 1, basal endogenous plasma testosterone levels were similar between the 2 groups. On days 1 and 2 after testosterone implantation (T1, T2), plasma testosterone levels rose in both groups to values higher than normally seen in the noncastrated male sheep (<1 ng/mL).28 These plasma testosterone levels were maintained at T8 and T9 but declined to physiological levels by T22 and T23. The MAP did not alter significantly from baseline on any day during testosterone replacement in either treatment group. In group S, these values were as follows: T1, 91±3 mm Hg; T2, 93±6 mm Hg; T8, 91±3 mm Hg; T9, 89±3 mm Hg; T22, 90±2 mm Hg; and T23, 89±2 mm Hg (Figure 2).

Effects of the Dexamethasone Treatment on Fetal Development

Body, organ, and placental weights at 130 days of gestation were not different between the 2 treatment groups (Table 2). The twin fetuses of ewes treated with saline (group SF; n=10) weighed 2.8±0.1 kg, whereas those from the dexamethasone group (group DF; n=8) were 2.8±0.2 kg.

Effects of the Dexamethasone Treatment on Gene Expression

Figure 3 shows the relative expression levels of AT1 and angiotensinogen in the medulla oblongata and hypothalamus.

Results

As described previously, dexamethasone treatment resulted in a significant increase in maternal glucose concentrations and suppressed plasma adrenocorticotrophic hormone concentrations to undetectable levels (all, P<0.05).23

TABLE 1. Plasma Testosterone Levels Before and After Testosterone Implant

<table>
<thead>
<tr>
<th>Animals</th>
<th>C</th>
<th>T1</th>
<th>T2</th>
<th>T8</th>
<th>T9</th>
<th>T22</th>
<th>T23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group S</td>
<td>0.2±0.05</td>
<td>1.3±0.31</td>
<td>1.0±0.25</td>
<td>1.4±0.24</td>
<td>1.4±0.27</td>
<td>0.8±0.13</td>
<td>0.7±0.12</td>
</tr>
<tr>
<td>Group D</td>
<td>0.3±0.05</td>
<td>1.6±0.21</td>
<td>1.5±0.21</td>
<td>1.9±0.21</td>
<td>1.7±0.26</td>
<td>0.4±0.06</td>
<td>0.4±0.05</td>
</tr>
</tbody>
</table>

Plasma testosterone levels (ng/mL) in adult wethers prenatally exposed to saline (group S; n=8) or dexamethasone (group D; n=9) for 48 hours from 26 days of gestation at control plasma testosterone levels (C) and at days 1 (T1), 2 (T2), 8 (T8), 9 (T9), 22 (T22), and 23 (T23) following testosterone implant. Results are presented as mean±SEM.
in group SF (n = 10) and group DF (n = 8) fetuses at 130 days of gestation.

There was higher expression of the AT$_1$ receptors in the medulla oblongata of group DF (2.6 ± 0.6 versus 1.1 ± 0.2 in group S fetuses; P < 0.01). Interestingly, higher expression of the AT$_1$ receptor was also found in the medulla oblongata of group DA (adult animals at 7 years of age) (2.6 ± 0.5 versus 1.1 ± 0.2 in group SA; P < 0.05) (Figure 3). There was no change in the expression of A'ogen in the medulla oblongata from both fetal and adult samples (Figure 3).

In the hypothalamus, group DF showed an increased expression for angiotensinogen (2.5 ± 0.3 versus 1.3 ± 0.3 in group SF; P < 0.05) (Figure 3). This effect of prenatal dexamethasone treatment was not evident in adult female offspring at 7 years of age (group DA, n = 5, 1.0 ± 0.1 versus 1.2 ± 0.1 in the group SA, n = 6) (Figure 3). There was no change in the expression of AT$_1$ receptors in the hypothalamus from both fetal and adult samples (Figure 3).

In addition, no expression of the AT$_2$ receptors was detected in any region of the fetal or adult brain examined in this study.

### Discussion

Exposure of pregnant ewes to pharmacological levels of dexamethasone, for only 2 days very early in gestation (at a mean age of 27 days of the 150-day gestation period), results in hypertensive female offspring at 3 to 4 months of age.$^{11}$ Subsequently, we demonstrated that this type of hypertension amplifies with age and is associated with increased cardiac output, increased left ventricular hypertrophy, and reduced
cardiac functional reserve. In the present study, we demonstrated that the effect of prenatal dexamethasone on blood pressure in the adult females is reproducible and that the same treatment also leads to high blood pressure in male offspring irrespective of testosterone status.

In other animal models, the long-term/programming effect of various prenatal perturbations on adult blood pressure is not always seen in both sexes. Undernutrition of the pregnant rat, confined to the preimplantation period (0 to 4.5d), produced hypertension only in male offspring. In addition, only the male offspring of rats exposed to maternal protein restriction (9% versus the normal 18% casein diet) early in gestation show a significant elevation in blood pressure. However, with low protein exposure later or throughout gestation, hypertension develops in offspring of both sexes.

It is also interesting that the male offspring had higher blood pressures than did the females in both the control (saline) group and the dexamethasone-treatment group. Although the males were slightly older than the females, it is unlikely that this accounts for the sex difference in blood pressure. It has been reported previously that blood pressure in females is not different between 19 months and 7 years of age. Epidemiological studies have shown men to have higher blood pressure than that of age-matched women. The male offspring of rats fed 70% of total nutrient requirements for pregnancy (0 to 18 days) developed elevated blood pressure earlier than did the female offspring, and the elevation was greater in the males than in the females at all ages tested (60, 100, and 200 days).

One obvious reason that might explain the gender differences is the presence of different sex hormones. In men, testosterone has been shown to have antiangiogenic effects, and endogenous levels have an inverse relationship to systolic blood pressure. Castration of rats at a young age (3 to 5 weeks) reduces the development of hypertension in spontaneously hypertensive rats (SHR), Dahl salt-sensitive rats, whereas treatment of castrated male and oophorectomized female SHR with testosterone exacerbates their hypertension. Furthermore, chronically blocking the androgen receptor with the antagonist flutamide attenuates blood pressure in male SHR to lower levels than those found in female SHR. However, we did not see any effect of testosterone on blood pressure in our castrated sheep. A recent study by Giussani et al demonstrated that basal arterial pressure is higher in male than female late gestational ovine fetuses, suggesting that in our study, high blood pressure in males might be determined before castration, perhaps in utero.

Although the exact mechanisms for programming the hypertension in adult offspring is not known, studies suggest that resetting of major hormonal axes, such as the hypothalamic pituitary adrenal axis and the renin-angiotensin system, might be involved. However, we have already reported that the hypothalamic pituitary adrenal axis is not the mechanism that accounts for high blood pressure seen in the adult offspring after short prenatal dexamethasone exposure. Another system, shown to be programmed by prenatal undernutrition and, potentially, glucocorticoid exposure, is the renin-angiotensin system. Maternal undernutrition in sheep causes upregulation of the AT1 receptor in a number of tissues, including kidney, adrenal, and lung. We have shown that the dexamethasone treatment used in the current study causes higher expression of angiotensinogen and at the AT1 and AT2 receptors in the kidney of the late gestation fetus. Studies in the rat have shown that administration of the ACE inhibitor captopril 2 to 4 weeks postpartum, prevents the development of hypertension, programmed by intrauterine exposure to a maternal low-protein diet, speculating that these effects may have been mediated by the local kidney and/or brain angiotensin systems.

In our adult dexamethasone-exposed animals, we have shown that circulating levels of components of the renin-angiotensin system are not different. In addition, short peripheral AT receptor blockade did not affect their blood pressure. Interestingly, when the human angiotensinogen gene is expressed solely in the kidney or brain, transgenic mice are hypertensive and do not have increased levels of the circulating angiotensin II. In addition, in these transgenic mice, blood pressure is not lowered by the peripheral AT receptor blockade. Most recently, it has been shown that overexpression of the AT receptors selectively in the brain of transgenic mice leads to enhanced cardiovascular responses to intracerebroventricular infusion of angiotensin II. In the same study, a fall in blood pressure was observed after intracerebroventricular infusion of losartan.

There is no information in the literature concerning the effects of various maternal perturbations on the expression of components of the angiotensin system in the brain. In the present study, we show that brief dexamethasone exposure at the end of the first month of gestation resulted in increased gene expression for AT1 in medulla oblongata and angiotensinogen in hypothalamus of the late gestational ovine fetus (4 months after the treatment ceased). These findings are remarkable given that at the time of dexamethasone treatment, the ovine fetal brain is quite immature. It is not known, however, if this very primitive brain expresses the genes for components of the angiotensin system. Components of the angiotensin system are present in the adult sheep brain, where they play a role in control of blood pressure, modulation of drinking behavior, salt appetite, sensory functions, memory and learning, and stimulation of pituitary hormone release. The fact that elevated expression of the AT receptor was also found in the dexamethasone-exposed sheep at 7 years of age suggests that alteration of the brain angiotensin system might play an important role in programming of adult hypertension after a brief prenatal exposure to dexamethasone.

**Perspectives**

In conclusion, we demonstrated that brief prenatal exposure of pregnant ewe to dexamethasone leads to hypertension found in both female and male adult offspring. In males, this type of hypertension was not modulated by the addition of testosterone. Most interestingly, the mechanism leading to programming of hypertension, after a short prenatal dexamethasone exposure, might be linked with the local kidney and brain angiotensin systems.

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References
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