Exaggerated sympathetic mediated responses to behavioral or pharmacological challenges following antenatal betamethasone exposure

Hossam A. Shaltout, Mark C. Chappell, James C. Rose, and Debra I. Diz

Hypertension and Vascular Research Center, Department of Obstetrics and Gynecology, Wake Forest University School of Medicine, Winston-Salem, North Carolina

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Shaltout HA, Chappell MC, Rose JC, Diz DI. Exaggerated sympathetic mediated responses to behavioral or pharmacological challenges following antenatal betamethasone exposure. Am J Physiol Endocrinol Metab 300: E979–E985, 2011. First published March 8, 2011; doi:10.1152/ajpendo.00636.2010.—Glucocorticoid administration to women at risk for preterm delivery is standard practice to enhance neonatal survival. However, antenatal betamethasone exposure (β-exposure) increases mean arterial pressure (MAP) in adult sheep (1.8 yr old) and results in impaired baroreflex sensitivity (BRS) for control of heart rate (HR). In the current studies we tested the hypothesis that enhanced sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis-mediated responses are evident at an early age in β-exposed sheep. Pregnant ewes were administered betamethasone (0.17 mg/kg twice over 24 h) or vehicle (Veh-control) on the 80th day of gestation, and offspring were delivered at full term. Female β-exposed and control offspring instrumented at age 42 ± 3 days for conscious continuous recording of MAP and HR had similar resting values at baseline. However, BRS was ~45% lower in β-exposed offspring. β-Exposed lambs allowed to suckle for 10 min had a greater elevation in MAP than Veh-control lambs (19 ± 1 vs 12 ± 2 mmHg; n = 4–5, P < 0.05). MAP was reduced by 20% from baseline via sodium nitroprusside infusion (SNP) over 10 min, which triggered a rebound increase in MAP only in β-exposed lambs. HR increased with the reduction in MAP during SNP infusion in Veh-control lambs, whereas there was no change in HR with the reduction in MAP in β-exposed lambs. Combined vasopressin-CRF injection caused greater increases in MAP in the β-exposed lambs. Cortisol and ACTH responses were higher in response to SNP hypotension in the β-exposed lambs. The data reveal enhanced sympathetic and HPA axis responses associated with impaired BRS preceding differences in resting MAP in preweanling female lambs exposed in utero to glucocorticoids. The consequences of these alterations at an early age include eventual development of higher blood pressure in this ovine model of fetal programming.

The intrauterine environment is extremely important in determining the health of the individual later in life, and the role of prenatal programming as a determinant of adult diseases has become increasingly clear (20). Prematurity and very low birth weight increase risk for cardiovascular disease later in life (2). Exposure of fetuses that are candidates for premature delivery in the early part of the third trimester to synthetic steroids accelerates lung development, activates the sympathetic nervous system, and protects neonates from early developmental problems (1). The increase in survival and fewer complications in the neonatal period has led to the widespread use of glucocorticoids as standard practice in threatened preterm delivery (1). However, recent studies have questioned whether the antenatal exposure to steroids has unintended consequences for cardiometabolic disease in young adulthood (12) despite the immediate benefits to survival. In addition, with the increase in use of the antenatal steroids, more infants delivered at full term experience betamethasone exposure (β-exposure) without the complication of prematurity, and this exposure have been shown to cause a reduction in size at birth independent of prematurity (14).

The most common time point in gestation for threatened premature delivery in women is in the third trimester during nephrogenesis. In an ovine model of fetal programming with β-exposure at 0.6 gestation during nephrogenesis but without the premature delivery, exposure of the fetus to synthetic steroids perturbs normal nephrogenesis and leads to reduced nephron number (16), elevated mean arterial pressure (MAP), and impaired baroreflex sensitivity (BRS) for control of heart rate (HR) in adult sheep at >6 mo of age (45, 52). It also causes a reduction in the ability of the betamethasone-exposed (β-exposed) animals to excrete a sodium load and reduces the natriuretic effect in response to Ang-(1–7) infusion at that age (51). Altered expression of enzymes of the renin-angiotensin system (RAS) (47) along with responses to angiotensin peptide receptor antagonists are consistent with higher angiotensin (Ang) II and lower Ang-(1–7) “tone” as contributors to the higher MAP and attenuation of reflex function in the β-exposed adult animals. Thus, the elevated pressure and impaired BRS associated with an increased sympathetic/parasympathetic ratio in adults following β-exposure may involve an imbalance in actions of these two opposing peptides of the RAS.

The mechanisms behind the beneficial activation of the sympathetic nervous system in the premature newborn in response to glucocorticoid exposure in utero (42) are not known. The brain RAS promotes development of the catecholamine-containing pathways in brain, and rats with low brain angiotensinogen have delayed development of sympathetic pathways (30). Ang II also participates in regulation of the hypothalamic and pituitary control of vasopressin and ACTH release (6, 18). Thus, an activated brain RAS associated with β-exposure has the potential to influence responsiveness of these stress-related pathways. Since it is not known how early the increase in MAP and impairment in BRS occurs in the sheep model of β-exposure described above, the current study was designed to determine MAP and BRS status at rest in conscious preweanling β-exposed animals. In addition, stressors known to activate the sympathetic nervous system or hypothalamic-pituitary-adrenal (HPA) axis were used to provide additional insight into the early functioning of these pathways. Therefore, we also studied the ACTH, cortisol,
MAP, and HR responses of control and β-exposed lambs to feeding (suckling), combined corticotropin-releasing factor (CRF)-vasopressin infusion, and hypotension, all known stimuli for activation of the sympathetic nervous system and the HPA axis (11). Elucidation of the functional pathways in β-exposed sheep at an early age is a key to understanding the cardiovascular changes that occur in the adults.

**MATERIALS AND METHODS**

**Animals.** Sheep were administered either betamethasone (2 doses of 0.17 mg/kg im, 24 h apart; beta group) or two vehicle injections, which contained 3.4 mg of monobasic sodium phosphate, 7.1 mg of dibasic sodium phosphate, 0.1 mg of sodium ethylene diamine tetraacetic acid, and 0.2 mg benzalkonium chloride/ml (control group) at 80–81 days of gestation. Animals were brought to term, and after delivery they were farm raised, and at 5 wk of age the preweaning lambs were brought to our Association for Assessment and Accreditation of Laboratory Animal Care-approved facility with their mothers. The ewes were maintained on a normal diet, and both the lambs and ewes had free access to tap water and were housed with a 12:12-h light-dark cycle (lights on 7 AM to 7 PM). All lambs in this study were female, and the experiments were performed at ~6 wk of age (42 ± 3 days). All experiments were initiated between 1100 and 1300, and experiments were conducted in a quiet environment. These procedures were approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee.

**Determination of MAP and BRS.** Sheep at 5 wk of age were anesthetized with ketamine and isoflurane, and catheters were inserted in the femoral artery and vein for blood pressure recording and drug administration, respectively. Sheep were housed in large metal cages with their mother after the surgical procedure. During 4 days recovery after surgery, the animals were placed in a supportive sling for 1 h every day to acclimate to the separation from mother and become accustomed to the sling. On the 5th day after surgery, lambs were put in a hanging sling and left undisturbed for 45 min, and after that blood pressure was recorded. The arterial catheter was connected to pressure transducers, and conscious pressure and HR were recorded using Biopac (Santa Barbara, CA) acquisition software. The arterial pressure and HR were digitized and recorded with Acknowledge software (version 3.8.1; Biopac). After the stabilization period of ~45 min, the evoked BRS was assessed in lambs using multiple doses of phenylephrine (1, 2, 4, and 8 µg/kg) and calculating the slope of the line-correlating changes in MAP and beat-to-beat interval (48).

**Protocol for challenges.** On consecutive days, lambs were again separated from the mother, put in the hanging sling, and connected to transducers to record blood pressure for 45 min to calculate baseline MAP. Each of the following procedures were then performed, after which arterial lines were filled with heparinized saline, closed and secured on the back of the lamb under a protective net, and the lamb was returned to the ewe.

**Nursing.** Lambs were then presented with a bottle of artificial milk. They were allowed to suckle for ≤10 min, and the pressure recording was continued for an additional 45 min.

**Sodium nitroprusside.** Sodium nitroprusside (SNP) was infused for 10 min at a rate that caused a 25% reduction in MAP compared with baseline (5–7 μg·kg⁻¹·min⁻¹). Then, pressure was recorded for 45 min after the SNP infusion was stopped.

**Oxine CRF and vasopressin.** An aliquot of oxine CRF (oCRF: 0.17 nM/kg iv) and vasopressin (0.17 nM/kg iv) was injected as a bolus (0.05 ml/kg), followed by 5 ml of saline flush, and blood pressure and HR were monitored as described above during the experiments.

**Cortisol and ACTH responses to the above challenges.** After collection of control arterial blood samples (4–6 ml, each sample) obtained after the acclimation period each day, samples were drawn at the time points indicated in each figure over the time course of the above studies for hormone assays. All samples were collected with EDTA as the anticoagulant into chilled tubes and centrifuged as quickly as possible for division into two portions. Plasma ACTH and cortisol were measured with commercial RIA kits (DSL, Webster, TX), with detection limits of 3.5 pg/ml (ACTH) and 0.6 ng/ml (cortisol) (7).

**Statistics.** The data were assessed by two-way analysis of variance (ANOVA) for group and treatment effects, followed by one-way ANOVA or Student-Newman-Keuls post hoc analysis where indicated. Student’s t-test was used to compare variables with only two conditions. The criterion for statistical significance was set at P < 0.05. (GraphPad Software, San Diego, CA).

**RESULTS**

The resting conscious blood pressure in female lambs at 6 wk of age was not significantly different between the β-exposed and control groups (Fig. 1, top). HR was also similar at baseline (Fig. 1, middle). In contrast, BRS was significantly lower in the β-exposed sheep (P < 0.05) at this age (Fig. 1, bottom). We used several stimuli to test sympathetic and HPA responsiveness in these animals.

**Nursing.** For the first test, lambs were allowed to suckle artificial milk for 10 min, and the pressor and HR responses were measured (Fig. 2, top). MAP was elevated above baseline in both groups within the first 5 min of suckling, remained elevated throughout the period of suckling, and returned to baseline levels by 25 min. The maximal increase of 19 ± 1 mmHg was higher in the β-exposed compared with control lambs (12 ± 2 mmHg; n = 4–5, P < 0.05), as was the value at 10 min. There was no significant change in HR in response to the suckling in either group. Whereas cortisol was higher in the control group at baseline and remained elevated throughout the study period, suckling significantly increased cortisol levels in β-exposed lambs at 10 min. Cortisol then returned to baseline by 25 min. There were no differences in plasma ACTH between groups at any time point. (Fig. 2, bottom).

**Hypotension.** For the second stimulus, MAP was reduced by 20% from baseline via SNP infusion over 10 min (Fig. 3, top left). The absolute values of MAP shown in Fig. 3 confirm that the MAP was lowered to similar levels in both groups. This reduction of MAP to the same level by SNP triggered a subsequent increase in MAP above baseline only in the β-exposed lambs. The rebound elevation in β-exposed animals started 5 min after the end of the SNP infusion, averaged 7 ± 1 mmHg (P < 0.05 vs. baseline), and lasted for 10 min. HR increased with the reduction in MAP during SNP infusion in control lambs, whereas there was no change in HR with the reduction in MAP in β-exposed lambs (Fig. 3, top right). Resting cortisol was higher in the control animals at baseline and remained at this level throughout the experiment, whereas it increased significantly in the β-exposed sheep at 10 min and declined by 55 min. There was no difference in ACTH levels between the two groups at baseline, but hypotension due to SNP infusion increased ACTH significantly in β-exposed lambs compared with control and then returned to baseline by 55 min (Fig. 3, bottom). oCRF plus vasopressin. In the third test, MAP and HR responses to a combined oCRF plus vasopressin infusion were assessed (Fig. 4). In Fig. 4, top left, the increase in MAP in response to the challenge in the β-exposed animals was significantly greater at 1, 10, and 15 min following the injection than that observed in the control lambs. HR declined in
response to the increase in MAP in both groups at 1 min, and the response was similar in β-exposed and control animals during the rebound (Fig. 4, top right). Both cortisol and ACTH increased in response to this challenge, but there was no difference in the responses for cortisol or ACTH between groups (Fig. 4, bottom).

**DISCUSSION**

Antenatal β-exposure on the 80th day of gestation is associated with an increase in blood pressure and impaired baroreceptor reflex control of HR in adult sheep at >6 mo of age (16, 48, 52). In the present study, we find that MAP and HR are not different between control and β-exposed lambs at 6 wk of age, but there is evidence of impaired BRS for control of HR in response to increases in pressure in the lambs exposed in utero to betamethasone. The impaired reflex function as assessed here is largely a measurement of vagal function. It has been shown that the BRS was impaired within a few hours of antenatal exposure to betamethasone in fetal sheep that were then delivered by cesarean section (40). Our studies at 6 wk of age and into adulthood (1.8 yr), as we have shown recently (48), demonstrate that effects on BRS persist in this model of fetal programming. Exogenous corticosteroids also enhance the sympathetic response to cold at birth in prematurely delivered lambs (42). This enhanced sympathetic activation effect seems to persist as shown in our studies, where increases in MAP in response to several stimuli that activate the sympathetic system [suckling, rebound MAP following an acute drop in MAP, and responses to CRF/arginine vasopressin (AVP) injection] were exaggerated in the β-exposed animals. The HR responses to these challenges were variable. The responses included no change in HR accompanying the increase in MAP in response to the suckling in either group, a similar reduction in HR in the face of the different increases in MAP following the CRF/AVP stimulus, and an impaired tachycardia in response to the reduction in MAP with SNP in the β-exposed animals. The data suggest that enhanced sympathetic nervous system responsiveness, perhaps resulting from baroreflex impairment at an early age, precedes the eventual development of higher blood pressure in this ovine model of fetal programming.

Feeding (suckling) has been shown to increase MAP in lambs at 6 wk of age. The increase in MAP lasts throughout feeding and is attributed to activation of sympathetic system activity since it was partially abolished by α-receptor blockade (4). Suckling also is usually accompanied by a transient tachycardia, and the transient nature suggests that it is terminated by baroreflex activation. We did not detect the transient increase in HR in either group in our study. The increase in MAP was also higher in the β-exposed lambs in response to the CRF/AVP injection. The pressor response was accompanied by a reduction in HR in both groups, but the fall in HR was similar in the two groups despite the larger increase in MAP in the β-exposed animals. This finding would be consistent with less activation of the vagus or less sympathetic withdrawal associated with reflex for control of HR. Finally, acute hypotension with the use of SNP infusion also results in a reflex activation of the sympathetic arc to return blood pressure to normal. In the current study, β-exposure resulted in exaggerated rebound increase in MAP, as evidenced by the enhanced sympathetic nervous system responses. Whereas we saw a trend for tachycardia at the 10-min time point in the control animals during the hypotension with SNP, there was no change in HR in the β-exposed animals. These findings suggest that activation of the sympathetic limb or suppression of the vagal limb of the cardiac baroreflex is also impaired, consistent with the impaired BRS, as shown by the evoked bradycardic response to pressor responses. A more complete investigation of both limbs of the reflex and the sympathovagal balance is required to fully understand the defects in reflex function accompanying the antenatal steroid exposure. In addition, SNP has direct effects on the reflex (8, 22), and this must be taken into account.
consideration in the interpretation of the HR changes observed in our experiments.

In contrast to the exaggerated increase in sympathetically mediated responsiveness, there did not appear to be enhanced activation of the HPA axis assessed by release of ACTH and cortisol in response to the various challenges. This is based on several observations. First, plasma cortisol was lower in β-exposed animals at the baseline time point on the first 2 days of study (suckling and SNP) but was similar at baseline on the third day of testing when the CRF + vasopressin injection was given (36). This may reflect a normal stress reaction in response to the separation from the ewe and acclimation to the procedures over the 4 days of study in the controls, whereas the lower level in the β-exposed animals suggests suppression of the response to this potential stressor. In previous studies conducted in adult sheep, CRF + vasopressin injections increased ACTH and cortisol release into plasma to a similar level as in the present study (36). In addition, the ACTH and cortisol responses to hypotension are similar to those observed previously (36). Since hypotension is known to result in release of AVP in dogs (17), and AVP also releases ACTH in a synergistic manner with CRF (9, 21, 26, 33, 34), it is not surprising that the hypotension and CRF + vasopressin challenges produced comparable hormonal responses. The fact that the cortisol response tended to be blunted in the control animals during hypotension may result from the elevated baseline levels in these animals. The lack of effect of antenatal betamethasone treatment in our model of fetal programming on HPA axis is somewhat different than what has been reported in newborns, where antenatal betamethasone altered the newborn lamb neuroendocrine and endocrine responses to hypoxia (15). This difference in effect could be due to age or treatment regimen differences.

The mechanisms by which fetal exposure to glucocorticoids would lead to greater activation of the sympathetic nervous system in response to challenges are not fully elaborated. We have shown that exposure of the ovine fetus to synthetic steroids on the 80th day of gestation, during peak nephrogenesis, perturbs normal nephrogenesis and leads to reduced nephron number, elevated MAP, increased left ventricular heart weight, and impaired BRS for control of HR and other indices of autonomic dysfunction in adult sheep at ~6 mo and 1.8 yr of age (48). The impairments in autonomic function include reductions in indices of parasympathetic and increases in indices of sympathetic tone. Certainly, the altered sympathovagal balance would preclude normal operation of the
baroreceptor reflex and, therefore, incomplete maintenance of blood pressure within narrow limits. Disturbances in BRS for control of HR, an index of vagal function, are associated with cardiac left ventricular hypertrophy, increased proteinuria, and increases in overall mortality (23, 49). BRS for control of renal sympathetic nerve activity and HR is highest at the very late phase of gestation in sheep (39, 41), and this decreases dramatically within the first week of postnatal life and over 4–6 wk (41). Thus, it is possible that impairment in reflex function at this critical stage in postnatal development contributes to the renal and cardiac dysfunction we see at later stages in this model of fetal programming. Whether the impairment in the vagal component of the control of HR as reflected in the lower BRS of the β-exposed group contributes to this response in neural pathways responsible for regulation of autonomic pathways and contributes to the eventual development of high MAP is not known. However, prior studies support the concept that impairment in BRS is a permissive factor in development of hypertension (50).

There is profound activation of the RAS during late gestation and the early postnatal period (1st wk in sheep), with dramatically increased plasma levels of Ang-(1–7) (29) and Ang II (35). Ang II is known to reduce the sensitivity or gain of the reflex control of HR (31, 32, 43, 44) and may contribute to the impaired BRS. However, long-term peripheral infusion of Ang-(1–7) is associated with an enhancement in the BRS in adult rats (3), and Ang-(1–7) replacement centrally mediates improvement in BRS (19). In previous investigations of the potential mechanisms for the fetal exposure glucocorticoid-mediated elevation in MAP in adult animals, decreases in the expression of enzymes of the RAS, such as angiotensin-converting enzyme 2 (ACE2), are evident in both kidney and the circulation of β-exposed animals (47). ACE2 is considered a major factor responsible for the normal balance of Ang II and Ang-(1–7) (10). Indeed, blockade of the AT1 receptor mitigates the higher MAP and impaired BRS at the late gestation and early postnatal periods, suggesting that higher Ang II “tone” is contributing to the elevation in pressure and increase in the ratio of sympathetic to parasympathetic function seen in the β-exposed animals (48). In contrast, studies using the Ang-(1–7) receptor antagonist indicate loss of the facilitation of the BRS in β-exposed adult sheep (46). Together these data provide evidence that in β-exposed lambs the positive influence of Ang-(1–7) on reflex function has been lost, whereas the negative effect of Ang II is retained or enhanced, which may reflect altered ACE2. Thus, the mechanisms for the impaired BRS and the increase in pressure in later life may be in part a result of an imbalance in the actions of these two opposing peptides of the RAS.

The brain RAS may also play a role in maturation of the sympathetic nervous system and the HPA axis. Development of catecholaminergic pathways is delayed in transgenic animals that express low brain angiotensinogen [TGR(ASRagen)] (30). The ASRagen rats also have a deficit in vasopressin (38), whereas (mRen2)27 rats with overexpression of brain renin have elevated brain tissue vasopressin (27). Stress-activated pathways descending from hypothalamic sites are known to involve AT1-dependent modulation of sympathetic outflow to the vasculature within medullary centers (13, 25). Previous work suggests differences in the resting blood pressure and response to stressors such as baroreflex activation and salt and water challenge in mice with brain overexpression of glial or neural components of the RAS (28, 37). Finally, ACTH is required for Ang II-stimulated release of cortisol, suggesting a direct action of Ang II within the hypothalamus or the pituitary on release of ACTH as the intermediary for cortisol release (5, 24). Therefore, if alterations in the brain RAS occur with fetal exposure to betamethasone, a subject of ongoing investigations, there is potential for dysfunction to develop in endocrine as well as cardiovascular systems as the animals age.

Perspectives. The current study examined the effect of antenatal betamethasone exposure in lambs on the activity of the sympathetic nervous system in response to pharmacological and behavioral challenges. The sympathetic nervous system responses were enhanced in lambs exposed to antenatal betamethasone. This could be due to direct actions of the steroid on fetal development that lead to an increase in sympathetic nervous system components or to a loss of the opposing forces of the parasympathetic system. That these animals exhibit lower baroreflex function for control of HR compared with control lambs further supports the loss of parasympathetic and increased sympathetic tone. These early alterations in auto-

Fig. 4. Antenatal betamethasone exposure leads to enhanced increases in arterial pressure in response to intravenous injection of ovine corticotropin-releasing factor (oCRF) and vasopressin. The change in MAP (top left) to intravenous injection of 0.17 nM/kg oCRF + 0.17 nM/kg vasopressin was greater in the antenatal β-exposed group. Baseline line MAP was 75.8 ± 2.2 mmHg in β-exposed lambs vs. 76 ± 4.8 mmHg in control lambs. HR was lower than baseline for ≤10 min postinjection in both groups, but there was no significant difference in the fall in HR between groups (top right). Plasma cortisol and ACTH were similar at baseline, and the increases in both hormones were similar in the 2 groups (bottom). Values are means ± SE; n = 5. *P < 0.05 vs. control group; #vs. own baseline.
nomic balance may be one of the mechanisms that contribute to development of hypertension in this model of fetal programming-induced hypertension. β-Treatment is standard care for threatened premature delivery, and very low birth weight resulting from prematurity is known to be associated with increased cardiovascular disease risk later in life. Whether steroid exposure ameliorates this risk or accentuates it is a matter of current study; however, brief elevation of glucocorticoids during midgestation prior to full term delivery is an increasing consequence of the current practice standards. Our observations should raise awareness for cardiovascular risk assessment in young adults with fetal glucocorticoid exposure and argue for close attention to cardiovascular risk factor development at earlier ages than the general population.

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REFERENCES