Angiotensin-(1-7) Deficiency and Baroreflex Impairment Precede the Antenatal Betamethasone Exposure-Induced Elevation in Blood Pressure

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Abstract—Betamethasone is administered to accelerate lung development and improve survival of premature infants but may be associated with hypertension later in life. In a sheep model of fetal programming resulting from exposure at day 80 of gestation to Betamethasone (Beta-exposed), adult sheep at 6 to 9 months or 1.8 years of age have elevated mean arterial pressure (MAP) and attenuated spontaneous baroreflex sensitivity (sBRS) for control of heart rate compared to age-matched controls associated with imbalances in angiotensin (Ang) II vs Ang-(1-7) tone. At 6 weeks of age, evoked BRS is already low in the Beta-exposed animals. In this study, we assessed the potential contribution of the renin-angiotensin system to the impaired sBRS. Female lambs (6 weeks old) with Beta exposure in utero had similar MAP to control lambs (78 ± 2 vs 77 ± 2 mm Hg, n = 4-5 per group), but lower sBRS (8 ± 1 vs 16 ± 3 ms/mm Hg; P < 0.05) and impaired heart rate variability. Peripheral AT1 receptor blockade using candesartan lowered MAP in both groups (~10 mm Hg) and improved sBRS and heart rate variability in Beta-exposed lambs to a level similar to control. AT7 receptor blockade by infusion of D-ala Ang-(1-7) (700 ng/kg/min for 45 minutes) reduced sBRS 46% ± 10% in Beta-exposed vs in control lambs (P < 0.15) and increased MAP in both groups (~6 ± 2 mm Hg). Our data reveal that Beta exposure impairs sBRS and heart rate variability at a time point preceding the elevation in MAP via mechanisms involving an imbalance in the Ang II/Ang-(1-7) ratio consistent with a progressive loss in Ang-(1-7) function. (Hypertension. 2012;59[part 2]:453-458.)

Key Words: baroreflex sensitivity ■ Betamethasone ■ fetal programming ■ heart rate variability ■ lambs ■ spectral analysis

Premature delivery occurs in ~10% of pregnancies and it is the most important cause of prenatal morbidity and mortality.1 Survival rates have been greatly improved since the implementation of glucocorticoids use prenatally to accelerate lung maturation.2 Betamethasone has been used to improve lung function in premature infants, but recent longitudinal studies have shown that prenatal exposure to Betamethasone caused an elevation in blood pressure in preterm children at 14 years of age.3 Enhanced femoral vascular resistance with alteration in responses to multiple dilators and constrictors has been shown in sheep exposed prenatally to Betamethasone, which can induce a sustained systemic blood pressure increase.4,5 Investigating the mechanism of the vascular changes and the blood pressure elevation associated with prenatal exposure to Betamethasone is of clinical importance to better-diagnose and possibly protect these children from the consequences of prenatal Betamethasone exposure.

The renin-angiotensin system plays a vital role in the development of the fetus, and alterations in the renin-angiotensin system have been linked to fetal programming-induced elevations in blood pressure in placental insufficiency,6 maternal protein deprivation,7 and other models of fetal programming.8 Shifts in the balance of angiotensin (Ang) II over Ang-(1-7) in the circulation or locally within tissues are associated with impairments in autonomic function that are known risk factors for target organ damage and increased mortality. Recently, we showed that exposure of the ovine fetus at day 80 of gestation to synthetic glucocorticoid perturbs normal nephrogenesis and leads to elevated mean arterial blood pressure (MAP) and impaired baroreflex sensitivity (BRS) for the heart rate control and increased left ventricle/body weight ratio in 2-year-old adult sheep. Beta-exposed sheep at 2 years of age exhibited changes in the renin-angiotensin system in many tissues, causing a shift favoring Ang II over Ang-(1-7), similar to what happens in other models of hypertension and in aging.9,10 We showed also that in adult sheep, the AT1 receptor was involved in mediating these cardiovascular effects because MAP and BRS were improved by acute AT1 receptor blockade using...
candesartan. Both the increase in MAP and impairment in spontaneous BRS (sBRS) were evident as early as 6 to 8 months of age and the imbalance between Ang II and Ang-(1-7) was evident at that time point. More recently, we reported that lambs at 6 weeks of age have normal resting MAP, but the BRS to phenylephrine-evoked increases in MAP are already reduced by ~50%.

The arterial baroreflex for control of heart rate (HR) is developmentally regulated, partly as a result of differential maturation rates of the parasympathetic and sympathetic branches of the autonomic nervous system. The role of the renin-angiotensin system in modulating cardiovascular homeostasis is greatest soon after birth (during first week) and decreases with postnatal maturation, stabilizing by 6 weeks of age at a level similar to that seen in adult sheep.

In this study, we examined the effect of antenatal Betamethasone treatment on MAP, HR, sBRS, heart rate variability (HRV), blood pressure variability (BPV) at this early time point (6 weeks) in lambs at a dose and time similar to the clinical steroid treatment given to mothers expected to have a premature delivery to determine the contribution of enhanced Ang II actions or Ang-(1-7) deficiency to the autonomic nervous system impairment at a time point that precedes the elevation in blood pressure.

Materials and Methods

Mixed-breed, time-dated, pregnant sheep obtained from local suppliers were maintained in open pasture with free access to food and water during pregnancy and lactation. Sheep were randomly assigned to 2 groups: 1 received 2 intramuscular injections of 0.17 mg/kg of a 1:1 mixture of Betamethasone acetate and Betamethasone phosphate (Celestone Soluspan; Schering, Kenilworth, NJ), whereas the other group received 2 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 tetra acetic acid, and 0.2 mg of benzyl alcohol per milliliter. Doses were given 24 hours apart at days 80 and 81 of gestation (term is ~145 days in our flock). The Betamethasone dose administered is analogous to that used in human pregnancy. Pregnancy was allowed to continue unimpeded and offspring were born naturally at term. All procedures were approved by the Institutional Animal Care and Use Committee.

Protocol

After delivery, animals were farm-raised and at 5 weeks of age the preweaning lambs were brought to our Association for Assessment and Accreditation of Laboratory Animal Care-approved facility with their mothers. Both the lambs and ewes had free access to tap water and were housed with a 12-hour light/dark cycle (lights on 7:00 AM to 7:00 pm). All lambs in this study were females (n=6 Beta and 4 control) and the experiments were performed at ~6 weeks of age (42±3 days). All experiments were initiated between 11:00 and 13:00 hours and experiments were conducted in a quiet environment as reported previously. Lambs were anesthetized with ketamine and isoflurane and catheters were inserted in the femoral artery and vein for blood pressure recording and drug administration. Lambs were housed in large metal cages with their mothers after the surgical procedure. Five days after surgery, conscious lambs were put in a hanging sling to acclimate while blood pressure was recorded. The arterial catheter was connected to pressure transducers and conscious and HR were recorded using BIOPAC acquisition software (version 3.8.1; BIOPAC, Santa Barbara, CA). Digitized MAP and HR were used for the measurements of sBRS (as low-frequency alpha index [LFA], high-frequency alpha index [HFA], sequence [seq] UP, seq DOWN and seq TOTAL), HRV (measured as the standard deviation of beat-to-beat interval [SDRR], and root mean square of successive differences [rMSSD] and the ratio of the power of RRI spectrum in the low frequency range to the high frequency range [LF_HF/HF], and BPV as measured the power of systolic arterial pressure (SAP) spectrum in the low frequency range (LF_SAP), and the standard deviation of mean arterial pressure (SDMAP). The sBRS, HRV, and BPV were calculated by the frequency-domain analysis or sequence-time domain methods as in our previous work and reported studies and using analysis software designed for large animals (Neurokord BRS; Medistar, Ljubljana, Slovenia). Measurements were made before and 45 minutes after AT1 receptor blockade using injection of 0.3 mg/kg (intravenous) candesartan or Ang-(1-7) receptor blockade using 700 ng/kg/min D-Ala7(7)-Ang-(1-7) continuous infusion over 1 hour. Complete blockade of AT1 receptor was tested using Ang II injection (20 ng/kg) intravenously before candesartan injection and ~60 minutes after candesartan injection at the end of the experiment. The 2 experiments were performed on separate days.

Statistical Procedures

All measurements were expressed as the mean ± SEM. All statistical analyses were performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Two-way repeated-measures analysis of variance with Student-Newman-Keuls post hoc analysis was used to compare between groups at baseline and before and after candesartan or D-alanine treatment. A Student t test was used to compare variables with only 2 conditions. The criterion for statistical significance was set at P<0.05.

Results

Effect of Candesartan and D-alanine on MAP and HR

There were no difference in resting MAP or HR between Beta-exposed lambs and control lambs at this age. Acute AT1 blockade using candesartan treatment (45 minutes after injection) lowered MAP in both groups but was statistically significant in the Beta-treated group only (Figure 1A) and had no effect on HR in either group (Figure 1B). Meanwhile, blockade of the Ang-(1-7) receptor (AT7) using D-alanine increased blood pressure in both groups (Figure 1C) with no effect on HR (Figure 1D).

Effect of Candesartan on Spontaneous Baroreflex Sensitivity, HRV, and BPV

The sBRS for HR control measured by spectral analysis as HFa (Figure 2A) and via sequence method as Seq-ALL (Figure 2B; measures for parasympathetic arm) were lower in Beta-exposed lambs on the day of the candesartan injections. The HRV measured as SDRR (Figure 2C) and rMSSD (Figure 2D) was similar in both groups. Candesartan treatment improved the sBRS in Beta-exposed lambs to values not different from those of the control lambs at baseline (Figure 2A, B) and had no effect on measures of HRV.

There was no effect of Beta exposure or candesartan treatment on sBRS measured as LFa (which is mostly a measure of sympathetic arm of the baroreflex; Figure 3A). Antenatal Betamethasone exposure had no effect on BPV measured as LF_SAP (Figure 3B), and candesartan injection had no effect on BPV in either group. LF_HF/HF, a measure of sympathetic balance, was similar between Beta-exposed lambs and control lambs. Candesartan treatment did not alter this ratio in either group (Figure 3C).
Effect of D-ala on Baroreflex Sensitivity, HRV, and BPV

The sBRS for HR control measured by spectral analysis as HF/HR25 (Figure 4A) and via sequence method as Seq-ALL (Figure 4B; which are measures for parasympathetic arm) were lower in Beta-exposed lambs on the day of the experiment for the D-ala infusions, again indicating impairment in the central control of the circulation. The HRV measured as either SDRR (Figure 4C) or rMSSD (Figure 4D) was lower in Beta-exposed lambs on this treatment day. D-ala treatment impaired all 4 measures of parasympathetic activity in control lambs to values not different from those of the Beta-exposed lambs baseline level (Figure 4). There was no further impairment by D-ala treatment in the Beta-exposed animals.

Effect of D-ala on Baroreflex Sensitivity, HRV, and BPV

The sBRS for HR control measured by spectral analysis as HFα (Figure 4A) and via sequence method as Seq-ALL (Figure 4B; which are measures for parasympathetic arm) were lower in Beta-exposed lambs on the day of the experiment for the D-ala infusions, again indicating impairment in

**Figure 1.** Beta-exposed lambs had similar mean arterial pressure (MAP) compared to control lambs at 6 weeks of age. AT1 receptor blockade using candesartan (CV 11974, 0.3 mg/kg, intravenous injection) lowered MAP in Beta exposed group (A), whereas AT1 blockade using 1-hour infusion of 700 ng/kg/min D-Ala—angiotensin (Ang)-(1-7) increased MAP in both groups. C, There were no effect of Beta-exposure on heart rate at this age and no effect of either of the blockers (B, D). Data are mean±SEM. *P<0.05 vs control at baseline; #P<0.05 vs Beta-exposed before candesartan or D-ala.

**Figure 2.** Antenatal Betamethasone exposure was associated with impaired spontaneous baroreflex sensitivity (sBRS) measured by spectral analysis methods such as (A) high-frequency alpha index (HFα), (B) sequence all (Seq-ALL), (C) with no significant change in heart rate variability measured by standard deviation of beat-to-beat intervals (SDRR), and (D) by root of mean square of successive differences (rMSSD). AT1 receptor blockade with candesartan injection (CV, 0.3 mg/kg) improved baroreflex measures in Beta-exposed lambs to a level that is not different from that of the control 45 minutes after injection. Data are mean±SEM. *P<0.05 vs control at baseline; #P<0.05 vs Beta-exposed before candesartan.
There was no effect of either Beta exposure or D-ala treatment on baroreflex measured as LF/HF (sympathetic arm; Figure 5A). Antenatal Betamethasone exposure had no significant effect on BPV at this time point measured as LFSAP (Figure 5B), and D-ala infusion had no effect in either group. LFRRI/HFRRI, a measure of sympathovagal balance, was similar between the 2 groups and D-ala treatment increased this ratio in control lambs to a value not different from that of the Beta-exposed lambs (Figure 5C).

Discussion

We have previously shown that antenatal exposure of sheep to Betamethasone at a time point developmentally similar to that when infants of mothers at risk for premature delivery were exposed to Betamethasone increased MAP and impaired BRS and HRV in adult sheep as early as 6 months of age.11,12 In addition to these cardiovascular changes, the Beta-exposed animals showed an enhanced Ang II action through the AT₁ receptor, and these changes were corrected by the AT₁ receptor blockade.11 At 6 months of age, the elevated MAP and impaired BRS for the control of HR in Beta-exposed sheep were accompanied by a loss of Ang-(1-7) tone.

The current study examined the effect of antenatal Betamethasone exposure in female lambs on MAP, sBRS, HRV, and BPV. We also studied the effect of AT7 blockade in addition to AT1 blockade on the hemodynamic parameters in both control and Beta-exposed lambs. Our data reveal for the first time that Beta exposure at day 80 of gestation followed by term delivery impairs sBRS and HRV at a time point preceding the elevation in MAP. This is consistent with our previous observations of an impaired BRS in response to phenylephrine-evoked increases in MAP and is accompanied by exaggerated response to several stress-related stimuli at this age, even though resting MAP is not elevated and in the absence of changes in resting ACTH and cortisol levels.16 Acute AT₁ blockade with candesartan in these lambs lowered MAP in both groups, revealing a contribution of
Ang II to resting MAP at this time point. Candesartan also increased sBRS in Beta-exposed lambs, with no effect on HRV in either group. These data support the hypothesis that antenatal Beta exposure is associated with enhanced actions of Ang II via \( \text{AT}_1 \) receptors to impair baroreflex control of heart rate at this early age. Moreover, these data also suggest that candesartan actions to lower MAP are independent of the effect on sBRS, because in control lambs candesartan lowered MAP without altering sBRS, but this does not completely rule out the possibility that the improvement in sBRS in Beta-exposed lambs is related to the MAP reduction. Whether the increased “tone” for Ang II via \( \text{AT}_1 \) receptors represents an actual increase in Ang II or \( \text{AT}_2 \) receptors was not investigated in this study.

Meanwhile, interruption of Ang-(1-7) actions via \( \text{AT}_7 \) receptors using D-alanyl increased MAP in both groups and significantly impaired sBRS and HRV measures in control lambs, illustrating that Ang-(1-7) contributes to both maintenance of resting MAP and autonomic balance at this age. Although there was a trend for slight reductions in sBRS and HRV in Beta-exposed lambs, suggesting that some Ang-(1-7) tone remains in the Beta-exposed lambs for improving BR and HRV at this age that is lost as the animals age. Previous data at 6 to 9 months of age indicate almost total loss of Ang-(1-7) tone for MAP, sBRS, and HRV in Beta-exposed sheep. Thus, the progressive loss in Ang-(1-7) function after Beta exposure may reflect an accelerated age-related decline resulting from this prenatal event.

Considerable evidence\textsuperscript{25–28} shows that Ang II and Ang-(1-7) act in an opposing manner to regulate parasympathetic control of heart rate and sympathetic control of BP, and the balance of actions is altered in aging.\textsuperscript{9} We have reported previously an increase in angiotensin-converting enzyme activity in proximal tubules isolated from Beta-exposed sheep at 1.8 years of age,\textsuperscript{29} which may shift the balance between Ang II and Ang-(1-7) toward higher Ang II and lower Ang-(1-7) in the kidney and circulation.\textsuperscript{29} This reduction in Ang-(1-7) appears to occur in other tissues such as brain\textsuperscript{30} and may explain the loss of Ang-(1-7) contribution to facilitate sBRS in the Beta-exposed sheep. The loss of Ang-(1-7) tone for sBRS and HRV in Beta-exposed sheep at an early age may argue that these phenomena may be at least 1 mechanism contributing to the increase in MAP.

**Perspectives**

Numerous studies indicate that impaired sBRS and HRV are associated with elevated MAP, cardiac hypertrophy, and increased risk of stroke,\textsuperscript{31} before and without frank hypertension. Antenatal Betamethasone exposure impairs sBRS for control of HR and reduces HRV before the elevation of MAP in lambs associated with a progressive loss in Ang-(1-7) function as a potential mechanism for the autonomic imbalance. These findings are relevant to the follow-up of children exposed prenatally to steroids to detect changes in cardiovascular control systems that may precede any elevation of MAP.

Many epidemiological studies have shown that the intrauterine environment is extremely important in determining the health of the individual later in life and that perturbations at critical points during development can lead to long-lasting programming effects, including the development of cardiovascular diseases.\textsuperscript{32} Preterm glucocorticoid administration confers a distinct prognostic advantage on infants delivered before full gestation and is an established method to reduce neonatal mortality and morbidity.\textsuperscript{2} Whereas prematurity alone is associated with reduced nephron number and increased risk of cardiovascular disease, it is not clear whether the exposure to antenatal steroids mitigates or aggravates the risk factors for early-onset cardiovascular problems over the long-term. Emerging evidence suggests that antenatal administration of glucocorticoids is associated with alteration in the development of the fetus and results in elevated MAP during the adolescent years\textsuperscript{3} and disturbances of metabolism in young adults,\textsuperscript{19} as well as in various models of fetal programming.\textsuperscript{4,8,33–39} Studies at earlier ages (~6 years old) indicate no difference in MAP between steroid-exposed and nonexposed groups,\textsuperscript{40} consistent with our observations here in lambs.

Because antenatal steroids provide such distinct advantages postnatally, current guidelines support their use in threatened premature delivery. Thus, the incidence of steroid exposure in the last trimester of gestation in human subjects at risk for premature delivery and who subsequently proceed to full-term has increased.\textsuperscript{41} Therefore, the question of whether in the

**Figure 5.** Antenatal Betamethasone exposure had no significant effect on the sympathetic arc of the baroreflex sensitivity measured as (A) low-frequency alpha index (LF\( \alpha \)) and (B) no effect on the blood pressure variability measured as power of the spectral density of systolic arterial pressure in the low frequency range (LF\( SAP \)) in normalized units (nu). (C) The sympathovagal balance measured by low-frequency (LF\_RRI) and high-frequency (HF\_RRI) ratio was similar in both groups at baseline. AT\(_2\) receptor blockade with 700 ng/kg/min D-Ala\(_7\)-angiotensin (Ang)-(1-7) infusion had no effect on LF\( \alpha \) or LF\( SAP \) and it increased LF\_HF ratio in control lambs. Data are mean±SEM. *P<0.05 vs control at baseline.
absence of prematurity. Beta-exposure has negative consequences is also of increasing importance and will help provide information relevant to the increasing number of human subjects exposed to steroids and delivered at full-term.41

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Disclosures
None.

References
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