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

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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 nephrilysin (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·mg⁻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 and control 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 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) 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 nephrilysin (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-His³-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 hypothe-
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 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 AT1/αMas receptor in the brain dorsal medulla (41, 67, 82). The current study utilized sheep that 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 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 AT1/αMas 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 electrophotorethoraphically 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 of the rat protein (25). An additional cytosine residue (Cys*) was added for covariant 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) detection system. Films were exposed for 90 s at 25 Hz and centrifuged at 28,000 g. The following day, the films were examined 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 μM 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 −20°C for Western blot analysis of soluble components of the RAS. The pellet was solubilized by resuspending in 10 μl/mg tissue of 0.5% Triton in reaction buffer and kept on ice overnight. The following day, samples were spun at 28,000 g for 5 min. The supernatant was collected and protein concentration was measured using a Bradford protein assay (66). Brush-border membrane preparation. Brush-border membranes (BBMs) were precipitated and solubilized from ChP4 tissue as previously described by Wittico et al. (78, 79). Briefly, tissue was homogenized in a buffer of mannitol (100 mM), KCl (100 mM), and HEPES (10 mM) pH 7.4 (MIKH buffer) using a Teflon tissue grinder and centrifuged at 300 g for 15 min. The pellet was discarded, and a fraction of the supernatant was saved as a nonenriched control. The remaining supernatant was precipitated with 10 mM CaCl2 for 20 min at 4°C. Samples were centrifuged at 2,500 g for 10 min to precipitate nuclei. 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 MIKH buffer. Both the nonenriched 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 (nonenriched) 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, SCH33970 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 MIKH 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 (Kι) 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, Kι) 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 peptidase 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...
ANGL-(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 ANGL-(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). Acetointrile (Optima grade) was obtained from Fisher Scientific (Fair Lawn, NJ). Lisinopril, a converting enzyme inhibitor, was provided by Merck (West Point, PA). SCH39370, a neprilysin 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 ANGL-(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 Al-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\(^{-1}\)-mg 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 (nonenriched) 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 ANGL-(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 ANGL-(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, ANGL-(1–7) exhibited the highest concentration of the three peptides (Fig. 5D). Moreover, the CSF content of ANGL-(1–7) was significantly lower in BMX sheep compared with the vehicle-treated group. Al-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 125I-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 ± 0.5 vs. 6.6 ± 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 125I-labeled ANG-(1–7) to 125I-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 $\mu$M and $V_{\text{max}}$ of 54.1 nmol·min$^{-1}$·mg$^{-1}$ in controls, and a $K_m$ of 4.1 $\mu$M and $V_{\text{max}}$ of 56.9 nmol·min$^{-1}$·mg$^{-1}$ for BMX animals. In contrast, the rate of 125I-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 $\mu$M in the pooled control CSF, we determined an inhibitory constant ($K_i$) for PCMB of 4.1 $\mu$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 difference in ANG II, ANG-(1–7), or ANG I content between control and BMX animals; however, ANG II was significantly higher than ANG I or ANG-(1–7). In contrast, CSF peptide levels of ANG-(1–7) were significantly lower in the BMX group. We also found that CSF ACE activity was higher in BMX animals, which may contribute to higher metabolism of ANG-(1–7) in the CSF. Finally, the metabolism studies revealed a thiol-sensitive peptidase that contributed to the majority of ANG-(1–7) metabolism in the CSF.

In this model of glucocorticoid-induced fetal programming, antenatal BM exposure is associated with an impaired BRS and increased MAP by 6 mo of age (19, 68, 70, 76). These functional changes are consistent with long-term alterations in the central and renal RAS (24, 41). Microinjection of the AT$_1$
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 AT\(_7\) receptor/Mas antagonist d-Ala\(^1\)-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 AT\(_7\) receptor protein Mas is significantly lower in the dorsal 71). Indeed, we recently showed that protein expression of the

**Fig. 7. Analysis of ANG-(1-7) metabolism in CSF.** \(^{125}\)I-labeled ANG-(1-7) was added to CSF from control or BMX animals in the presence of no inhibitors, 0.5 mM of the thiol agent p-chloromercuribenzoic acid (PCMB), or 0.5 mM PCMB and 10 \(\mu\)M of the ACE inhibitor lisinopril (LIS). Left: representative chromatographs of ANG-(1-7) metabolism with no inhibitors, PCMB alone, or PCMB and LIS. Reactions were terminated at 30, 60, or 120 min and the % of ANG-(1-7) remaining was determined. ○, Control animals no inhibitors; ●, BMX animals no inhibitors; □, control animals + PCMB; ■, BMX animals + PCMB; □, control animals + PCMB and LIS; ●, BMX animals + PCMB and LIS. BMX animals have significantly more metabolism at the 120-min time point in the presence of no inhibitors, PCMB alone, and PCMB with LIS (n = 5 per group). *\(P < 0.05\), **\(P < 0.01\).

**Table 1. Changes in ANG-(1-7) metabolism following treatment with inhibitors;**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ANG-(1-7) Remaining [%]</th>
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<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>+PCMB</td>
<td><strong>80</strong></td>
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<tr>
<td>+PCMB, LIS</td>
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**Table 2. Changes in ANG-(1-7) metabolism following treatment with inhibitors;**

- Control: 100%
- +PCMB: 80%
- +PCMB, LIS: 80%

The ChP from the fourth ventricle includes both polarized epithelial cells connected by tight junctions, and fenestrated endothelial cells that provide nutrients to the basolateral membrane of epithelial cells (56, 81). 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 NH\(_2\)-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 observe a 60-kDa band that is abolished upon exposure to renin, indicating the specificity of the NH\(_2\)-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. ImmunobLOTS 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. ○, With solid lines, control animals; ●, 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/mg protein. For BMX animals K_m = 4.1 µM ANG-(1–7); V_max = 56.9 nmol/min/mg protein.

Fig. 9. Diagram of potential ChP4 and CSF localization of renin-angiotensin system (RAS) components. AI-Aogen, ANG peptides, and prorenin were detected in 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 kcat values in BMX animals. ACE hydrolyzed the Ile3-His6 bond of ANG-(1–7) to form ANG-(1–5), whereas the thiol peptidase cleaved the Tyr4-Ile5 bond to generate ANG-(1–4). Kinetic analysis of the CSF peptidase revealed an apparent Kcat 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 form ANG-(1–7) from ANG I or ANG II, little is known about the enzymatic pathways that metabolize the peptide other than ACE (3). We previously reported that NEP cleaved the Tyr4-Ile5 bond of ANG-(1–7) in the BBM fraction from rat kidney (3); however, NEP is a metalloproteidase that is insensitive to thiol inhibitors such as PCMB, and the specific NEP inhibitor SCH39370 did not attenuate the metabolism of ANG-(1–7) (data not shown). Other thiol-sensitive peptidases localized in CSF include cystatin C, cathepsins, and papain, but their participation in the hydrolysis of ANG-(1–7) to ANG-(1–4) has not been described in CSF (45, 63, 80). Moreover, we have not established whether the thiol-sensitive peptidase is released from choroid or brain tissues. Studies are in progress to isolate the peptidase from sheep CSF and complete the kinetic characterization for ANG-(1–7) and other peptides using the purified enzyme, and to assess the distribution of the peptidase in choroid, brain, and other peripheral tissues.

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.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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