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Fetal betamethasone exposure attenuates angiotensin-(1-7)-Mas receptor expression in the dorsal medulla of adult sheep

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ABSTRACT

Glucocorticoids including betamethasone (BM) are routinely administered to women entering into early preterm labor to facilitate fetal lung development and decrease infant mortality; however, fetal steroid exposure may lead to deleterious long term consequences. In a sheep model of fetal programming, BM-exposed (BMX) offspring exhibit elevated mean arterial pressure (MAP) and decreased baroreflex sensitivity (BRS) for control of heart rate by 0.5-years of age associated with changes in the circulating and renal renin-angiotensin systems (RAS). In the brain solitary tract nucleus, angiotensin (Ang) II actions through the AT1 receptor oppose the beneficial actions of Ang-(1-7) at the Mas receptor for BRS regulation. Therefore, we examined Ang peptides, angiotensinogen (Aogen), and receptor expression in this brain region of exposed and control offspring of 0.5- and 1.8-years of age. Mas protein expression was significantly lower (>40%) in the dorsal medulla of BMX animals at both ages; however, AT1 receptor expression was not changed. BMX offspring exhibited a higher ratio of Ang II to Ang-(1-7) (2.30 ± 0.36 versus 0.99 ± 0.28; $p < 0.01$) and Ang II to Ang I at 0.5-years. Although total Aogen was unchanged, Ang I-intact Aogen was lower in 0.5-year BMX animals (0.78 ± 0.06 vs. 1.94 ± 0.41; $p < 0.05$) suggesting a greater degree of enzymatic processing of the precursor protein in exposed animals. We conclude that in utero BM exposure promotes an imbalance in the central RAS pathways of Ang II and Ang-(1-7) that may contribute to the elevated MAP and lower BRS in this model.

1. Introduction

Antenatal glucocorticoid (GC) therapy decreases respiratory distress syndrome and infant mortality when administered to women at risk for preterm delivery [22]. Numerous randomized, controlled trails confirm the efficacy of this therapy [8,38], and organizations such as the National Institutes of Health and the American College of Obstetricians and Gynecologists have recommended antenatal GC treatment for women at risk for delivery before 34 weeks of gestation [1].

The long term consequences of fetal GC exposure are not well characterized, particularly their influence on cardiovascular events. At 14 years of age, preterm children exposed to GCs exhibit higher blood pressure than children born preterm with no exposure [9]. Experimental studies by our group and others have begun to elucidate the potential mechanisms for altered blood pressure associated with GC exposure [2,5,40]. These mechanisms include alterations in kidney development [41], a significant reduction in nephron number [2,44], impaired neural control [28,30,33], and alterations to the circulating and local renin-angiotensin systems (RAS) [2,5,29,40]. In the present study, pregnant ewes were exposed to a clinically relevant dose of BM during the early third trimester, a critical window of kidney and brain development in the fetus. This time corresponds to the period at which GC therapy is administered to women entering into preterm labor [29]. Therefore, we investigated the role of both BM exposure and age on the expression of the brain RAS. We hypothesize that the balance between Ang II and Ang-(1-7) pathways within the brain are altered in a way that is consistent with the chronic elevation in blood pressure and reduction in BRS in this sheep model of fetal programming.

Abbreviations: Aogen, angiotensinogen; Ang, angiotensin; Ang I, [Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-Leu^{10}]; Ang II, [Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8]; Ang-(1-7), [Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7]; AI-Aogen, antibody against angiotensinogen residues 25–34; Int Aogen, antibody against angiotensinogen residues 42–57; GC, glucocorticoid; BM, betamethasone; BMX, betamethasone exposed; RAS, renin-angiotensin system.

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2. Materials and methods

2.1. Animals

Sheep received saline or betamethasone acetate: phosphate 1:1 mixture (IM, 2 doses of 0.17 mg/kg, 24 h apart) at the 80th day of gestation. After term delivery, animals were farm raised and weaned at 3 months of age. At 0.5- or 1.8-years, male offspring were brought to our Association for Assessment of Laboratory Animals Care (ACUC) approved facility, where they were maintained on a normal diet, with free access to tap water and a 12-h light/dark cycle (lights on 7 AM to 7 PM). Sheep were anesthetized with ketamine and isoflurane and euthanized by exsanguination. Brain medullas were removed and immediately covered in Clear Frozen Section Compound (VWR West Chester, PA) and stored at −80 °C. Tissue from a total of 21 animals was used in this study. These procedures were approved by the Wake Forest University School of Medicine ACUC for animal care.

2.2. Western blot analysis

Brain medullas were cut 4 mm rostral and 2 mm caudal to the obex and divided in half along the dorsoventral axis to isolate the dorsal medulla including the NTS. Isolated membrane or cytoplasmic fractions of brain dorsal medulla (10 and 35 μg, respectively) were added to Laemmli buffer containing β-mercaptoethanol. Proteins were separated on 12% SDS polyacrylamide gels for 80 min at 120 V in Tris-glycine buffer and electrophoretically transferred onto polyvinylidene difluoride membranes. Immunodetection was performed on blots blocked for 1 h with 5% dry milk (Bio-Rad, Hercules, CA) and Tris-buffered saline containing 0.05% Tween and probed with antibodies against Mas (1:250 dilution; Alomone AAR-013, Jerusalem, Israel), AT1 receptor (1:200; Alomone AAR-011) and both total and Ang I-intact forms of rat angiotensinogen (Aogen: 1:2000). Mas and AT1 receptor antibodies were probed against proteins separated using the Criterion Cell and Blotter (Bio-Rad) on 12% Tris–HCl 26 lane gels (Bio-Rad 345-0016). Specificity of Mas and AT1 receptor antibodies was validated by preabsorption of the antibody with the immunizing peptide (ratio of 1 μg peptide to 1 μg antibody) on proteins run on 12% Mini-PROTEAN TGX gels. The two Aogen antibodies were raised against residues 25–34 [Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Cys*, Ang I sequence] and residues 42–57 [Cys-Ala-Gln-Leu-Glu-Asn-Pro-Ser-Val-Glu-Thr-Leu-Pro-Glu-Pro-Thr] of the rat protein [18]. An additional cysteine residue (C*) was added for covalent coupling of the Ang I peptide to keyhole limpet hemocyanin to enhance antigenicity. Both rat and sheep contain the identical Ang I sequence while the sheep 42–57 sequence [Cys-Asp-Gln-Leu-Glu-Lys-Pro-Ser-Val-Glu-Thr-Ala-Pro-Asp-Pro-Thr] shares similar identity to the rat [18]. Aogen antibodies were probed on 12% Mini-PROTEAN TGX gels. Reactive proteins were detected with PerkinElmer ECL substrate (Waltham, MA) and exposed to Amersham Hyperfilm enhanced chemiluminescence (Piscataway, NJ). Gels were stripped and probed with β-Actin (Sigma–Aldrich A5441) as a loading control. Band density was calculated using MCID Elite 7.0 (Cambridge, England).

2.3. Angiotensinogen measurement

Renin isolated from sheep kidney cortex (100 mg) was added to a cocktail of inhibitors (aprotinin, bestatin, PCMB, soybean trypsin inhibitor, 10 μM each) in the presence or absence of aliskiren (10 μM) on ice. Nephrectomized sheep plasma as the source of intact Aogen was added and the reaction was transferred to a 37 °C water bath. Aliquots of the reactions were removed at 30, 60, 120, and 240 min, added to Laemmli buffer containing β-mercaptoethanol, and put on ice. All samples were boiled and loaded on a gel for Western blot analysis. Separate gels were run and the blots probed with antibodies against the Ang I sequence (Ang I-intact or AI-Aogen) and the internal sequence (Int-Aogen), representing both intact and Ang I-cleaved forms of angiotensinogen (total Aogen).

2.4. Peptide measurement

Ang peptides in the medullary tissues were measured in the Hypertension Center Core Assay Laboratory utilizing multiple radioimmunoassays (RIAs) [4,6,29]. Frozen medullas were homogenized in acid ethanol (80%, vol/vol 0.1 N HCl) containing the peptidase inhibitors EDTA, phenanthroline, phenylmethylsulfonyl fluoride (PMSF), p-Chloromercuribenzoic acid (PCMB), and a renin inhibitor [4]. Total protein content was analyzed in aliquots from the acid ethanol homogenate using the Bradford protein assay with BSA as a standard. Homogenates were centrifuged at 30,000 × *g* for 20 min, and supernatant was decanted and acidified with 1% heptafluorobutyric acid. The solution was precipitated overnight at 4 °C and centrifuged at 30,000 × *g* for 20 min. The supernatants were concentrated in a vacuum centrifuge and applied to activated Sep-Paks C18 columns (Waters, Milford, MA), washed with 0.1% HFBA, and eluted with 5 ml of 80% methanol, and 0.1% HFBA. Recovery of Ang peptides was determined by addition of ^{125}I-Ang-(1-7) to homogenates by comparing total counts applied to the Sep-Pak to that recovered in the eluate [6,29]. The Ang peptide content of each fraction was determined by separate RIAs for Ang I, Ang II, and Ang-(1-7) that fully recognize each peptide but cross-react less than 0.01% with each other [29]. Minimum detection levels for the assays are 1 fmol/ml, 0.8 fmol/ml, and 2.8 fmol/ml for Ang I, Ang II, and Ang-(1-7), respectively. Peptide content in the medulla is expressed as fmol/mg protein.

2.5. Statistics

Data are expressed as mean ± SEM. Unpaired *t* tests and two-way ANOVA with Bonferroni posttests were used for the statistical analysis of the data with GraphPad Prism 5.01 (GraphPad Software, San Diego, CA). The criterion for statistical significance was set at $^{*}p < 0.05$. We are able to detect a difference of 45% between group means with an $N = 4$ in each group, and a difference of 55% between group means for $N = 3$ with a standard deviation equal to 15% of the total value, and a Beta error of 80%.

3. Results

The protein expression for Mas and AT1 receptors was determined by Western blot analysis normalized to β-Actin. Both antibodies revealed double bands at the expected molecular weights (Mas antibody = 50 kDa, AT1 receptor antibody = 40 kDa) using the Criterion Cell apparatus and 12% Tris–HCl gels (Fig. 1, upper panel). Direct comparisons between control and BMX animals as well as for 0.5- and 1.8-years of age were achieved with a 26 lane gel. As shown in Fig. 1 (middle panel, left), Mas receptor expression was significantly lower in the BMX animals ($p < 0.05$) at both 0.5- and 1.8-years of age. In contrast, there was no difference in AT1 receptor protein expression in homogenates of the dorsal medulla at 0.5-years (Fig. 1, lower panel, left). We noted a large variability in AT1 receptor expression at 1.8-years; however, there was no trend toward increased AT1 receptor expression in BMX animals, indicating that altered AT1 receptor expression may not contribute to the phenotype at this time. Preabsorption of the primary antibody with the appropriate immunizing peptide was performed on tissue extracts separated on 12% Mini-PROTEAN TGX gels. This abolished