Alterations in Circulatory and Renal Angiotensin-Converting Enzyme and Angiotensin-Converting Enzyme 2 in Fetal Programmed Hypertension

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Abstract—Antenatal betamethasone treatment is a widely accepted therapy to accelerate lung development and improve survival in preterm infants. However, there are reports that infants who receive antenatal glucocorticoids exhibit higher systolic blood pressure in their early adolescent years. We have developed an experimental model of programming whereby the offspring of pregnant sheep administered clinically relevant doses of betamethasone exhibit elevated blood pressure. We tested the hypothesis as to whether alterations in angiotensin-converting enzyme (ACE), ACE2, and nephrilysin in serum, urine, and proximal tubules are associated with this increase in mean arterial pressure. Male sheep were administered betamethasone (2 doses of 0.17 mg/kg, 24 hours apart) or vehicle at the 80th day of gestation and delivered at term. Sheep were instrumented at adulthood (1.8 years) for direct conscious recording of mean arterial pressure. Serum and urine were collected and proximal tubules isolated from the renal cortex. Betamethasone-treated animals had elevated mean arterial pressure (97±3 versus 83±2 mm Hg; P<0.05) and a 25% increase in serum ACE activity (48.4±7.0 versus 36.0±2.7 fmol/mL per minute) but a 40% reduction in serum ACE2 activity (18.8±1.2 versus 31.4±4.4 fmol/mL per minute). In isolated proximal tubules, ACE2 activity and expression were 50% lower in the treated sheep than in control sheep. We conclude that antenatal steroid treatment results in the chronic alteration of ACE and ACE2 in the circulatory and tubular compartments, which may contribute to the higher blood pressure in this model of fetal programming–induced hypertension. (Hypertension. 2009;53[part 2]:404-408.)

Key Words: fetal programming ■ hypertension ■ renin-angiotensin system ■ sheep ■ angiotensin-converting enzyme 2 ■ proximal tubules and serum

In a landmark article, Liggins and Howie demonstrated that antenatal glucocorticoid therapy administered to women at risk for preterm delivery reduced the incidence of respiratory distress syndrome and mortality in their offspring. The efficacy of antenatal glucocorticoid therapy has been confirmed subsequently by many randomized, controlled trials. The reduction in the severity and incidence of respiratory distress syndrome has resulted in decreased requirements for surfactant therapy, lower concentrations of supplemental oxygen, and a decreased need for prolonged mechanical ventilation in the neonatal period.

Indeed, various organizations, including the National Institutes of Health and the American College of Obstetricians and Gynecologists, have recommended antenatal glucocorticoid treatment for women at risk for preterm delivery before 34 weeks of gestation. However, a recent report showed that, at 14 years of age, blood pressure was higher in a group of children born preterm whose mothers received betamethasone before preterm delivery compared with a similar group who were not exposed to corticosteroids. The mechanism for this increase in blood pressure is not fully known, but several possibilities include the interruption of kidney development, reduction in nephron numbers, and long-term alteration in the renin-angiotensin system (RAS). The development of hypertension later in life as a result of prenatal events has been established in many models of fetal programming. In the present study we examined the effect of antenatal betamethasone given to sheep at a dose and gestational time point similar to the therapy received by women at risk for preterm delivery. We investigated the hypothesis that key enzymes of the RAS, including angiotensin-converting enzyme (ACE), ACE2, and nephrilysin, are altered to contribute to an elevation in blood pressure in this sheep model of fetal programming.

Methods

Animals

Date-mated sheep received at the 80th day of gestation saline or betamethasone acetate:phosphate 1:1 mixture (IM, 2 doses of 0.17 mg/kg, 24 hours apart). After delivery at term, the animals were farm...
Blood Pressure Measurement
Sheep were anesthetized with ketamine and isoflurane, and catheters were inserted in the femoral artery and vein for blood pressure recording and drug administration. Sheep were housed in a study care after the surgical procedure. Five days after surgery, the arterial catheter was connected to a pressure transducer. Arterial pressure and heart rate were recorded and digitized with Acknowledge software 3.8.1 (BIOPAC). Mean arterial pressure (MAP) was recorded continuously before and up to 45 minutes after IV bolus injection of candesartan (0.3 mg/kg).

Tissue Collection
Two days after the experiment, sheep were anesthetized with ketamine and isoflurane, and blood was obtained from a venous catheter. The blood was centrifuged at 3000g for 20 minutes, and the isolated serum was stored at −80°C. The urine was collected directly from the bladder, concentrated 10-fold on a Millipore 5000-Da cutoff filter with the metabolism assay buffer, and immediately used for metabolism experiments or frozen at −80°C. Kidneys were removed immediately, and the renal cortex was dissected on ice for isolation of proximal tubules using a modified method of Shaltout et al., as described previously.

Determination of Angiotensin Metabolism
Metabolism assays were conducted at 37°C in 10 mmol/L of HEPES, 125 mmol/L of NaCl, and 10 μmol/L of ZnCl2 (pH 7.4), with 50-μg protein of tubule membranes (centrifugation of homogenized frozen tubules at 28 000g for 10 minutes at 4°C and suspension of the pellet in assay buffer), 50 μL of serum, or 7 μL of urine (50 μg of protein) in a final volume of 1.0 mL with or without the indicated inhibitors and 0.5 mmol/L iodinated [125I]-angiotensin (Ang) I or [125I]-Ang II. The reaction was stopped by the addition of ice-cold 1.0% phosphoric acid, centrifuged at 16 000 g, and the supernatant was stored at −20°C. Samples were filtered before separation by reverse-phase high-performance liquid chromatography, and the [125I] products were monitored by a Bioscan flow-through γ-detector as described. Products were identified by comparison of retention times to [125I]-standard peptides. The following inhibitors, based on previous studies to distinguish ACE2 activity using Ang II as a substrate, composed the inhibitor mixture in the assay: amastatin (2 μmol/L), benzyl succinate (10 μmol/L), benzamidine (10 μmol/L), bestatin (10 μmol/L), chymostatin (10 μmol/L), chymotrypsinogen A (0.5 mmol/L), and para-chloro-mercuribenzoic acid (0.5 mmol/L). We subsequently added lisinopril to block ACE activity and SCH39370 to inhibit nephrilysin using Ang I as a substrate.

Kinetic Analysis
Metabolism assays were conducted as described above with 50 μL of serum in a final volume of 1.0 mL with the indicated inhibitors and 0.5 mmol/L iodinated [125I]-Ang I or [125I]-Ang II in the presence of 0, 1, 10, 25, 50, and 100 μmol/L of unlabeled Ang I or Ang II, respectively. The ACE and ACE2 maximum velocity constants were determined from plots of the enzyme activity versus the total peptide concentration.

Western Blot
Immunoblots for ACE2 were determined with an NH2 terminally directed rabbit polyclonal antibody (Hypertension and Vascular Disease Center No. A2405). The membrane fraction of the proximal tubules was diluted in sodium dodecyl sulfate/β-mercaptoethanol solution and applied to 10% sodium dodecyl sulfate polyacrylamide gels (Bio-Rad) for 60 minutes at 120 V in Tris-glycine sodium dodecyl sulfate. Proteins were transferred onto a polyvinylidene fluoride membrane and blocked with 5% nonfat dry milk in 0.1% Tween 20 in Tris-buffered saline for 60 minutes at room temperature before incubation with the ACE2 antibody (1:5000). Immunoblots were then resolved with Pierce Super Signal West Pico Chemiluminescent substrate, as described by the manufacturer, and exposed to Amersham Hyperfilm ECL. The gels were stripped and probed with a mouse anti-annexin II monoclonal antibody.

Materials
Angiotensin peptides were purchased from Bachem. Acetonitrile (Optima grade) was obtained from Fisher Scientific. Lisinopril, a converting enzyme inhibitor, was provided by Merck. SCH 39370, a nephrilysin inhibitor, was provided by Schering-Plough. The ACE2 inhibitor MLN4760 was provided by Millennium Pharmaceuticals. Candesartan was provided by Takeda Chemical Industries, Ltd. Mouse anti-annexin II monoclonal antibody was from BD Biosciences. All of the other reagents were obtained from Sigma.

Statistical Procedures
All of the measurements were expressed as the means ± SEMs. All of the statistical analyses were performed with GraphPad Prism 4.02 (GraphPad Software). Differences in the generation of [125I]peptides under various conditions were assessed by 1-way ANOVA with a Student-Newman-Keuls post hoc analysis. A Student t test was used to compare studies with only 2 conditions. The criterion for statistical significance was set at P<0.05.

Results
MAP measured directly from the femoral artery was significantly elevated in adult male sheep that received antenatal betamethasone rather than vehicle (97±3 versus 83±2 mm Hg; P<0.05; Figure 1). Blockade of the Ang II type 1 receptor by bolus administration of 0.3 mg/kg of candesartan normalized MAP in the betamethasone-treated sheep to the control values in 45 minutes (Figure 1). There was no effect on MAP in the control animal, suggesting that elevation of blood pressure in the betamethasone-treated sheep may be attributed to enhanced Ang II action through the Ang II type 1 receptor.

We assessed the activity of several enzymes (ACE, ACE2, and nephrilysin) that play critical roles in maintaining the circulating and renal levels of Ang II and Ang-(1-7). There was a trend to higher ACE activity in the serum of betamethasone-treated sheep compared with vehicle (48.4±7.0 versus 36.0±2.7 fmol/mL per minute), whereas ACE2 activity was significantly lower (18.8±1.2 versus 31.4±4.4 fmol/mL per minute; n=4 to 5; P<0.05; Figure 2A), and serum nephrilysin activity was not detectable. Both ACE and ACE2 activities in serum were highly correlated with MAP,
as shown in Figure 2B and 2C. ACE exhibits a positive and ACE2 a negative relationship with MAP, with ACE/ACE2 revealing a stronger correlation with MAP (Figure 2D).

Kinetic analysis to determine Michelis-Menten constants in serum revealed that the betamethasone-treated sheep had a higher maximum velocity for ACE compared with vehicle (14.7 ± 1.2 versus 7.8 ± 1.3 nmol/mL per minute; n = 4; P < 0.05) but a lower maximum velocity for ACE2 (1.4 ± 0.14 versus 1.98 ± 0.09 nmol/mL per minute; n = 4; P < 0.05), consistent with the activity measurements.

Because these enzymes are highly expressed in the kidney, we determined their activities in isolated tubules from the renal cortex. Antenatal betamethasone treatment was associated with lower ACE2 activity in adult betamethasone-treated sheep compared with control (81 ± 7 versus 145 ± 23 fmol/mg per minute; n = 4 to 5; P < 0.05) in the proximal tubules. In contrast to serum, we found no significant change in tubular ACE or nephrilysin activities (Figure 3A). The immunoblot of ACE2 in proximal tubules (predominant band at 120 kDa) normalized to annexin 2 revealed a 68% reduction in ACE2 expression in the tubular fraction from the betamethasone-treated sheep compared with control (Figure 3B). In the urine, antenatal betamethasone treatment was associated with lower ACE2 activity compared with control (6.9 ± 1.1 versus 14.3 ± 3.1 fmol/mg per minute; Figure 3C), with no changes in ACE or nephrilysin activities. Similar to serum, urinary ACE2 was negatively correlated with MAP (R = -0.69; P = 0.10).

**Figure 2.** Antenatal betamethasone increased ACE activity and reduced ACE2 activity in betamethasone-treated animal serum vs control values. A, MAP was positively correlated with ACE activity in serum. B, MAP was negatively correlated with ACE2 activity. C, The ACE:ACE2 ratio was positively correlated with MAP. D, Data are means ± SEM. *P < 0.05 vs control.

**Discussion**

Increasing evidence supports the concept that adverse events in the perinatal environment predispose an individual to disease later in life. In this study, we investigated the effect of antenatal betamethasone given to sheep at a dose and gestational time point similar to the therapy that women at risk for preterm delivery receive. We found that antenatal betamethasone treatment increased MAP in the adult offspring, and this increase in pressure was blocked by the Ang II type 1 receptor antagonist candesartan. This suggests a higher vasoconstrictor tone in the betamethasone-treated animals than in the control animals. We further evaluated several key enzymes that are critical to maintain the balance between Ang II and Ang-(1-7). Betamethasone treatment was associated with higher serum ACE activity but lower serum ACE2 activity, which supports a shift toward greater synthesis (ACE) and reduced metabolism (ACE2) of Ang II, as well as reduced formation of Ang-(1-7). The alterations in the ratio of these peptides would favor an elevation of blood pressure. Antenatal betamethasone treatment also was associated with reduced ACE2 activity in the isolated proximal tubules and the urine. The reduction in ACE2 activity in both of these compartments suggests reduced expression of the enzyme rather than increased shedding of the enzyme into the tubular fluid. Moreover, an ACE2 immunoblot of the proximal tubules revealed lower protein levels consistent with the lower ACE2 activity in the tubules. ACE and nephrilysin activities in the proximal tubules were not altered by the antenatal betamethasone treatment.
Glucocorticoids are potent regulators of fetal growth and development, because they can alter the expression of various proteins, including receptors, enzymes, ion channels, and transporters. They also alter various growth factors, cytoarchitectural proteins, binding proteins, and components of the intracellular signaling pathways. Overexposure to either endogenous or exogenous glucocorticoids during fetal life programs a number of organ systems and increases the predisposition to several disease states in later life, including hypertension.

The RAS plays a critical role in regulating arterial pressure and body fluid balance through both the systemic and intrarenal actions of angiotensin. In the developing kidney, components of the RAS are highly expressed and are important in mediating proper nephrogenesis. ACE2 and ACE are coexpressed in many tissues, and alterations in their activities may participate in hypertension programming. Although glucocorticoid exposure can acutely increase ACE expression in vascular smooth muscles, chronic upregulation of RAS components may also occur as a result of inappropriate exposure to glucocorticoids in utero.

There are many possible explanations for how this elevation in MAP develops in this model. Recent studies suggest that intrarenal Ang II can contribute to the development of hypertension in the absence of an increase in components of the systemic RAS, because the kidney has all of the components of the RAS, including angiotensinogen, and the majority of intrarenal Ang II may be formed within the kidney. Tissue-specific overexpression of renal angiotensinogen in transgenic mice resulted in marked elevations in blood pressure despite unchanged levels of circulating Ang II. Hypertension in lambs exposed to low protein in utero was associated with enhanced renal ACE protein expression. In a model of fetal programming induced by early dexamethasone exposure in the sheep, elevations in blood pressure were not associated with elevations in peripheral components of the RAS; however, the intrarenal RAS was not assessed in that study. What role the intrarenal RAS plays in the etiology of hypertension in our sheep model of fetal programming needs further investigation. Current studies are in progress to determine whether the circulating and renal tissue levels of angiotensin peptides (Ang I, Ang II, and Ang-(1-7)) are altered in this model. In addition, other possible mechanisms include increased sensitivity to Ang II and alterations in Ang II receptor expression, as observed previously in offspring from other animal models of fetal programming.

It is worth noting that ACE2 activity was detectable in the serum and at comparable levels to ACE activity in the control animals. In human serum, ACE2 activity is not easily detectable, and a recent report suggests that the lack of activity is attributable to the presence of an endogenous inhibitor that binds the catalytic site of ACE2. Moreover, treatment of the plasma sample with a strong anion exchange column was necessary to dissociate the inhibitor from the enzyme and to recover ACE2 activity.

We do not know whether the ACE2 inhibitor exists in sheep or other species beside humans; however, the level of activity in serum does not support the presence of an inhibitor. Finally, although we readily measured neprilysin activity in the proximal tubule and urine of sheep, neprilysin activity was not detectable in serum. To our knowledge, there are no reports of neprilysin inhibitors in serum, and it is likely that neprilysin is not readily shed from the vascular surface, as is ACE. Vascular neprilysin does contribute to circulating levels of Ang-(1-7) by the direct conversion of Ang I. In this regard, additional studies are required to assess the impact of
betamethasone treatment on the vascular expression of nephrilysin activity.

Perspectives
The tenet that fetal programming events lead to cardiovascular pathologies in the adult is becoming more widely accepted. Several well-characterized experimental models demonstrate alterations in the RAS in association with reduced nephron number and an increase in blood pressure. The current demonstration of reduced ACE2 activity within the adult kidney of betamethasone-treated sheep provides additional evidence that programming influences the expression of this key enzyme that participates in maintaining the balance between Ang II and Ang-(1-7). Moreover, that urinary ACE2 activity was reduced to a similar extent as the tubular enzyme and correlated with blood pressure may provide a unique biomarker to screen children at risk for early changes in activity before obvious changes in blood pressure.

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Disclosures
None.

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