Antenatal betamethasone exposure is associated with lower ANG-(1–7) and increased ACE in the CSF of adult sheep

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Submitted 1 July 2013; accepted in final form 7 August 2013

Marshall AC, Shaltout HA, Pirro NT, Rose JC, Diz DL, Chappell MC. Antenatal betamethasone exposure is associated with lower ANG-(1–7) and increased ACE in the CSF of adult sheep. Am J Physiol Regul Integr Comp Physiol 305: R679–R688, 2013. First published August 15, 2013; doi:10.1152/ajpregu.00321.2013.—Antenatal betamethasone (BM) therapy accelerates lung development in preterm infants but may induce early programming events with long-term cardiovascular consequences. To elucidate these events, we developed a model of programming whereby pregnant ewes are administered BM (2 doses of 0.17 mg/kg) or vehicle at the 80th day of gestation and offspring are delivered at term. BM-exposed (BMX) offspring develop elevated blood pressure; decreased baroreflex sensitivity; and alterations in the circulating, renal, and brain renin-angiotensin systems (RAS) by 6 mo of age. We compared components of the choroid plexus fourth ventricle (ChP4) and cerebral spinal fluid (CSF) RAS between control and BMX male offspring at 6 mo of age. In the choroid plexus, high-molecular-weight renin protein and ANG I–intact angiotensinogen were unchanged between BMX and control animals. Angiotensin-converting enzyme 2 (ACE2) activity was threefold higher than either neprilysin (NEP) or angiotensin I-converting enzyme (ACE) in control and BMX animals. Moreover, all three enzymes were equally enriched by approximately 2.5-fold in ChP4 brush-border membrane preparations. CSF ANG-(1–7) levels were significantly lower in BMX animals (351.8 ± 76.8 vs. 77.5 ± 29.7 fmol/mg; P < 0.05) and ACE activity was significantly higher (6.6 ± 0.5 vs. 8.9 ± 0.5 fmol-min⁻¹·ml⁻¹; P < 0.05), whereas ACE2 and NEP activities were below measurable limits. A thiol-sensitive peptidase contributed to the majority of ANG-(1–7) metabolism in the CSF, with higher activity in BMX animals. We conclude that in utero BM exposure alters CSF but not ChP RAS components, resulting in lower ANG-(1–7) levels in exposed animals.

fetal programming; sheep; choroid plexus; cerebral spinal fluid; angiotensin peptidases

THE ADMINISTRATION OF ANTENATAL glucocorticoids to women at risk for early preterm labor greatly decreases the risk of respiratory distress syndrome and improves infant survival (39). Indeed, organizations such as the National Institutes of Health and the American College of Obstetricians and Gynecologists recommend the use of antenatal glucocorticoids for women at risk for delivery before 34 wk of gestation (45a). Although the short-term effects of antenatal treatment are clearly of benefit to the newborn, the long-term consequences of glucocorticoids may be detrimental regarding metabolic and cardiovascular health (15, 64). Antenatal exposure to betamethasone (BM) in sheep elicits decreased baroreflex sensitivity (BRS) by 6 wk (65, 70), and an elevated mean arterial pressure (MAP) by 6 mo of age (19, 67). The loss of ANG-(1–7) (Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷) action appears to be an early event in the BM-induced fetal programming alterations of the renin-angiotensin system (RAS) (67, 68, 70). Microinjection of the AT₁- and ANG-(1–7)-receptor antagonists into the solitary tract nucleus (NTS) of the dorsal brain stem of betamethasone-exposed (BMX) sheep reveal an increase in ANG II (Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸) and a decrease in ANG-(1–7) pathways mediating BRS at 6 wk of age (69). We recently reported that expression of the ANG-(1–7) AT₁/Mas receptor protein in the dorsal brain stem was lower in adult male BMX sheep (41).

The interface between the peripheral vascular system and brain parenchyma is the choroid plexus (ChP), a polarized epithelial structure that produces cerebrospinal fluid (CSF) (55, 56). The ChP of the fourth ventricle (ChP4) is in close proximity to the medulla, an important site for the regulation of autonomic and cardiovascular function (6, 13). The ChP also exhibits a local RAS, including the expression of renin, angiotensinogen (Aogen), and angiotensin-converting enzyme (ACE) (29, 30, 62). Arregui and Iversen reported that ACE protein expression in the ChP was 50-fold higher than any other brain region of the rat (5), suggesting a pathway for ANG II formation in the choroid or in the adjacent CSF compartment (5, 10, 58). Moreover, the choroid exhibits neprilysin (NEP) and angiotensin-converting enzyme 2 (ACE2) proteins that may be indicative of the formation of ANG-(1–7) (12).

CSF is secreted from the apical membrane of the ChP and serves to protect and stabilize the brain. Components of the RAS previously described in CSF include ACE, Aogen, ANG I (Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸-His⁹-Leu¹⁰), ANG II, and cathepsin D (17, 20, 57, 62); however, renin is typically absent in this central compartment (32). Intracisternal ANG II elicits an immediate increase in blood pressure, suggesting that CSF-borne ANG II can target receptors within the brain to influence blood pressure (14). Therefore, we hypo-
esized that the ChP4 and CSF RAS components are influenced by BM-induced programming events to support a higher ratio of ACE-ANG II to ACE2-ANG-(1–7) within the CSF and ChP tissue surrounding the sheep brain.

MATERIALS AND METHODS

Animals. Sheep were exposed to saline or BM acetate:phosphate 1:1 mixture (2 doses of 0.17 mg/kg im, 24 h apart) at the 80th day of gestation via injections given to a pregnant ewe. After term delivery, animals were farm-raised and weaned at 3 mo of age. At 6 mo of age, male offspring were brought to our Association for Assessment of Laboratory Animals Care-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 A.M. to 7 P.M.). Tissues and CSF for this study were collected from 6-mo-old sheep that were anesthetized with ketamine and isoflurane and euthanized by exsanguination. ChP4 was removed and immediately covered in Clear Frozen Section Compound (VWR, West Chester, PA) and stored at −80°C. CSF was extracted (∼3 ml per animal), taking care to avoid contamination with blood, and tubes were stored at −80°C. Samples from a total of 32 animals were used in this study. Experimental measurements were performed in CSF or choroid tissue from control and exposed sheep groups with four to five animals per group. The age of 6 mo was chosen because these animals exhibit a lower nephron number, higher blood pressure, lower BRS, and reduced expression of the AT-1/Mas receptor in the brain dorsal medulla (41, 67, 82). The current study utilized tissue from 10 sheep included in these previous publications. All procedures were approved by the Wake Forest University School of Medicine animal care and use committee.

Western blot analysis. Isolated cytosolic fractions of ChP (35 µg) and CSF (5 µl) were added to Laemmli buffer containing β-mercaptoethanol. Proteins were separated on 12% Mini-PROTEAN TGX 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 then probed with antibodies against renin (1:1,000, Inagami antibody No. 826) and the ANG I-intact form of rat Aogen (1:2,000). The Aogen antibody was raised against residues 25–34 (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Cys*; ANG I sequence) of the rat protein (25). An additional cytosine residue (Cys*) 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 (25). Reactive proteins were detected with a PerkinElmer (Waltham, MA) enhanced chemiluminescence (ECL) substrate and exposed to Hyperfilm ECL (Amersham, Piscataway, NJ). The protein bands for Aogen, renin, and β-actin were quantified at several different exposures and the ratios were comparable, indicating that the films were not saturated.

ChP tissue preparation. Approximately 40 mg of ChP4 tissue was added to 0.5 ml of reaction buffer (25 mM HEPES, 125 mM NaCl, 10 mM ZnCl₂ pH 7.4). Aliskiren and pepstatin (10 µM) were added to each sample. Samples were homogenized using a TissueLyser (Qiagen, Valencia, CA) for 90 s at 25 Hz and centrifuged at 28,000 g for 10 min. The supernatant was collected and frozen at −80°C. The supernatant was removed and centrifuged at 35,000 g for 30 min. The resulting pellet, enriched for the BBM, was resuspended in a minimal volume of MHK buffer. Both the supernatant fraction and the BBM-enriched fraction were solubilized overnight with 0.5% Triton. The following day, samples were centrifuged at 28,000 g for 5 min and the supernatants were saved for metabolism reactions.

HPLC separation. Metabolism reactions were conducted at 37°C in reaction buffer protein from the total membrane (nenonriched) or BBM-enriched fractions (2.5 µg) or CSF (2.5 µl) in a final volume of 250 µl. Each reaction includes 0.5 nM 125I-labeled ANG I or 125I-labeled ANG II, and 1 µM noniodinated ANG I or ANG II (66). The reaction was stopped after 120 min by addition of ice-cold 1.0% phosphoric acid and centrifuged at 16,000 g. The supernatant was immediately filtered for separation by reverse-phase HPLC. The 125I-labeled products were monitored by a Bioscan flow-through γ detector as described (18). Products were identified by comparison of retention times to 125I-labeled standard peptides. Peptides were iodinated by the chloramine T method and purified by HPLC to a specific activity >2,000 Ci/mmol (8). The following inhibitors, on the basis of previous studies to distinguish ACE2 activity using ANG II as a substrate, comprised the inhibitor cocktail in the assay: amastatin (2 µM), bestatin (10 µM), chymostatin (10 µM), benzyl succinate (10 µM), and p-chloromercuribenzoic acid (PCMB; 0.5 mM). We subsequently added lisinopril to block ACE activity, SCH39370 for NEP activity, or MLN4760 for ACE2 activity (all at 10 µM final concentration) (66).

PCMB-sensitive peptidase. Metabolism of 125I-labeled ANG-(1–7) in CSF was determined in the presence of no inhibitors, PCMB (0.5 mM), or PCMB and lisinopril (10 µM) following 30-, 60-, and 120-min incubation periods at 37°C. Reactions were stopped as previously described and separated using HPLC (66).

PCMB-peptidase ANG-(1–7) kinetics. CSF was pooled separately from control and BMX animals and centrifuged using molecular-weight filtration tubes (50 kDa, Millipore Bedford, MA). Concentrated CSF was resuspended in 500 µl of MHK buffer and protein concentrations were measured using a Bradford protein assay. Saturation curves were performed with 125I-labeled ANG-(1–7) as a substrate and increasing concentrations of either unlabeled ANG-(1–7) or ANG II (0–50 µM) as previously described by Shaltout et al. (66). Reaction velocities for ANG-(1–4) formation from ANG-(1–7) were expressed as nmol·min⁻¹·mg protein⁻¹. The assays included amastatin (2 µM), bestatin (10 µM), chymostatin (10 µM), benzyl succinate (10 µM), and lisinopril (10 µM) to prevent the contribution of other peptidases in the CSF and preserve the ANG-(1–4) product. The inhibitory constant (Kᵢ) of PCMB was determined by adding varying concentrations of PCMB (50 nM to 500 µM) to a pool of control CSF. Kinetic constants (Kₘ, Vₘₐₓ, Kᵢ) were determined by the Prism 5 statistical program.

Peptide measurements. ANG peptides in ChP4 and CSF were measured by the Hypertension Center Core Assay Laboratory utilizing multiple radioimmunoassays (RIAs) (2, 8, 66). Frozen ChP4 tissues were homogenized in acid ethanol (80% vol/vol, 0.1 N HCl) containing the peptidease inhibitors EDTA, phenanthroline, phenylmethylsulfonyl fluoride (PMSF), PCMB, and a renin inhibitor (2). Homogenates were centrifuged at 30,000 g for 20 min, and the supernatant was decanted and acidified with 1% n-heptanfluorobutyric acid (HFBA). 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. ANG peptides were measured directly in the CSF. 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 (66). 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 is expressed as fmol/mg protein in ChP4 and fmol/ml in CSF. Total protein content was determined in the acid ethanol homogenate by Bradford protein assay with BSA as a standard.

Materials. ANG peptides were purchased from Bachem (Torrance, CA). Acetonitrile (Optima grade) was obtained from Fisher Scientific (Fair Lawn, NJ). Lisinopril, a converting enzyme inhibitor, was provided by Merck (West Point, PA). SCH93970, a nephrilysin inhibitor, was provided by Schering-Plough (Madison, NJ). The ACE2 inhibitor MLN4760 was provided by Millennium Pharmaceuticals (Baltimore, MD). All other reagents were obtained from Sigma (St. Louis, MO) and BioRad (Hercules, CA).

Statistics. Data are expressed as means ± SE. Unpaired t-tests were used to determine significant between two groups. Two-way ANOVA was used to analyze time-course experiments. Values below the minimal detectable limit for each RIA were assigned the minimal detectable value [1.0, 0.8, and 2.8 fmol for ANG I, ANG II, and ANG-(1–7), respectively]. All statistical analyses were performed with GraphPad Prism software (GraphPad, San Diego, CA). The criterion for statistical significance was set at P < 0.05.

RESULTS

Choroid plexus RAS. Protein expression of Aogen was detected using the ANG I-intact (AI)-Aogen antibody as described previously (41). Two bands were observed at 50 and 60 kDa. Bands were analyzed separately and together. We found a trend toward higher expression of AI-Aogen at 50 kDa in exposed sheep (P = 0.058; Fig. 1A). There was no difference in either the 60-kDa band or the density of the combined bands (Fig. 1, B and C, respectively). High-molecular-mass renin protein was detected as a single band at ~60 kDa, possibly indicating the presence of prorenin; however, there was no difference between the control and BMX groups (Fig. 2).

ACE, ACE2, and NEP activities were determined by HPLC analysis of the hydrolysis of 125I-labeled ANG I and 125I-labeled ANG II (Fig. 3A). There were no differences in enzyme activities between the control and BMX animals (Fig. 3B). The combined activity data from BMX and control animals (n = 10) revealed that ACE2 activity is significantly higher than either ACE or NEP (Fig. 3C). Activity values (expressed as fmol-min⁻¹-ng protein⁻¹) averaged 9.0 ± 1.4 for ACE, 31.6 ± 3.4 for ACE2, and 13.0 ± 1.9 for NEP (Fig. 3B).

We then prepared a BBM-enriched fraction from the ChP4 using ACE as a marker of the BBM to determine the localization of ACE2 and NEP in the Chp (7, 10, 58). ACE, ACE2, and NEP activities in the total solubilized homogenate (nondenriched) were compared with the solubilized BBM-enriched fraction (Fig. 4). All three enzyme activities were enriched by approximately 2.5-fold in the BBM fraction. Representative chromatographs for ACE2 activity in the Chp4 total membrane homogenate and the BBM-enriched fractions are shown in Fig. 4, top and bottom insets, respectively. Note the larger peak of ANG-(1–7) from ANG II in the BBM fraction, indicating higher ACE2 activity (Fig. 4, bottom inset). Again, we found no differences in the BBM enzyme activities between control and BMX animals.

Finally, the tissue concentrations of ANG I, ANG II, and ANG-(1–7) were quantitated in the ChP4 from both groups (Fig. 5A). There were no significant differences between BMX and control animals for the individual peptides; however, ANG II was the predominant peptide in the ChP4 of both groups (Fig. 5B).

CSF RAS. In addition to the ChP4 peptide content, we assessed ANG peptides in clear CSF samples from 6-mo-old male sheep (Fig. 5C). In contrast to the choroid data, ANG-(1–7) exhibited the highest concentration of the three peptides (Fig. 5D). Moreover, the CSF content of ANG-(1–7) was significantly lower in BMX sheep compared with the vehicle-treated group. AI-Aogen content of the control and BMX groups is shown in Western blots in Fig. 6. In contrast to the
choroid, the CSF levels of AI-Aogen were similar between BMX and control groups.

We then assessed the overall rate of $^{125}$I-labeled ANG-(1–7) metabolism in the CSF from both control and BMX animals over 120 min (Fig. 7). The time-course studies revealed a significantly higher rate of metabolism in the BMX animals at 120 min of reaction (Fig. 7, right). The thiol peptidase inhibitor PCMB blocked the majority of ANG-(1–7) metabolism in CSF in both groups. In the presence of PCMB there was a significant difference in ANG-(1–7) metabolism between the BMX and control groups. Addition of the ACE inhibitor lisinopril abolished any difference between BMX and control animals and completely inhibited the metabolism of ANG-(1–7) to ANG-(1–5) in CSF. Assessment of ACE alone (conversion of ANG I to ANG II) revealed higher activity in the BMX group compared with controls ($8.9 \pm 0.5$ vs. $6.6 \pm 0.5$ fmol·min$^{-1}$·mg protein$^{-1}$; $P = 0.02$). Because the PCMB-sensitive enzyme contributed to the majority of ANG-(1–7) metabolism, we performed saturation studies on the activity in pooled and concentrated CSF.
samples from BMX and control animals. As shown in Figure 8A, the rate of metabolism of $^{125}$I-labeled ANG-(1–7) to $^{125}$I-labeled ANG-(1–4) appeared to plateau with increasing concentrations of unlabeled ANG-(1–7). Kinetic analysis of the activity curves revealed an apparent $K_m$ for ANG-(1–7) of $5.4 \pm 0.9$ M and $V_{max}$ of $54.1 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ in controls, and a $K_m$ of $4.1 \pm 0.9$ M and $V_{max}$ of $56.9 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for BMX animals. In contrast, the rate of $^{125}$I-labeled ANG-(1–7) metabolism did not appear to plateau with increasing concentrations of unlabeled ANG II in control CSF (Fig. 8B). These data suggest that over this concentration range ANG II does not compete for the PCMB-sensitive peptidase to metabolize ANG-(1–7). Finally, on the basis of an apparent $K_m$ for ANG-(1–7) of $5.4 \pm 0.9$ M in the pooled control CSF, we determined an inhibitory constant ($K_i$) for PCMB of $4.1 \pm 0.9$ M.

**DISCUSSION**

In the present study, we characterized components of the RAS in the ChP4 and CSF of control and BMX male sheep at 6 mo of age. We report a trend toward higher expression of AI-Aogen in BMX animals. There was no difference in the high-molecular-weight renin expression between the two groups. The relative activities of ACE, ACE2, and NEP were localized to the brush-border fraction of the ChP4 and were not different between groups, although ACE2 activity was the predominant peptidase activity in this compartment. Peptide analysis of the ChP4 revealed no differences in ChP peptide levels exist between control and BMX animals for any of the three peptides ($n = 5$). B: control and BMX peptide values were combined for analysis, and ANG II levels were higher than those for ANG I or ANG-(1–7) in ChP ($n = 9$). C: in the CSF, ANG-(1–7) was significantly lower in BMX animals ($n = 5$). D: control and BMX peptide values were combined for analysis, and ANG-(1–7) levels were significantly higher than those for ANG I or ANG II in CSF ($n = 10$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
receptor antagonist candesartan into the NTS improved BRS in both control and BMX animals at 6 wk of age (69). In contrast, ANG-(1–7) receptor blockade with the selective AT7 receptor/Mas antagonist d-Ala7-ANG-(1–7) (d-Ala, A779) in the NTS inhibited the reflex only in the unexposed animals, suggesting an imbalance in the actions of ANG II and ANG-(1–7) for baroreflex control of heart rate (69). Systemic infusion of candesartan improved BRs and lowered MAP in BMX sheep, whereas systemic d-Ala increased MAP and lowered BRS only in control sheep at 6 wk, 6 mo, and 1.8 years of age (67, 70, 71). Indeed, we recently showed that protein expression of the AT7 receptor protein Mas is significantly lower in the dorsal 71). Indeed, we recently showed that protein expression of the control sheep at 6 wk, 6 mo, and 1.8 years of age (67, 70, 71). Furthermore, we have shown that systemic d-Ala increased MAP and lowered BRS only in control sheep at 6 wk, 6 mo, and 1.8 years of age (67, 70, 71). Indeed, we recently showed that protein expression of the AT7 receptor protein Mas is significantly lower in the dorsal 71). Indeed, we recently showed that protein expression of the control sheep at 6 wk, 6 mo, and 1.8 years of age (67, 70, 71). Therefore, our analysis of RAS components includes the blood supply fueling CSF production, and the cells that are directly responsible for CSF secretion. We examined the precursor protein angiotensinogen (Aogen) by Western blot analysis using an antibody directed against the NH2-terminal sequence of ANG I (41). Two protein bands were evident in ChP tissue that likely represent multiple isoforms of Aogen. In nephrectomized sheep plasma, we observed a 60-kDa band that is abolished upon exposure to renin, indicating the specificity of the NH2-terminally directed antibody (25, 41). Because the ChP is composed of both an epithelial and vascular layer, the two forms of Aogen may reflect the contribution of both compartments. In support of this concept, Western blot analysis of CSF Aogen revealed a single band at 50 kDa. There was a trend toward greater Aogen expression in BMX animals that may reflect higher Aogen expression or altered processing of the protein mediated by active renin. Immunobots with the renin antibody revealed a prominent band around 60 kDa; others have found that prorenin has a similar molecular weight (44, 54) and that prorenin concentrations are altered in the plasma of BMX sheep (34). Prorenin is the precursor to renin, in which a 43-amino acid prosegment blocks the active site of renin (42). In circulation and brain tissue, the prorenin protein is 10-fold higher than renin (38, 47). We did not detect the active form of renin (35–40 kDa), possibly due to the sensitivity of this Western blot analysis. In future studies, renin activity and (pro)renin receptor (PRR) expression in the ChP of control and
BMX animals will be measured to determine the role of fetal programming on central renin activity.

The localization and activity of RAS enzymes were also studied in these experiments. Mammalian ACE activity in the ChP4 was high and localized to the BBM (5, 7, 59). Although ACE2 and NEP mRNA were detected in the ChP, we are not aware of any studies measuring their relative activity (21, 72). We found that ACE2 activity was 3.5-fold higher than ACE and 2.4-fold higher than NEP. Although there were no differences in relative enzyme activity between BMX and control animals in a total membrane fraction, we investigated the potential for specific alterations in enzyme activity in the BBM compartment. ACE was used as a BBM marker as previous studies localized ACE exclusively to the BBM of both ChP and renal proximal tubules (3, 7, 74). All three peptidases were enriched 2.5-fold in BBM fractions; however, there were no overall changes in activity in the BMX group. Localization of these peptidases on the BBM may be important because the apical membrane contacts the CSF and these exopeptidases could directly contribute to CSF peptide processing (Fig. 9) (20, 62, 75).

To further characterize the ChP RAS, we determined ANG I, ANG II, and ANG-(1–7) peptide concentrations in the ChP4 tissue and CSF. The peptide contents in ChP4 tissue were similar between BMX and control animals. ANG II was the predominant angiotensin peptide despite ACE2 activity being higher than ACE or NEP, likely reflecting that ACE2 is located in the BBM facing the CSF rather than an intracellular location. Thus the higher ANG II content in ChP4 may reflect different processing compartments or pools within ChP4. Indeed, it has been shown that ChP is compartmentalized into a stromal core with fenestrated capillaries and the tight-junction epithelium (43). Alternatively, there may be greater uptake and protected sequestration of ANG II from CSF or blood via AT1 receptor internalization (22, 40). Studies have identified AT1 receptor protein (22, 77) and shown AT1 receptor binding in ChP tissue (33).

ANG-(1–7) was the predominant peptide in the CSF compared with ANG I or ANG II, and we found lower levels of ANG-(1–7) in BMX animals. Past studies by our group report reduced ANG-(1–7) tone in the brain, circulation, and kidney of BMX sheep (24, 41, 68–70). In this regard, intracerebroventricular (icv) administration of ANG-(1–7) increased BRS in DOCA-salt and (mRen2)27 transgenic rats, as well as a rabbit model of heart failure (23, 35, 46). Conversely, the ANG-(1–7) antagonist D-Ala given icv reduced BRS in normotensive Wistar rats and spontaneously hypertensive rats (SHR) treated with an ACE inhibitor, but not in control SHR (27, 48). These studies suggest that CSF ANG-(1–7) may potentially influence BRS, and that a decrease in the peptide could contribute to alterations in pressure and BRS in BM-exposed sheep (70). ACE activity, determined by conversion of ANG I to ANG II or ANG-(1–7) to ANG-(1–5), was significantly higher in the exposed group. Again, this is consistent with previous data showing a functional shift toward a higher ANG II to ANG-(1–7) ratio in BMX animals (24, 66). However, we could not detect generation of ANG-(1–7) from either

Fig. 8. Saturation curves for ANG-(1–7) and ANG II metabolism by PCMB-sensitive enzyme in CSF. Pooled CSF from control or BMX sheep was concentrated by a 50-kDa filtration tube. (A) With solid lines, control animals; (B) with dashed lines, BMX animals. K_m and V_max for ANG-(1–7) metabolism were calculated with a GraphPad Prism 5 statistical program. For control animals K_m = 5.4 μM ANG-(1–7); V_max = 54.1 nmol·min⁻¹·mg protein⁻¹. For BMX animals K_m = 4.1 μM ANG-(1–7); V_max = 56.9 nmol·min⁻¹·mg protein⁻¹.

Fig. 9. Diagram of potential ChP4 and CSF localization of renin-angiotensin system (RAS) components. AI-Aogen, ANG peptides, and prorenin were detected in the ChP tissue. ACE, ACE2, and NEP were detected in the BBM of the ChP. CSF peptide levels indicate potential BBM processing because ANG-(1–7) levels were markedly higher than those of ANG II and ANG I. Metabolism of ANG-(1–7) to form ANG-(1–5) was mediated by ACE, whereas a PCMB-sensitive peptidase was the major activity that metabolized ANG-(1–7) to ANG-(1–4).
ANG I or ANG II in CSF either alone or with PCMB and lisinopril to prevent ANG-(1–7) metabolism. It is likely that CSF ANG-(1–7) is formed by BBM-localized ACE2 from ANG II or released by ChP and/or brain tissue. Smith and colleagues have proposed that CSF angiotensin peptides may be of ChP or brain tissue origin (73).

Numerous studies have investigated the presence and origin of soluble forms of ACE and ACE2 (1, 16, 36, 50). Both peptidases undergo proteolysis or shedding in which the juxtamembrane stalk is cleaved and releases a soluble form of the enzyme from the cell membrane that is catalytically active (36, 53). Shedding of the active forms of ACE and ACE2 is mediated by distinct members of the secretase family. ACE2 shedding is mediated by a disintegrin and metalloprotease (ADAM) 10 or 17 (1, 36, 52). In contrast, the ACE sheddase is very similar to α-secretase, a sheddase that cleaves the amyloid precursor protein involved in the pathogenesis of Alzheimer’s disease (1, 51). α-Secretase is present in the CSF of both healthy individuals and patients with Alzheimer’s disease (49), suggesting that a similar sheddase that cleaves ACE may also be present in the CSF. Although we did not investigate proteolytic shedding of ACE and ACE2 in this study, it is possible that ACE is shed from the BBM of the ChP at a far higher rate than ACE2, which may explain the relative activities of ACE and ACE2 in the BBM and CSF.

Although we could not detect an ANG-(1–7)-forming pathway in the CSF, the present study revealed that ACE and a thiol peptidase accounted for the total extent of ANG-(1–7) degradation. The PCMB-sensitive enzyme and ACE metabolized ~23% of ANG-(1–7) in controls and 30% of ANG-(1–7) in BMX animals. ACE alone metabolized ~10% of ANG-(1–7) in control and 15% of ANG-(1–7) in BMX animals. Differences in ANG-(1–7) metabolism between groups is likely due to higher ACE activity in BMX animals, and slightly higher PCMB-sensitive enzyme activity, as suggested by the higher apparent $V_{\text{max}}$ in BMX animals. ACE hydrolyzed the Ile$^3$-His$^5$ bond of ANG-(1–7) to form ANG-(1–5), whereas the thiol peptidase cleaved the Tyr$^4$-Ile$^5$ bond to generate ANG-(1–4). Kinetic analysis of the CSF peptidase revealed an apparent $K_m$ of 4–5 μM for ANG-(1–7), which is comparable to that of ACE (9). To our knowledge, this is the first report of a thiol-peptidase involved in the metabolism of ANG-(1–7) in CSF or other tissue compartments in sheep. Although there is a wealth of evidence on the enzymes that cleave ANG-(1–7) in CSF or other tissue compartments in sheep, it is very similar to ADAM 10 or 17 (1, 36, 52). In contrast, the ACE sheddase is mediated by a disintegrin and metalloprotease (ADAM) 10 or 17 (1, 36, 52). In contrast, the ACE sheddase is very similar to α-secretase, a sheddase that cleaves the amyloid precursor protein involved in the pathogenesis of Alzheimer’s disease (1, 51). α-Secretase is present in the CSF of both healthy individuals and patients with Alzheimer’s disease (49), suggesting that a similar sheddase that cleaves ACE may also be present in the CSF. Although we did not investigate proteolytic shedding of ACE and ACE2 in this study, it is possible that ACE is shed from the BBM of the ChP at a far higher rate than ACE2, which may explain the relative activities of ACE and ACE2 in the BBM and CSF.

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**Perspectives and Significance**

The present study established the expression of the RAS components in the sheep ChP and the CSF, and the potential changes in a BM-induced model of fetal programming. Because the brain and ChP are in direct contact with CSF, it is likely that these tissues regulate CSF enzymes that lead to the differences in the predominant peptides. CSF peptides are known to exert cardiovascular effects (11, 28), and the levels of ANG-(1–7) relative to BRS are functionally relevant. Thus dysregulation of RAS components in epithelial elements of both brain and kidney resulting from fetal programming events represents at least one target contributing to the observed cardiovascular and autonomic dysfunction. Although we do not explore the mechanisms responsible for these changes, it is possible that epigenetic modifications play an important role in initiating the long-term programming effects. Investigation of epigenetic programming of the RAS components in brain tissue and the ChP warrants further study.

**ACKNOWLEDGMENTS**

The authors gratefully acknowledge Ellen Tommasi and Eric LeSaine for their technical and surgical support.

This work represents partial fulfillment of the requirements for the degree of Doctorate of Philosophy in the Department of Physiology and Pharmacology, Wake Forest University School of Medicine, for A. C. Marshall.

**GRANTS**

Support for this study was provided by National Institutes of Health Grants HD-047584, HD-017644, and HL-51952; the Groskot Heart Fund; and the Wake Forest Venture Fund.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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AJP-Regul Integr Comp Physiol • doi:10.1152/ajpregu.00321.2013 • www.ajpregu.org


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