ALTERATIONS IN THE BRAIN RENIN-ANGIOTENSIN SYSTEM IN A MODEL OF FETAL PROGRAMMING

BY

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LIST OF ABBREVIATIONS

11β-HSD = 11β-hydroxysteroid dehydrogenase

ACE = Angiotensin converting enzyme

AM = Amastatin

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}]

Ang-(1-4) = [Asp^{1}-Arg^{2}-Val^{3}-Tyr^{4}]

APMA = aminophenylmercuric acetate

BBM = Brush border membrane

BM = Betamethasone

BMX = Betamethasone exposed

BRS = Baroreflex sensitivity

BS = Bestatin

BSC = Benzylsuccinate

ChP = Choroid plexus

ChP4 = Choroid plexus of the fourth ventricle

CHYM = Chymostatin

CSF = Cerebrospinal fluid

DEAE = Diethylaminoethyl sepharose

DTT = Dithiothreitol

EDTA = Ethylenediaminetetraacetic acid

EGTA = Ethylene glycol tetraacetic acid

GC = Glucocorticoid

HPLC = High performance liquid chromatography

JMV-390 = N-[3-[(hydroxyamino) carbonyl]-1-oxo-2(R)-benzylpropyl]-L-leucine

LIS = Lisinopril

MAP = Mean arterial pressure

NEP = Neprilysin

NTS = Solitary tract nucleus

PCMB = P-chloromercuribenzoic acid

PRR = (Pro)renin receptor

RAS = Renin-angiotensin system

SDS = Sodium dodecyl sulfate

SEM = Standard error of the mean

TOP = Thimet oligopeptidase

ABSTRACT

Allyson Catherine Marshall

ALTERATIONS IN THE CENTRAL RENIN ANGIOTENSIN SYSTEM IN A MODEL OF FETAL PROGRAMMING

Dissertation under the direction of

Mark C. Chappell, Ph.D., Professor

AND

Debra I. Diz, Ph.D., Professor

Antenatal betamethasone (BM) is an approved therapy for women threatening early preterm labor between 24 and 34 weeks gestation. The immediate effects of BM therapy are of benefit to the offspring, as fetal steroid exposure decreases infant mortality by accelerating lung and gastrointestinal tract development and activating the sympathetic nervous system. However, fetal steroid exposure may lead to elevated mean arterial pressure and decreased autonomic function in young adults. Baroreflex sensitivity for control of heart rate is an important marker of autonomic function and is reduced in conditions associated with hypertension. Indeed, sheep exposed *in utero* to BM develop decreased baroreflex sensitivity by 6-weeks of age and increased mean arterial pressure by 6-months of age. These changes in blood pressure and autonomic function are associated with alterations in components of the circulating and intra-renal renin-angiotensin systems (RAS) that shift the RAS to the pro-hypertensive peptide, angiotensin (Ang) II. In the brain, the Ang II AT_1 receptor opposes the beneficial actions of Ang-(1-7) at the Mas receptor for control of baroreflex sensitivity and blood pressure

regulation. Our goal was to identify BM induced alterations in the central RAS that are consistent with functional data reporting increased pressure and decreased baroreflex function. This dissertation focuses on the RAS peptides, receptors, and enzymes that act as possible sites of BM induced alterations.

Ang-(1-7) tone was assessed in BM-exposed (BMX) and control animals by measuring angiotensin receptor expression, peptide levels, and processing enzymes at 0.5-years of age in the brain dorsal medulla containing the solitary tract nucleus, choroid plexus (ChP), and cerebral spinal fluid (CSF). We demonstrate reduced Ang-(1-7) tone in BMX offspring as shown by lower Mas receptor expression and an increased ratio of Ang II/Ang-(1-7) in the brain dorsal medulla, and decreased Ang-(1-7) peptide levels accompanied by increased Ang-(1-7) metabolism in the CSF. This increase in Ang-(1-7) metabolism is a result of increased angiotensin-converting enzyme (ACE) activity, and increased activity of an unidentified peptidase.

Further studies investigate the Ang-(1-7) metabolising peptidase in the CSF and characterize the peptidase as a novel activity due to the unique inhibitor profile, substrate specificity, and optimal pH. We identify high activity of the Ang-(1-7) peptidase in the brain medulla and utilize this tissue as a source of activity for purification. After purifying the peptidase approximately 2000-fold, we confirm the unique inhibitor profile and substrate specificity originally reported in the CSF. These findings support the hypothesis that there is a novel Ang-(1-7) metabolising peptidase present in the CSF and brain medulla of sheep. Collectively, the findings in this dissertation support the hypothesis that *in utero* BM exposure shifts the central RAS towards the ACE-Ang II-AT_1 receptor axis and away from the ACE2-Ang-(1-7)-Mas receptor axis. Additionally,

a novel peptidase involved in endogenous Ang-(1-7) regulation was uncovered and may play an important role in the development of pathological conditions involving central Ang-(1-7) peptide levels.

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BM induced alterations in the central RAS that are consistent with functional data reporting increased pressure and decreased baroreflex function. This dissertation focuses on the RAS peptides, receptors, and enzymes that act as possible sites of BM induced alterations.

Ang-(1-7) tone was assessed in BM-exposed (BMX) and control animals by measuring angiotensin receptor expression, peptide levels, and processing enzymes at 0.5-years of age in the brain dorsal medulla containing the solitary tract nucleus, choroid plexus (ChP), and cerebral spinal fluid (CSF). We demonstrate reduced Ang-(1-7) tone in BMX offspring as shown by lower Mas receptor expression and an increased ratio of Ang II/Ang-(1-7) in the brain dorsal medulla, and decreased Ang-(1-7) peptide levels accompanied by increased Ang-(1-7) metabolism in the CSF. This increase in Ang-(1-7) metabolism is a result of increased angiotensin-converting enzyme (ACE) activity, and increased activity of an unidentified peptidase.

Further studies investigate the Ang-(1-7) metabolising peptidase in the CSF and characterize the peptidase as a novel activity due to the unique inhibitor profile, substrate specificity, and optimal pH. We identify high activity of the Ang-(1-7) peptidase in the brain medulla and utilize this tissue as a source of activity for purification. After purifying the peptidase approximately 2000-fold, we confirm the unique inhibitor profile and substrate specificity originally reported in the CSF. These findings support the hypothesis that there is a novel Ang-(1-7) metabolising peptidase present in the CSF and brain medulla of sheep. Collectively, the findings in this dissertation support the hypothesis that *in utero* BM exposure shifts the central RAS towards the ACE-Ang II-AT_1 receptor axis and away from the ACE2-Ang-(1-7)-Mas receptor axis. Additionally,

a novel peptidase involved in endogenous Ang-(1-7) regulation was uncovered and may play an important role in the development of pathological conditions involving central Ang-(1-7) peptide levels.

CHAPTER ONE

INTRODUCTION

Adminstration of the glucocorticoid betamethasone (BM) is a recommended therapy for women with threatened early preterm labor (1). Antenatal BM improves infant survival by stimulating lung development and decreasing the risk of Respiratory Distress Syndrome, a severe complication of preterm birth and the primary cause of preterm infant mortality, as well as maturation of other fetal physiological systems (128). A landmark study by Liggins and Howie demonstrated antenatal glucocorticoid exposure prevents Respiratory Distress Syndrome by stimulating the synthesis of surfactant and lowering surface tension in immature fetal lungs (96). Indeed, subsequent studies by several groups report antenatal glucocorticoids stimulate the development of numerous organ systems in animal models (119, 168). While the immediate effects of antenatal steroids are of clear benefit to the offspring in the prenatal period (51), the long term consequences are not well studied. Studies in animal models suggest offspring exposed to excess glucocorticoids *in utero* develop elevated blood pressure and an increased risk of cardiovascular disease later in life (24, 59, 173); however, human studies are less conclusive (42, 52).

Premature births account for approximately 12% of all live births in the United States, and since 1994 nearly all early preterm infants are exposed *in utero* to glucocorticoids. The number of individuals exposed to glucocorticoids such as BM or dexamethasone is growing, yet the long term consequences of these exposures are still

unknown. The pathological alterations induced by BM exposure must be identified and corrected in order to address the needs of this population. While several systems may contribute to the alterations observed in animal models of preterm BM exposure, the renin-angiotensin system (RAS) is an important endocrine system and a likely target of glucocorticoid-induced programing events.

1. Fetal Programming and Developmental Plasticity

The fetal programming hypothesis portends that alterations in the fetal environment induce developmental adaptations that predispose offspring to cardiovascular and metabolic diseases later in life. In 1962, the geneticist James Neel proposed the concept of a "thrift hypothesis" to explain the increased prevalance of diabetes mellitus in populations with a surplus of food (113). Accordingly, 'thrifty' genes, those selected during the hunter-gather period, increase an individual's capacity to store fat. Although expression of these genes was deemed advantageous during nomadic times, in modern society thrifty genes may lead to an increased body mass and risk of diabetes (65).

In 1990, the epidemiologist David Barker noted correlations between low birth weight and high rates of ischemic heart disease in adulthood (16). Indeed, Barker and Hale reported an association between low birth weight and several chronic diseases including hypertension, ischemic heart disease, type 2 diabetes, obesity, and hyperlipidemia (18, 73). These investigators proposed the thrifty phenotype hypothesis, which postulates that the fetus undergoes adaptative changes in development dependent on the maternal nutrient supply. These adaptations maximize survival to postnatal life,

but may place the individual at risk for certain diseases in adulthood (65). Often, low birth weight indicates poor *in utero* development. Stressors such as maternal illness and improper nutrition during a critical period in development may have permanent consequences on physiology and metabolism in the offspring. It is proposed that pathological changes arise from a mismatch between fetal conditions and conditions experienced later in life (69). Since Barker's report was first published in 1990, numerous studies have identified potential inducers of fetal programming including nutrient and growth restriction, environmental stressors, and administration of drugs during pregnancy, all of which are associated with increased rates of heart disease, hypertension, and diabetes (17, 77). While numerous stressors may induce long term programming in the offspring, the mechanisms through which this happens are not well established.

Additionally, the timing and severity of the insult are critical in mediating the physiological extent of the adaptive response. There are distinct periods or windows in development when the fetus is particularly sensitive to programming events. A famine in the Netherlands during late World Word II, referred to at the Dutch Hunger Winter, sheds light on the importance of timing in the programming of adult disease. Offspring exposed to the famine during early gestation exhibit higher rates of obesity and cardiovascular disease in adulthood. Rooij et al. discuss the development of central nervous system structures during early pregnancy with regard to appetite regulation and cognitive function whose formation may be altered by low maternal caloric intake (44). Those exposed during midgestation exhibit reduced renal function corresponding to midgestational renal development, and offspring exposed to the famine during late

gestation are born small and exhibit reduced rates of obesity throughout adulthood (53, 130). Animal models confirm the importance of gestational development in exposure to programming events (140).

Glucocorticoids

Glucocorticoids are steroid hormones that bind to the ubiquitously expressed glucocorticoid receptor. Endogenous glucocorticoids, cortisol in humans and corticosterone in rats and mice, are produced primarily by the adrenal cortex and modulate immune and metabolic pathways. In late gestation, fetal cortisol production increases and activates physiological maturation in animals, mandatory for functional lung maturation (30, 86, 95). Glucocorticoids act on receptors in the lung to stimulate production of surfactant and surfactant associated proteins by increasing the activity of phosphatidylcholine (28). Alveolar surfactant decreases surface tension and increases lung compliance. Glucocorticoids also stimulate cell maturation and differentiation, and lead to activation of the sympathetic nervous system.

In 1994, the National Institutes of Health issued a consensus statement recommending the use of the synthetic glucocorticoids BM or dexamethasone to pregnant women threatening early preterm labor, between 24 and 34 weeks gestation. Synthetic glucocorticoids such as BM have increased potency and a more extensive half life compared to the endogenous glucocorticoid cortisol. Two doses of BM (12 mg, 24 hours apart) or four doses of dexamethasone (6 mg, 12 hours apart) are recommended to occupy 75% of the available corticosteroid receptors and induce a near maximal receptor mediated response in target tissues (1). Historically, BM and dexamethasone are used

interchangeably, but BM is generally preferred, as in the current World Association of Perinatal Medicine guidelines (110). Synthetic and endogenous glucocorticoids readily cross the placenta and act on fetal glucocorticoid receptors to facilitate development and stimulate lung maturation. The immediate results of antenatal glucocorticoid therapy are clearly of benefit to the fetus; glucocorticoid exposure may increase survival by approximately 40% in premature infants (128). However, the long term consequences of prenatal glucocorticoid exposure are not well studied in humans. A randomized controlled trial published in the New England Journal of Medicine confirms exposed offspring have no differences in blood pressure, body size, use of health services, or respiratory morbidity than nonexposed children at 2 years of age (40). Another study investigates the hypothalamic-pituitary adrenal (HPA) axis in six to eleven year old children, and finds increased cortisol reactivity to acute psychosocial stress in exposed individuals compared to controls (3). Studies involving long-term followup into adulthood are necessary to identify programmed alterations. Numerous animal studies present evidence for glucocorticoid induced programming in response to one round of BM or dexamethasone treatment. These reports demonstrate alterations in the HPA axis (88), glucose metabolism (141), and renin-angiotensin system (24, 146, 161), as well as other neuroendocrine disorders in animal models (142).

11β-Hydroxysteroid Dehydrogenase

11β-hydroxysteriod dehydrogenase (11β-HSD) is an enzyme that catalyzes the conversion of biologically active corticosteroids (cortisol or corticosterone) to their inactive keto-metabolites (cortisone or 11-dehydrocorticosterone). Mammalian tissues have at least two distinct 11β-HSD isozymes involved in glucocorticoid conversion.

These enzymes have different physiological roles, regulation, and tissue distribution. 11β-HSD type 1 (11β-HSD1) is a NAD(P)H-dependent enzyme that is highly expressed in metabolic tissues such as the liver, adipose tissue, and central nervous system. It reduces cortisone to the hormone cortisol which activates glucocorticoid receptors. Conversely, 11β-HSD type 2 (11β-HSD2) is a high affinity NAD-dependent dehydrogenase that oxidizes endogenous glucocorticoids and serves to protect the mineralocorticoid and glucocorticoid receptors from excess stimulation (155). 11β-HSD2 is widely distributed but is especially important in the placenta during pregnancy, as maternal cortisol levels are ten to fifty times higher than fetal cortisol levels, depending on the stage of gestation (68). Placental conversion of maternal cortisol to cortisone creates a "hypo-cortisolic milieu" in the fetus that may be necessary for HPA axis formation and regulation of endogenous steroid production (156). Indeed, studies show that placental inhibition of 11β-HSD2 allows cortisol to directly cross from maternal to fetal circulation (23).

While 11β-HSD2 serves as a barrier for endogenous glucocorticoids, BM is a poor substrate for 11β-HSD2 oxidation and crosses the placenta more readily than cortisol. BM exposure may also inhibit or alter 11β-HSD2 activity, allowing increased fetal exposure to maternal cortisol in addition to BM (87, 154). As a result of HPA axis negative feedback, decreased 11β-HSD2 inhibits the generation of steriods through the fetal HPA axis. Numerous studies report glucocorticoid treatment during late pregnancy is associated with low birth weight, indicating that glucocorticoids alter fetal tissue maturation (141). Indeed, lower placental 11β-HSD2 activity is correlated with low birth weight in rodent models (23). Because maternal cortisol levels are much higher than

fetal cortisol levels, small changes in 11β-HSD2 activity may lead to relatively greater changes in cortisol in the fetal circulation.

Renin-Angiotensin System as a Target of Programming Events

The RAS is an important regulator of blood pressure and body fluid balance that reflects the tissue and cell specific actions of angiotensin (Ang) peptides (2). During gestation, RAS components play a critical role in mediating proper morphological development and physiological function in numerous tissues (176). Woods and Rasch demonstrated that inhibition of the Ang II type 1 receptor (AT_1 receptor) during nephrogenesis was associated with decreased nephron number (approximately 25%), increased arterial pressure, and reduced renal funciton in a rat model (174). Additional studies find that administration of angiotensin converting enzyme (ACE) inhibitors to the offspring of protein-restricted dams prevent the development of hypertension (100, 148). Indeed, lambs exposed *in utero* to a low protein diet during early development demonstrate enhanced ACE protein expression (66), increased sensitivity to Ang II (106, 133), and alterations in AT_1 receptor expression in the kidney (132, 133, 163). Maternal undernutrition also causes upregulation of AT_1 receptors in numerous tissues including the kidney, adrenal, and lung (171). These studies suggest a role for RAS involvement in the development of hypertension following a fetal programming insult.

In a model of fetal programming induced by overexpression to synthetic glucocorticoids, numerous tissue specific alterations in RAS components have been observed. Dodic and colleagues find that sheep exposed to prenatal dexamethasone during early gestation exhibit increased angiotensinogen expression in the hypothalamus

and higher AT_1 receptor expression in the brain medulla (49). Fetal exposure to BM during late gestation leads to systemic and organ specific changes in the RAS. Shaltout et al. report increased ACE and decreased ACE2 activity in the serum and decreased proximal tubule ACE2 activity in exposed animals compared to controls (143). Gwathmey and colleagues note a reduced functional response of the Mas receptor to produce nitric oxide in exposed offspring (71, 72). These studies are significant in that they demonstrate differential regulation of RAS components that comprise the ACE-Ang II-AT_1 receptor and ACE2-Ang-(1-7)-AT_7/Mas receptor pathways (34).

2. The Central Renin-Angiotensin System

The RAS was originally identified as a circulating hormone system; however, it is now understood that angiotensin peptides are produced and metabolized locally in numerous organs such as the brain, kidney, heart, blood vessels, testis, and eye (12). The experiments in this dissertation focus on alterations in the central RAS associated with functional alterations induced by fetal exposure to BM.

The Brain Medullary RAS

The brain contains the precursors, enzymes, and receptors required for the processing and signaling of angiotensin peptides. Long before the proposal of a brain specific RAS, Bickerton and colleagues demonstrated that infusion of Ang II into the brain increased blood pressure, suggesting the presence of central Ang II receptors (26). Importantly, Fischer-Ferraro et al. provided evidence for renin in the brain (60), and Gaten et al. identified renin-like activity in the central nervous system (64). While there

was initial controversy over the existence of a brain RAS separate from that in the circulation, advanced technology and use of transgenic animals allowing for localized expression of RAS components strongly supports a brain localized RAS (11). Several important components and functions of the brain RAS are summarized below.

Brain angiotensinogen is synthesized predominantly in astrocytes, processed to smaller peptide fragments including Ang II and Ang-(1-7), and incorporated into neurons where these peptides function as neurotransmitters (105). Ang II actions are mediated through AT_1 and AT_2 receptors. AT_1 receptors are highly expressed in organs with cardiovascular function such as the heart, kidney, and brain (4, 122). Major outcomes of AT_1 receptor activation include increased vasoconstriction and water retention, adrenal aldosterone release, supression of baroreflex function, increased cell proliferation, inflammation, fibrosis, and increased production of reactive oxygen species (4). Ang II signaling through the AT_1 receptor is inhibited by selective AT_1 receptor antagonists such as losartan or candesartan. The AT_2 receptor also mediates the actions of Ang II and is ubiquitously expressed in fetal, but shows distinct localization in adult tissues. Its actions typically counteract those of Ang II at the AT_1 receptor, leading to increased vasodilation, decreased proliferation, and antioxidant like actions (167). The AT_2 receptor antagonist PD123319 specifically inhibits Ang II-AT_2 receptor signaling. Overall, the activation of the ACE-Ang II-AT_1 receptor axis induces increased blood pressure and sympathetic nervous system activity.

Similar to its actions in peripheral tissues, central Ang II signaling is primarily mediated by the AT_1 receptor. Autoradiographical studies confirm that AT_1 receptors are expressed in brain areas known to be targets of Ang II actions such as the subfornical

organ, paraventricular nucleus, solitary tract nucleus, area postrema, and circumventricular organs (4, 94, 122). AT_1 receptor expression is altered in specific regions of the brain in response to distinct physiological challenges such as dehydration, stress, or hypertension (21, 36). Autoradiographical studies localize AT_2 receptors in the brains of rat and sheep (80, 131).

While AT_2 receptors may partially counterbalance the actions of Ang II at the AT_1 receptor, the actions of Ang-(1-7) at the Mas receptor are well characterized and oppose Ang II-AT_1 receptor signaling (181). The ACE2-Ang-(1-7)-Mas receptor axis is known as the non-classical RAS, due to the relatively recent discovery of these components (34). Ang-(1-7) is endogenously expressed in many tissues including brain cardio-regulatory centers such as the hypothalamus, medulla, and amygdala (32). It was characterized as the first amino-terminal angiotensin product with cardiovascular functions. Ang-(1-7) was noted to have non-AT_1, non-AT_2 receptor dependent stimulation of prostaglandin release and nitric oxide production (118, 160). Khosla and colleagues described D-Ala^7-Ang-(1-7) (A-779), an antagonist against the antidiuretic and blood pressure actions of Ang-(1-7) (136). Almost a decade later, the orphan G protein-coupled receptor Mas was identified as an endogenous receptor for Ang-(1-7) (137). Numerous studies demonstrate Ang-(1-7) signaling through the Mas receptor induces nitric oxide and prostaglandin release inducing vasodilation, decreased cell proliferation, and increased baroreflex sensitivity for control of heart rate (35, 56, 134).

Choroid Plexus RAS

The choroid plexus (ChP) is a polarized epithelial tissue that regulates the transport of ions and proteins from the blood to the cerebrospinal fluid (CSF). Unlike the capillaries that constitute the blood-brain barrier, ChP endothelial cells are fenestrated, allowing for the free movement of molecules into ChP epithelial cells. Tight junctions between ChP epithelial cells comprise the blood-CSF barrier (BCFSB) and regulate the passage of molecules into the CSF (93, 125). ChP epithelial cells resemble renal proximal tubules, as both tissues act to transport fluids and ions across their epithelium and regulate the chemical composition of CSF (ChP) or blood (renal proximal tubules). The ChP actively produces CSF, in contrast to renal tubules that act exclusively to facilitate transport (13, 123, 125). ChP transport is generally from the basolateral to the apical side or brush border membrane (BBM). Gasses and lipid soluble molecules diffuse into the CSF while large and polar molecules undergo mediated or active transport. The apical membrane is also able to filter metabolites out of the CSF for eventual elimination by the kidney or liver (13). Organic acids, halides, and potassium are actively cleared out of the CSF and into the ChP.

The RAS is known to regulate epithelial transporters in both the kidney and ChP and accordingly plays a role in the development of certain types of hypertension (74, 81). Amin et al. reported that expression of epithelial sodium channels (ENaC) is higher in the ChP of salt-sensitive as compared to salt-resistant rats (8). Moreover, the CSF sodium content is higher in the Ang II-deoxycorticosterone (DOCA) salt model of hypertension compared to normotensive controls. The ChP contains RAS components including angiotensinogen and renin (76). Early studies show ACE activity in the ChP, although

the degree of activity is species-dependent with very high enzyme activity in rabbits and lower activity in humans (9). Rix and colleagues localized ACE protein to the BBM of the ChP by an immunocytochemical approach (127). ACE is localized to the BBM of other epithelial tissues including proximal tubules of the kidney, lung (6), eye (157), and intestines (14). Membrane bound ACE2 and neprilysin activities are not well characterized in the ChP; however, both proteins are detected in this tissue (39).

Cerebrospinal Fluid RAS

The CSF is secreted from the apical membrane of the ChP and protects the brain while providing necessary ions and proteins. Although the blood-brain barrier is the main site of entry for oxygen, carbon dioxide, and glucose, the BCSFB regulates the passage of calcium, sodium, and proteins. The protein concentration of CSF is roughly 0.4% that of plasma, reflecting the high degree of selectivity of the BCSFB. Components of the RAS have previously been described in the CSF including ACE, ACE2, neprilysin, angiotensinogen, Ang I, Ang II, and cathepsin D (54, 63, 104, 138). Notably, renin is typically not detectable in the CSF (60, 82). Intracisternal administration of Ang II elicits an increase in blood pressure, suggesting CSF-borne Ang II targets receptors in the brain that influence blood pressure (45). While Schelling and colleagues demonstrate Ang II is present in the CSF, the angiotensin receptor antagonist saralasin and the ACE inhibitor captopril escape from CSF into circulation, when high doses are applied intraventricularly. They conclude that CSF Ang II is not from plasma origin (138). Thus, they hypothesize that RAS components are synthesized in brain tissue and released into the brain ventricles.

Interestingly, numerous studies have investigated the role of CSF RAS components in the progression of neurological diseases. Decreased CSF ACE activity is correlated with the degree of dementia in patients suffering from Alzheimer's and Parkinson's diseases (184). Conversely, ACE activity is increased in brain tissue from Alzheimer's patients indicating a difference in ACE regulation between brain tissue and CSF (109). The metalloendopeptidase neprilysin is also implicated in Alzheimer's disease; neprilysin is located in presynaptic nerve terminals and degrades the amyloid-β peptide (99). Accumulation of amyloid-β peptides leads to the deposition of amyloid plaques and is directly linked to neurodegeneration and dementia (112). Reduced CSF neprilysin activity is evident in the early stages of Alzheimer's disease, suggesting a relationship between neprilysin activity and amyloid plaque deposition (99, 108). Less data exists on the relationship between the CSF RAS and cardiovascular diseases. However, ACE and neprilysin activities are susceptible to pathological regulation and may contribute to fetal programming induced central alterations.

3. Angiotensin Peptide Metabolism by Endogenous Peptidases

Metabolism of angiotensin peptides, by membrane bound or soluble peptidases, is a critical step in regulating the activity of the vasoactive peptides. Metabolism of Ang-(1-7) deactivates the peptide and decreases the functional role of Ang-(1-7). The Ang II derivatives Ang III and Ang IV exert vasoactive actions; however, extended carboxy- or animo-terminal metabolism of Ang II results in non-vasoactive peptides. Angiotensin metabolizing peptidases are distinguished from one another based on their sensitivity to

specific inhibitors, substrate specificity, and the sites of cleavage in the angiotensin peptide sequence.

Ang I Metabolism

Ang I metabolism is well studied and leads to the production of the vasoactive peptides Ang II and Ang-(1-7). Ang I is cleaved by ACE to produce Ang II, and by neprilysin or thimet oligopeptidase to produce Ang-(1-7) (34).

ACE, a zinc dependent dipeptidyl carboxypeptidase, cleaves the carboxy-terminal dipeptide of Ang I to produce Ang II (35). Human ACE contains two homologous catalytically active sites, a large extracellular domain, and a short intracellular carboxy-terminal domain (27). ACE activity is observed in central nervous system areas including neuronal cell bodies in the hypothalamus, hippocampus, brainstem, ChP, and CSF (29, 129, 166). Autoradiography, using radiolabelled ACE inhibitors, established the presence of ACE protein in the ChP, blood vessels, subfornical organ, and organum vasculosum (27). Furthermore, ACE colocalized with renin in synaptosomal fractions of the brain (120). Like many other neuropeptidases, ACE is primarily membrane bound with a short hydrophobic membrane spanning domain. Although ACE is a membrane bound peptidase, its presence in CSF suggests a protolytic cleavage from the transmembrane stalk (90). Indeed, an unknown secretase proteolytically cleaves ACE from the luminal surface of vascular endothelial cells in close contact with the circulation, renal proximal tubules, and the ChP (22, 124). ACE is critically involved in blood pressure regulation due partially to Ang II production; accordingly ACE inhibitors are used as a first-line therapy for cardiovascular and renal diseases.

Ang I is also a precursor for the vasoactive heptapeptide Ang-(1-7). Neprilysin, thimet oligopeptidase, and to a lesser degree neurolysin, cleave the Pro^{7}-Phe^{8} bond of Ang I to produce Ang-(1-7). Neprilysin is a membrane bound zinc-metallopeptidase that cleaves peptide bonds surrounding hydrophobic amino acids, particularly aromatic residues including Phe or Tyr at the P1 site (150). Neprilysin is highly expressed in brain tissue and the ChP and directly cleaves Ang I to Ang-(1-7) (89, 175). Soluble neprilysin activity in the CSF is also widely studied with regard to neurological diseases such as Alzheimer's disease (103).

While neprilysin is primarily membrane bound, thimet oligopeptidase is a soluble metallopeptidase originally isolated from bovine pituitary and rat brain. Thimet oligopeptidase hydrolyzes the carboxyl side of aromatic and basic residues (78, 79, 117). Pereria and colleagues report that thimet oligopeptidase is the main enzyme involved in the generation of Ang-(1-7) in the rat hippocampus (121). Alzayadneh et al. demonstrate thimet oligopeptidase dependent Ang-(1-7) formation in the proximal tubule NRK-52E cell line (7). Analysis of recombinant thimet oligopeptidase shows recognition of peptide substrates ranging from six to seventeen amino acids in length (31, 116). It was later demonstrated that approximate 30% of thimet oligopeptidase activity was associated with lipid rafts in plasma membranes, endosomes, and synaptic vesicles, while the remaining 70% was cytosolic (75). Thimet oligopeptidase undergoes basal and secreted release into extracellular space, accounting for its presence both within and outside of the cell (58). The metallopeptidase is optimally active at neutral pH, inhibited by chelating agents, and is reactivated by low concentrations of Zn^{2+}.

Neurolysin, a neutral metallopeptidase localized in the central nervous system and periphery, also metabolizes Ang I to produce Ang-(1-7) (15, 37). Checler and colleagues purified the neurotensin-degrading peptidase from rat brain synaptic membranes (37). Neurolysin is largely membrane bound in neurons, but mainly cytosolic in astrocytes (165). Neurolysin cleaves the endogenous peptide neurotensin at the Pro^{10}-Tyr^{11} bond and Ang I at the Pro^{7}-Phe^{8} bond suggesting the preference for hydrophobic and aromatic residues at the cleavage site (43). There is evidence that neurolysin can hydrolyze the Tyr^{4}-Ile^{5} bond of Ang II but not Ang-(1-7) to form Ang-(1-4) (41, 135). Like other brain metalloendopeptidases, neurolysin is optimally activate at a neutral pH, sensitive to chelating agents, and utilizes small peptides substrates (149).

Ang II Metabolism

ACE2 is a major Ang II metabolizing peptidase and directly converts Ang II to Ang-(1-7) through cleavage of the carboxy-terminal Phe^{8} residue. While ACE2 is able to act on Ang I to produce Ang-(1-9), it exhibits a 500-fold higher catalytic efficiency for the conversion of Ang II to Ang-(1-7) (164). ACE2 plays a critical role in the central RAS by allowing for direct conversion between the two major vasoactive RAS peptides (50). By reducing AT_1 receptor stimulation by Ang II and augmenting Mas receptor stimulation by Ang-(1-7), ACE2 may play a part in opposing the development of neurogenic hypertension (179). ACE2 is widely expressed in the brain and localized in neuronal cell bodies and glial cells (50). ACE2 selective antibodies reveal high ACE2 expression in cardiorespiratory neurons of the brainstem suggesting the carboxypeptidase may play a role in central blood pressure regulation (50). Numerous groups have shown reduced ACE2 activity and expression in the development of hypertension.

Overexpression of central ACE2 leads to a reduction in sympathetic drive, improvement in baroreflex funciton, and a reduction in blood pressure in models of hypertension (55, 177, 178).

Similar to ACE, ACE2 is primarily membrane bound with a short carboxy-terminal cytoplasmic domain, a transmembrane domain anchoring it to the plasma membrane, and a long amino-terminal segment consisting of a single catalytic zinc binding domain (170). Although ACE and ACE2 share 42% sequence homology, the two peptidases are distinguishable through the use of specific inhibitors and have marked differences in substrate specificity. While ACE2 is primarily membrane bound, soluble ACE2 in the circulation and CSF indicates cleavage of active ACE2 from the transmembrane stalk (83). Tumor necrosis factor-alpha convertase (ADAM17) is identified as a secretase involved in ACE2 shedding (92). A recent study by Xia et al. reports increased ADAM17 mediated ACE2 shedding in a salt-sensitive rat model resulting in lower ACE2 compensatory activity (179). Urinary ACE2 levels increase in diabetes, suggesting that ACE2 shedding from tubular cells may be upregulated in this model of renal damage (180). Reductions in membrane bound ACE2 activity may correspond to increased ADAM17 mediated shedding and reduced local production of Ang-(1-7).

Ang-(1-7) Metabolism

Compared to Ang I and Ang II, little is known about Ang-(1-7) metabolism. Because the balance between Ang II and Ang-(1-7) is critical for normal RAS function, reductions in the activity of Ang-(1-7)-producing peptidases and elevations in Ang-(1-7)-

metabolizing peptidases are of equal importance. Furthermore, unlike metabolites of Ang I and Ang II, the products of Ang-(1-7) metabolism do not appear to have vasoactive actions. The dipeptidyl carboxypeptidase ACE plays an important role in the hydrolysis of Ang-(1-7) in circulation, kidney proximal tubules, and the brain (33, 102, 147). ACE inhibition decreases Ang I to Ang II processing while increasing the half-life of Ang-(1-7) six-fold in circulation (182). The mechanism for increased Ang-(1-7) peptide levels following treatment with the ACE inhibitor reflects protection of the peptide from ACE hydrolysis and shunting of Ang I to Ang-(1-7) through endopeptidase pathways such as neprilysin or thimet oligopeptidase. Although ACE is a transmembrane protein, it is proteolytically shed from the cell surface by a secretase resulting in soluble forms circulating in the blood and CSF (5).

While neprilysin facilitates the conversion of Ang I to Ang-(1-7), the endopeptidase may also hydrolyze Ang-(1-7) to inactive peptide fragments (84, 183). Past studies have shown neprilysin metabolism of Ang-(1-7) to Ang-(1-4) in the kidney (6, 70). Other mechanisms of Ang-(1-7) metabolism are investigated in Chapters three through five of this dissertation. Because the balance of Ang II and Ang-(1-7) regulates RAS actions, the production and metabolism of both peptides is important to understand.

4. The RAS and Blood Pressure Control

The RAS is a potent feedback system for control of blood pressure and volume homeostasis. Clinical therapeutics highlight the significance of Ang II on blood pressure control. ACE inhibitors, first developed in the late 1960s, and AT_1 receptor antagonists are two major classes of anti-hypertensive medications that decrease Ang II signaling and

consequently decrease blood pressure in many patients (57). ACE inhibitors prevent the conversion of Ang I to Ang II and increase circulating Ang-(1-7) levels due to decreased ACE dependent metabolism (35). Additionally, ACE inhibitors may lead to downregulation of AT_1 receptor expression (139). AT_1 receptor antagonists prevent Ang II binding at the AT_1 receptor and inhibit downstream signaling events including activation of phospholipase C, phosphoinositide hydrolysis leading to sustained muscle contraction, arachidonic acid production, and inhibition of adenylyl cyclase (107). AT_1 receptors are under tight negative feedback control by the agonist Ang II, as well as growth factors and cytokines that can upregulate or downregulate receptor expression in certain cell types. Peripheral administration of Ang II elicits a pressor response leading to increased arterial pressure (61). This response is partially mediated by an increase in sympathetic nervous system activity. Additionally, Ang II reduces the sensitivity of the baroreflex receptors for control of heart rate and shifts the sympathetic outflow setpoint to higher pressures (10). In the solitary tract nucleus, Ang-(1-7) has depressor responses and increases baroreflex control of heart rate. The mechanism of Ang-(1-7) manipulation of baroreflex sensitivity may arise from its ability to decrease sympathetic tone and modulate local norepinephrine effects in the brain (46, 67).

The Autonomic Nervous System

The autonomic nervous system is a division of the peripheral nervous system that acts autonomously to control biological processes such as breathing, blood pressure, and digestion. The autonomic nervous system innervates all internal organs and stimulates or inhibits biological processes. It is divided in two major branches, the sympathetic and the parasympathetic nervous systems, that act in a dynamic balance to regulate autonomic

function. The sympathetic nervous system mediates the hormonal stress response and is involved energy mobilization. It is activated during emergency situations and also known as the fight-or-flight response. Ang II exerts actions on the sympathetic nervous system to increase sympathetic outflow and stimulate neurotransmission (126). A stimulation of the sympathetic nervous system causes increased heart rate, expansion of the airways, pupil dilation, and release of stored energy. Conversely, the parasympathetic nervous system conserves energy and restores the body to a resting state. The parasympathetic nervous system stimulates digestion, slows heart rate, and decreases blood pressure. The reflex arcs regulating the sympathetic and parasympathetic nervous systems are regulated by sensory and motor neurons in the autonomic brainstem nuclei.

The autonomic nervous system undergoes rapid development during gestation and continues to develop during the first year of postnatal life (169). Autocorrection of fetal heart rate is used as a noninvasive measurement of fetal autonomic nervous system activity; the time period from 21 to 31 weeks gestation shows large improvements in fetal heart rate correction and is likely critical in fetal autonomic nervous system development (169). Fetal stressors, such as intrauterine growth restriction, delay maturation of the fetal autonomic system (62). The RAS is also active during intrauterine life. Fetal tissues contain receptors for the angiotensin peptides, and levels of Ang II are similar between the mother and fetus (97). Indeed, the central RAS plays an important role in regulating fetal cardiovascular responses, body fluid balance, and neuroendocrine control (101). In the fetal rat brain, choroid plexus, and ependymal cells lining the third ventricle, angiotensinogen immunoreactivity is present by the nineteenth day of gestation (111, 152). Additional studies also identify ACE in the choroid plexus, subfornical

organ, and posterior pituitary (158), renin (151), Ang II, and the AT_1 and AT_2 receptors by the nineteenth day of gestation in rats (153). Due to the early development of RAS components in the fetal brain, alterations in fetal Ang II or Ang-(1-7) levels may influence the subsequent development of the sympathetic and parasympathetic nervous systems.

Angiotensin Peptides and Autonomic Function

One of the most important reflex arcs in the autonomic nervous system for control of cardiovascular function is the baroreceptor reflex arc. The baroreceptor reflex is responsible for the regulation of blood pressure over a timeframe of seconds to minutes. Baroreceptors are stretch-sensitive mechanoreceptors located in the aortic arch and carotid bodies that activate autonomic feedback loops to regulate short term cardiovascular homeostasis. Numerous clinical studies demonstrate an association between hypertension and impairment of the baroreceptor reflex (10). This disruption is largely due to increased levels of Ang II. Ang II disruption of the baroreceptor reflex arc is two-fold. First, Ang II impairs baroreflex sensitivity through the AT_1 receptor and an apparent interaction with substance P. Second, Ang II increases the blood pressure set point maintained by the baroreceptors (10). Blockade of the RAS, and particularly Ang II, resets the baroreflex curve to normotensive pressures and improves baroreflex sensitivity by decreasing sympathetic nerve activity (25, 91). The organization of the baroreflex arc in the central nervous system begins with peripheral afferent fibers from the arterial baroreceptors in the solitary tract nucleus. The solitary tract nucleus contains a dense concentration of AT_1 receptors located on cell bodies and Ang II-containing nerve fibers (47, 159). Microinjection of angiotensin peptides and receptor antagonists in

the solitary tract nucleus has shed light on the opposing roles of Ang II and Ang-(1-7) for control of baroreflex function. Intracerebroventricular injection of Ang-(1-7) improves baroreflex sensitivity while the Mas receptor antagonist A779 depresses the baroreflex (115). Endogenous levels of Ang II and Ang-(1-7) contribute to basal baroreflex function as demonstrated by microinjection and intracerebroventricular studies of peptide and receptor antagonists.

5. Rationale

Glucocorticoids are typically administered to women entering early preterm labor to increase fetal lung development, activate the sympathetic nervous system, and improve infant survival (52, 162). At 14 years of age, blood pressure was reported to be higher in a group of children who were born preterm and whose mothers had received BM before delivery, compared to a preterm group who had not received steroid treatment (38, 162). It has become increasingly apparent that the prenatal administration of steroids has postnatal consequences on the cardiovascular health of the offspring. The concept of fetal programming was first introduced by David Barker, an epidemiologist who noted a correlation between low birth weight and adult cardiovascular disease in populations from England and Wales (16, 19). Lumbers et al. suggested that activation of the HPA axis, together with increased sympathoadrenal outflow in the fetus, can retard growth and lead to low birth weight (98). Over the past 20 years, various models of prenatal stress have reported increased incidences of cardiometabolic disorders including obesity, heart disease, hypertension, and insulin resistance (16, 85, 114). Although many studies utilize rodents and have identified potential mechanisms that contribute to fetal programming, we use a sheep model of pregnancy that parallels human gestation. In contrast to rodents,

sheep undergo similar intrauterine kidney development and have a comparable exchange of nutrients and gasses through the placenta to humans (20). Indeed, the similar renal development is particularly important as the kidneys are the major long-term regulator of mean arterial pressure and blood volume (172). In humans, synthetic glucocorticoid administration between 24 and 34 weeks gestation coincides with a key timepoint in kidney development. In fact, BM administration during this period decreases nephron number by approximately 25% (48, 173). BM is given to pregnant ewes at 80 and 81 days gestation, corresponding to a similar point in nephrogenesis, and yields a similar reduction in nephron number (173). Past studies by our group utilized this model of fetal programming in order to establish the mechanism for increased blood pressure. BM exposed (BMX) offspring exhibit reduced baroreflex sensitivity for control of heart rate by 6-weeks of age (146), increased mean arterial pressure by 6-months of age (59, 144), and increased sympathetic tone (142). Microinjections of AT_1 and Mas receptor antagonists into the solitary tract nucleus of the dorsal brain stem reveal an increase in Ang II and a decrease in Ang-(1-7) pathways mediating baroreflex sensitivity in 6-week old BMX offspring compared to controls (145). Similarly, Gwathmey et al. reported a reduction in Mas and AT_2 receptors and an increase in AT_1 receptors in renal cortical plasma membranes of BMX sheep (71). A summary of known alterations in BMX offspring in the circulation, brain, and kidney are shown on the following page.

Compartment	**Alteration**
Systemic	
Mean Arterial Pressure	Increased (6-months)
ACE2	Decreased (1.8-years)
Brain	
Baroreflex Sensitivity	Decreased (6-weeks)
Heart Rate Variability	Increased (6-weeks)
Hypothalamic-Pituitary-Adrenal Response	Increased (6-weeks)
Response to AT_1 receptor antagonist	Increased (1.8-years)
Response to Mas antagonist	Decreased (6-weeks)
Kidney	
AT_1 receptor sensitive sites	Increased (1-1.5-years)
AT_2 receptor sensitive sites	Decreased (1-1.5-years)
Ang II reactive oxygen species response	Increased (1-year)
Ang-(1-7) nitric oxide response	Decreased (1-year)
Nephron number	Decreased (fetal)
Glomerular filtration rate	Decreased (6-months)

Our ongoing studies focus on regulation of the RAS in the central nervous system of BMX animals and how these data correlate with the functional measures of baroreflex sensitivity and mean arterial pressure. Evidence provided by preliminary data suggests a shift in the central RAS towards a pro-hypertensive phenotype favoring the ACE-Ang II-AT_1 receptor pathway. Whether the pro-hypertensive phenotype is a result of higher

contribution of the ACE-Ang II-AT_1 receptor axis, lower contribution of the ACE2-Ang-(1-7)-Mas receptor axis, or a combination of both is unknown. Our goal in these studies was to identify BM induced alterations in angiotensin peptides, receptors, and metabolizing enzymes in the central nervous system.

The importance of the solitary tract nucleus for regulation of autonomic function prompted us to begin our analysis in dorsal medullary tissue. Mas and AT_1 receptors are highly expressed in the solitary tract nucleus. Microinjection of the Mas receptor antagonist A779 into this brain area of control sheep reduces baroreflex sensitivity. However, microinjection of A779 did not further reduce the impaired baroreflex sensitivity of BMX sheep. These data indicate a functional shift in Ang-(1-7) signaling mediated at the level of the Mas receptor in the solitary tract nucleus (145). Because the brain is in constant contact with the CSF and may interact with circulating RAS components in this compartment, we investigated the RAS in both CSF and ChP tissue. Little is known about the ChP RAS, so we aimed to localize components of the RAS within the tissue and investigate BM induced alterations. These studies led us to investigate an Ang-(1-7) metabolizing peptidase in the CSF. The peptidase is responsible for the majority of Ang-(1-7) processing in the CSF and correlated with Ang-(1-7) peptide levels, suggesting a physiological role in peptide expression. Due to its unique inhibitor sensitivity and selectivity for Ang-(1-7) as a substrate, it is likely that this is a novel peptidase. We identified high concentrations of the Ang-(1-7) peptidase in brain medullary tissue, and used it as a source for purification. Using a combination of ion exchange chromatography and molecular weight exclusion filtration, we purified the peptidase approximately 2000-fold. This preparation was used to derive apparent kinetic

constants, assess substrate specificity, and determine the inhibitor sensitivity. The aims for this dissertation are as follows:

Aim 1: Does betamethasone exposure induce a shift in the central renin-angiotensin system towards the ACE-Ang II-AT_1 receptor axis in the brain medulla of adolescent and adult sheep?

Aim 2: Are renin-angiotensin system components altered in the choroid plexus and cerebrospinal fluid of betamethasone exposed sheep?

Aim 3: Is there a novel peptidase involved in Ang-(1-7) degradation in the cerebrospinal fluid and brain medulla that contributes to lower Ang-(1-7) peptide levels in betamethasone exposed sheep?

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CHAPTER TWO

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.

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 (11, 36), 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 (12). Experimental studies by our group and others have begun to elucidate the potential mechanisms for altered blood pressure associated with GC exposure (5, 27, 38). These mechanisms include alterations in kidney development (41), a significant reduction in nephron number (27, 42), ENREF_7 impaired neural control (9, 29, 31), and alterations to the circulating and local renin-angiotensin systems (RAS) (5, 27, 30, 38). 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 (30). 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.

Materials and methods

Animals

Sheep received saline or betamethasone acetate: phosphate 1:1 mixture (IM, 2 doses of 0.17 mg/kg, 24 hours 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-hour 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.

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: 1:2,000). 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 1μg peptide to 1μg 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 (16). 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 (16). 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)

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) 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 minutes, 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).

Peptide Measurement

Ang peptides in the medullary tissues were measured in the Hypertension Center Core Assay Laboratory utilizing multiple radioimmunoassays (RIAs) (3, 10, 30). 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 (3). 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 (10, 30). 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 (30). 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.

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%.

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 (Figure 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 Figure 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 (Figure 1, lower panel, left). We noted a large variability in AT1 receptor expression at 1.8-years; however, there was no trend towards 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 the protein band for the Mas receptor, and attenuated expression of the AT1 receptor band (Figure 1, middle and lower panels, right).

The specificity of the Aogen antibodies in sheep was validated by measuring AI-Aogen and total Aogen in samples with and without the renin inhibitor aliskiren. The cytoplasm from kidney cortex homogenates provided active renin, and plasma from nephrectomized sheep was the source of the intact AI-Aogen substrate. The upper blot of Figure 2 reveals a time dependent decrease in the AI-Aogen samples lacking the renin inhibitor. Addition of aliskiren essentially abolished the disappearance of the AI-Aogen band up to the 4 hour time point (240 mins). In contrast, the lower blot probed with the Int-Aogen antibody demonstrates no change in the band over time regardless of the presence of aliskiren suggesting this antibody measures both Ang I and des-Ang I forms of Aogen (total Aogen). Utilizing these antibodies, we then quantified the relative expression of Aogen in the brain medulla. At 0.5-years of age, there was no difference in total Aogen protein expression between control and BMX animals (data not shown); however, AI-Aogen was 44% lower in the BMX animals (Figure 3, upper panel). These data suggest that greater processing of Aogen may occur in the dorsal medulla of exposed

sheep at this age. In contrast, AI-Aogen was 280% higher in the dorsal medulla of BMX animals (Figure 3 lower panel) with no change in total Aogen levels (data not shown) at 1.8-years of age.

Tissue concentrations of Ang I, Ang II, and Ang-(1-7) were determined in the dorsal medulla of both 0.5- (Figure 4, upper panel) and 1.8- (Figure 5, upper panel) year old animals. Although there were no statistical differences between BMX and control animals for any of the individual peptides, expression of the data as peptide ratios revealed significant differences between control and exposed animals. At 0.5-years of age, BMX animals exhibit a significantly higher ratio of Ang II to Ang-(1-7) as well as a higher Ang II/Ang I (Figure 4, lower panel). The ratio of Ang-(1-7) / Ang I was not different between groups. Moreover, there were no differences in the peptide content or ratios between BMX and control animals at the 1.8-year time point (Figure 5, lower panel).

As shown in Figure 6, there was a significant positive correlation between Ang-(1-7) peptide levels and the Mas receptor ($p = 0.02$; $r = 0.92$) at 0.5-years, while the levels of Ang II peptide trended towards a negative correlation with the AT1 receptor at this age ($p = 0.07$; $r = -0.84$).

Discussion

In the sheep model of GC-induced fetal programming, antenatal BM exposure is associated with impaired BRS as early as 6-weeks of age, with reduced BRS and

increased MAP by 0.5-years of age (9, 29, 31). Furthermore, microinjection of the AT1 receptor antagonist Candesartan (CV) into the nucleus tractus solitarius improved BRS in both control and BMX animals at 6-weeks of age (31). In contrast, Ang-(1-7) receptor blockade with the selective Mas antagonist D-Ala^7-Ang-(1-7) (D-Ala, A779) inhibits the reflex only in the unexposed animals (9, 14, 31). In BMX sheep at 0.5- and 1.8-years of age, AT1 receptor blockade also improved the reflex and lowered arterial blood pressure to that of controls (9, 32). However, there was little effect of bilateral microinjection of D-Ala on BRS or blood pressure in BMX sheep (9). Overall, these data suggest an imbalance in the actions of Ang II and Ang-(1-7) for baroreflex control of heart rate in the brain medulla that occurs as early as 6-weeks and persists to adulthood (17). The current study demonstrates that lower expression of the Mas receptor in the dorsal medulla at both 0.5- and 1.8- years of age. At 0.5-years of age there is also a higher ratio of Ang II to Ang-(1-7), suggesting decreased Ang-(1-7) tone in this brain region may contribute to the impaired BRS and dysregulation of blood pressure. Indeed, these data reflect the functional imbalance between Ang II and Ang-(1-7) in the kidney of BMX animals. We previously showed that young male BMX sheep exhibit a decreased renal vascular response to Ang-(1-7) (13). Ang-(1-7) infusion inhibits sodium reabsorption in control males, but prenatal BM also attenuated the natriuretic response to Ang-(1-7) (13). Binding studies also revealed a greater proportion of AT1 sites in the renal cortex of adult BMX sheep, as well as a reduced proportion of AT7 or D-Ala sensitive sites (3). Thus, BMX may induce the loss of Ang-(1-7) receptors resulting in the inability of the kidney to produce a natriuretic response to Ang-(1-7) (13).

In addition to altered receptor expression, AI-Aogen expression was lower in the BMX animals at 0.5-years, suggesting an enhanced degree of Aogen processing to form Ang peptides. At the same age, the Ang II: Ang-(1-7) and Ang II: Ang I ratios were higher in BMX animals, but there was no difference in the ratio of Ang-(1-7) to Ang I. This suggests an increased role for Ang II in 0.5-year BMX animals relative to Ang-(1-7) that may likely contribute to the suppression of BRS at this age (32). Moreover, the greater extent of Aogen processing in the medulla of BMX sheep may contribute to higher levels of Ang II without alterations in the tissue content of Ang-(1-7). Thus, it is possible that changes in ACE and ACE2 activities further influence the relative levels of the peptides. The balance between Ang II and Ang I may reflect higher ACE activity, while the balance between Ang II and Ang-(1-7) may reflect both ACE and ACE2 activities. Our previous study demonstrated that the ratio of ACE to ACE2 activity was significantly higher in the circulation of the BMX sheep reflecting changes in both peptidase activities (30). Proximal tubular and urinary ACE2 activities were also reduced in the kidneys of the exposed animals (33). Studies are in progress to assess the enzyme activities of ACE and ACE2 in the dorsal medullary tissues of control and BMX animals.

At 1.8-years of age, there is less AI-Aogen processing in BMX compared to control animals. While administration of CV reduces blood pressure and enhances BRS in 1.8-year old BMX animals (29, 30), we now report that Ang II content and AT1 receptor expression are no different than controls. It is possible that a reduction in the Mas receptor may lead to less Ang-(1-7) tone in the functional antagonism of the Ang II-AT1 receptor axis. Kostenis et al show that the Mas receptor heterodimerizes with the AT1 receptor and significantly impairs the Ang II mediated elevation of intracellular Ca^{2+}

with no change in AT1 protein expression or pharmacological characteristics of the receptor (21). Therefore, the loss of the Mas receptor may result in greater AT1 receptor signaling in the absence of overall changes in the AT1 receptor protein or Ang II levels. This may explain the sensitivity of the BMX sheep to acute administration of the AT1 antagonist CV at 1.8 years of age (28, 34).

Finally, we correlated the Ang II and Ang-(1-7) peptide levels to the AT1 and Mas receptors, in the dorsal medullas of 0.5-year old animals. This analysis revealed a trend for the negative correlation between Ang II and the AT1 receptor consistent with previous reports that Ang II attenuates expression of the AT1 receptor (6, 26). Interestingly, we found a positive correlation between Ang-(1-7) and expression of the Mas receptor. Indeed, several studies have documented changes in the Mas receptor in various pathological conditions (15, 18, 34). There are conflicting studies regarding the effect of Ang-(1-7) on Mas receptor expression; however, our study would support the findings of Tan et al that demonstrate the feed forward or positive regulation of the Mas receptor by Ang-(1-7) (28, 40). Therefore, a reduction in tissue levels of Ang-(1-7) may attenuate expression of Mas and lead to an imbalance favoring greater Ang II-AT1 receptor tone. In this regard, the nitric oxide response to Ang-(1-7) was reduced in the renal cortex of BMX sheep at 1.8-years of age which was associated with a lower proportion of D-Ala-sensitive binding sites (25). Moreover, our studies find that the Ang-(1-7) antagonist D-Ala increases blood pressure and inhibits the BRS in control but not BMX sheep at 0.5-years of age (9). These data suggest that attenuated expression of Ang-(1-7) and the Mas receptor may contribute to the cardiovascular phenotype of BMX sheep.

GCs are strong regulators of fetal growth and development which may influence a myriad of target proteins including growth factors, cytoarchitectural proteins, receptors, binding proteins, as well as various components of cell signaling pathways (39). Overexposure to GCs during fetal development influences numerous organ systems and predisposes the individual to disease states later in life (20, 41). However, we find that as early as 6-weeks of age, BM exposure is associated with an altered BRS despite the fact that blood pressure is not changed. Therefore, it is possible that the cardiovascular centers in the brain may be an early and key target for programming events following BM exposure in utero. In this regard, maternal protein deprivation in late gestation also resulted in increased mRNA expression of Aogen and ACE, but reduced levels of AT2 receptor mRNA levels in fetal rat brains (23). These data lend additional support to the concept that the brain undergoes almost immediate alterations in response to a stressful in utero environment. Further characterization of the timing of changes that take place in 6-week old brains is required. Importantly, this time period will allow for the assessment of the RAS components when BRS is altered but MAP is not changed between control and BMX animals.

In addition to the influence on the RAS, in utero overexposure to GCs reduced placental expression of 11β-hydroxysteroid dehydrogenase (11β-HSD2), an enzyme which oxidizes active GCs to their inactive derivates (37). The enzyme plays a protective role in pregnancy, where 11β-HSD2 is highly concentrated in the placenta and shields the fetus from overexposure to the circulating maternal GCs (4). During development the expression of 11β-HSD2 is evident throughout the brain, suggesting that the enzyme may protect sensitive tissues from overexpression to GCs before the placental barrier has been

fully established (7). While 11β-HSD2 expression decreases greatly after birth, recent studies have revealed 11β-HSD2 mRNA in the NTS of adult rat brains using histochemistry and RT-PCR (7). The effect of prenatal GC exposure on brain 11β-HSD2 activity has not been well characterized; however, studies in the kidney reveal lower 11β-HSD2 expression in rats exposed to maternal low protein diet (24). It is possible that fetal exposure to GCs alters 11β-HSD2 activity in the brain, thus allowing an excess of active steroids to reach key brain areas regulating blood pressure and the stress response.

Alterations in GC content and receptor expression may contribute to fetal programming through epigenetic mechanisms. Both hypertension and fetal programming exhibit altered methylation patterns and modified histones in the brain. Goyal et al (23) found that antenatal maternal protein deprivation leads to epigenetic changes and alterations in the RAS within fetal mouse brains. In this model, the mRNA levels of both Aogen and the AT2 receptors were higher in exposed offspring. Moreover, maternal protein deprivation was associated with decreased methylation of CpG islands in the promoter regions of the ACE-1 gene, and upregulation of miRNAs that regulate ACE-1 mRNA translation in the fetal brain. DNA methylation at CpG islands and histone acetylation are also known to limit nephron development (19). Reduced nephron number during development or shortly after birth is correlation to the development of essential hypertension later in life (8). The 11βHSD-2 gene is under epigenetic control and plays an important regulatory role in fetal exposure to maternal glucocorticoids (2). High 11βHSD-2 promoter methylation is associated with hypertension in patients treated with glucocorticoids (35, 43). It is possible that epigenetic changes such as histone

modification or DNA methylation play a role in the development of the functional changes evident in this model of fetal programming.

Conclusion

It is widely accepted that events that take place in utero have the ability to impact the long term cardiovascular health of an individual. The current study uses a GC induced model of fetal programming to investigate the role of the brain RAS in the development and maintenance of hypertension. These data provide evidence that BMX sheep undergo programming events that alter the receptor levels and peptide ratios in the brain dorsal medulla that may functionally change the balance between Ang II and Ang-(1-7). Targeted therapies that restore the balance of these two peptidergic systems within the brain RAS may be clinically important in the fetal programming of cardiovascular disease.

Conflict of interests

The authors declare that there are no competing financial interests in the work described.

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Glossary of terms

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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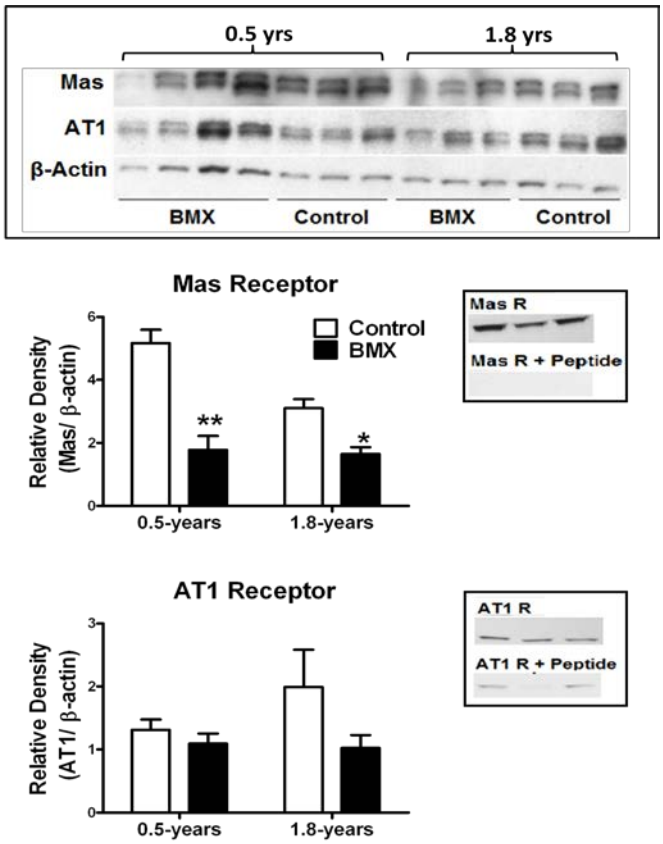

Figure 1. The AT7/Mas receptor protein expression was significantly reduced in the dorsal medulla of 0.5- and 1.8-Year old betamethasone exposed (BMX) animals. Upper panel: Western blots for Mas and AT1 receptors, as well as β-Actin expression in the dorsal medulla from 0.5 and 1.8 year olds. Quantification of receptor expression revealed reduced levels of the Mas protein at both ages and the protein band was abolished by preabsorption of the primary antibody with the antigenic peptide. There was no reduction in AT1 receptor expression and the protein band was diminished by preabsorption with the antigenic peptide. Mas and AT1 receptor densities were normalized to β-Actin. Mas protein was detected at 50 kDa, AT1 at 40 kDa, and β-Actin at 42 kDa. Data are mean ± SEM [0.5-Year: N = 4 BMX; N = 3 control and 1.8-Year: N = 3 BMX; N = 3 control]; *P < 0.05 vs. control

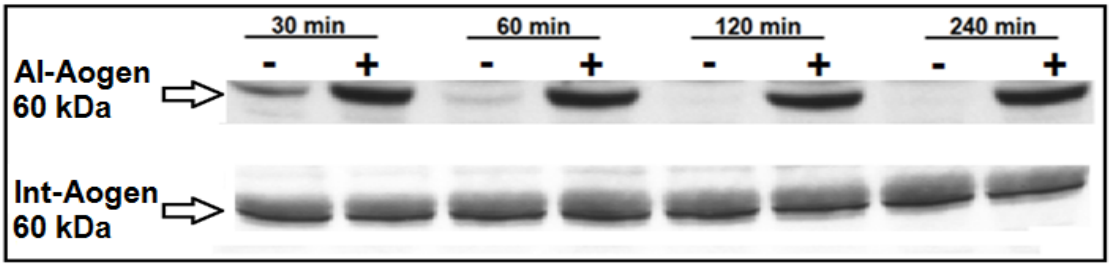

Figure 2. The renin inhibitor aliskiren preserves Ang I-intact angiotensinogen (AI-Aogen). Upper blot: immunoblot of samples without (-) or with (+) the renin inhibitor aliskiren probed with the AI-Aogen antibody. Lower blot: immunoblot of samples (-/+ aliskiren) probed with the Int-Aogen antibody. Samples contained nephrectomized sheep plasma incubated with the cytosolic fraction of sheep renal cortex from 30 to 240 minutes at 37°C. The 60 kDa band is shown.

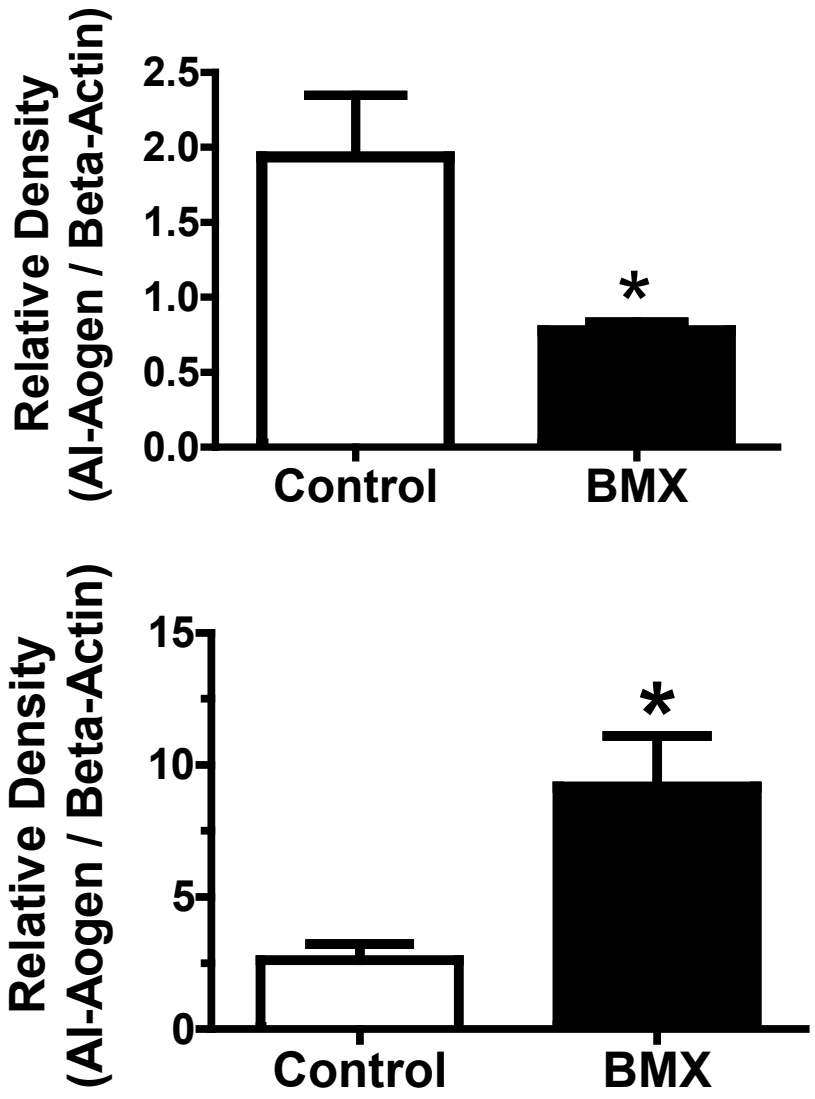

Figure 3. AI-Aogen expression was decreased in betamethasone exposed (BMX) animals at 0.5-years of age. Western blot analysis of AI-Aogen in dorsal medulla of 0.5- (upper panel) and 1.8- (lower panel) year old animals is shown. AI-Aogen protein was detected at 60 kDa. Band density was normalized to β-Actin. Data are mean ± SEM [0.5-Year N = 4 control; N = 4 BMX and 1.8-Year N = 3 control; N = 3 BMX]; * $P < 0.05$ vs. control.

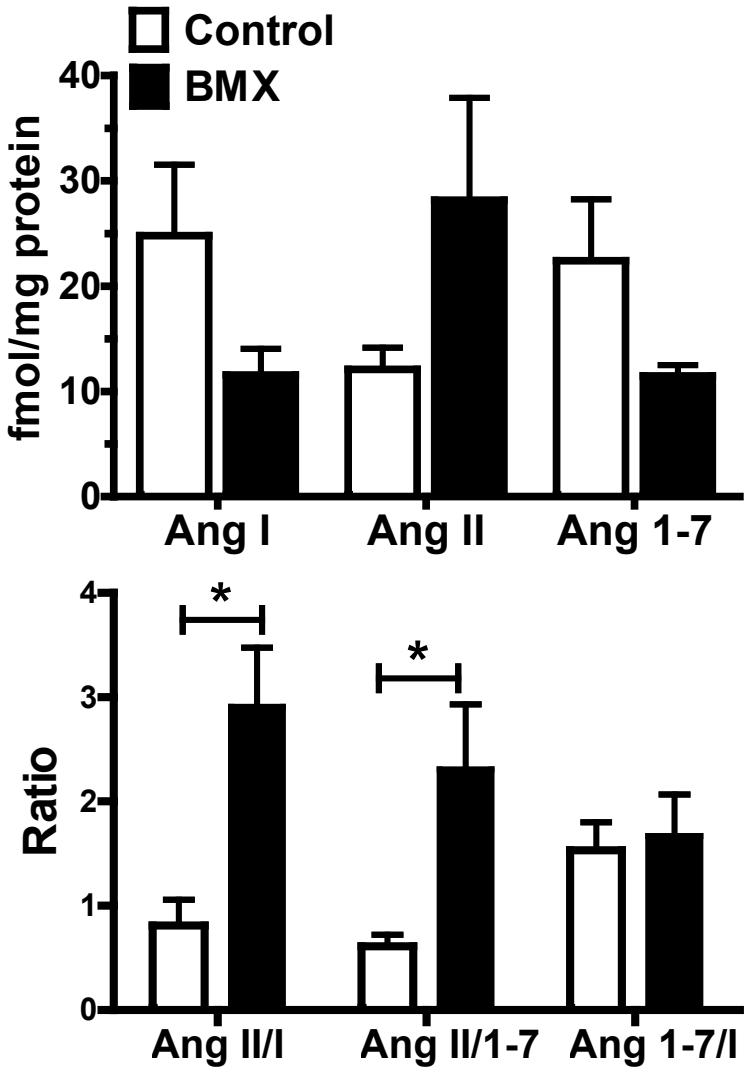

Figure 4. Angiotensin tissue content and the peptide ratios in control and betamethasone exposed (BMX) animals at 0.5-Years of age. The tissue content of Ang I, Ang II, and Ang-(1-7) in dorsal medulla of 0.5-year old animals was not significantly different between control and BMX groups (upper panel). Significantly higher peptide ratios of Ang II: Ang-(1-7) and Ang II: Ang I but not Ang-(1-7):Ang I were evident in BMX animals compared to controls (lower panel). Data are mean ± SEM [N = 5 control; N = 4 BMX]; *P < 0.05 vs. control.

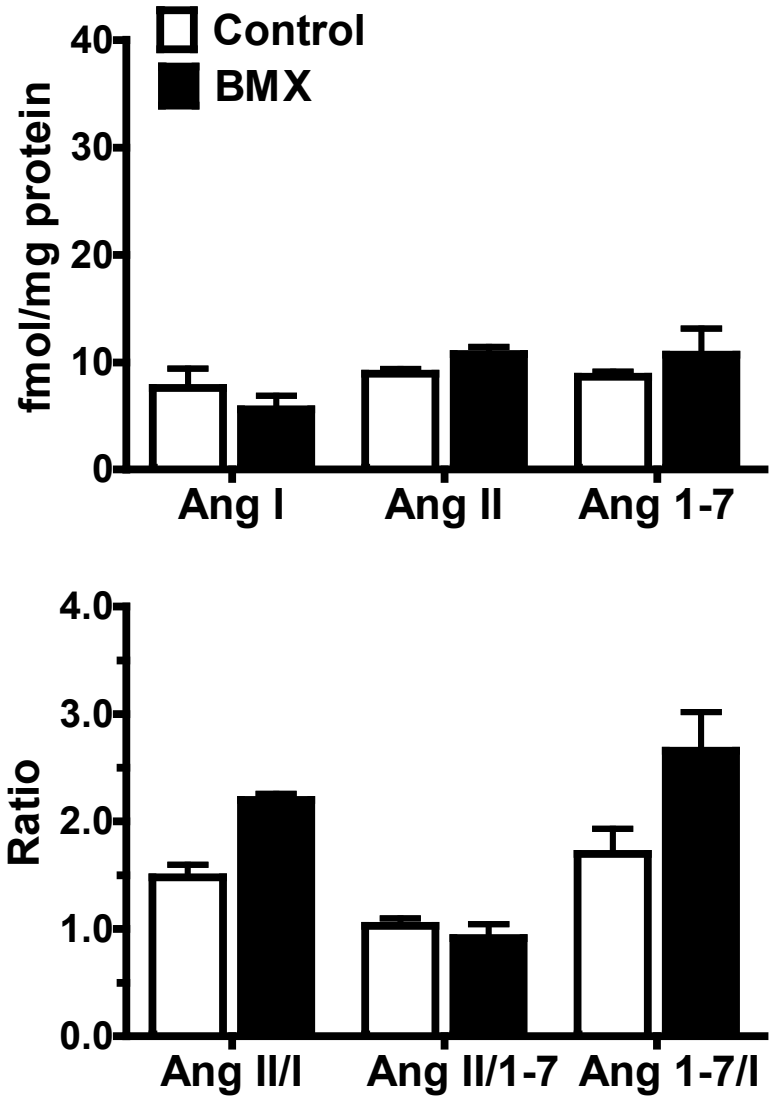

Figure 5. Angiotensin tissue content and the peptide ratios in control and betamethasone exposed (BMX) animals at 1.8-Years of age. Tissue content of angiotensin peptide (upper panel) and the peptide ratios (lower panel) were not significantly different between BMX and control groups at this age. Data are mean ± SEM [N = 3 control; N = 4 BMX].

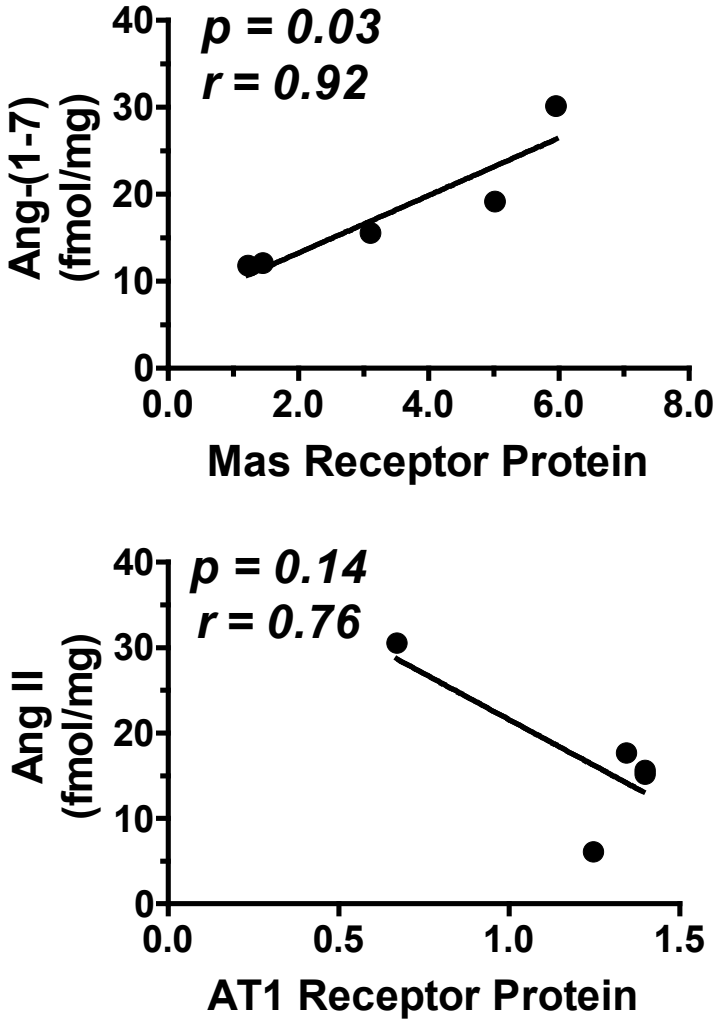

Figure 6. Correlation of Mas protein expression with Ang-(1-7) peptide levels in the brain dorsal medulla. Control and betamethasone exposed (BMX) animals are both used in this correlation. There was a positive correlation between Mas receptor and Ang-(1-7) peptide concentration (upper panel), and a trend for negative correlation between AT1 receptor and Ang II peptide concentration (lower panel). Correlation analysis was performed using GraphPad Prism 5.01 plotting and statistical software. Data are mean ± SEM [N = 5 in each group of control and BMX sheep].

CHAPTER THREE

ANTENATAL BETAMETHASONE EXPOSURE IS ASSOCIATED WITH LOWER ANG-(1-7) AND INCREASED ACE IN THE CSF OF ADULT SHEEP

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Abstract:

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. The 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-months of age. We compared components of the choroid plexus 4th ventricle (ChP4) and CSF RAS between control and BMX male offspring at 6-months of age. In the ChP, high molecular weight renin protein and Ang I-intact angiotensinogen were unchanged between BMX and control animals. ACE2 activity was 3-fold higher than either NEP or ACE in control and BMX animals. Moreover, all three enzymes were equally enriched ~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/ml; $P<0.05$) while ACE2 and NEP activities were below measurable limits. A p-chloromercuribenzoic acid (PCMB)-sensitive enzyme was identified as the major source of Ang-(1-7) metabolism in the CSF, with significantly higher activity in the 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.

Keywords: fetal programming, sheep, choroid plexus, CSF, angiotensin, enzymes

Introduction

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 weeks gestation (1). 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-weeks (65, 70), and an elevated mean arterial pressure (MAP) by 6-months of age (19, 67). The loss of Ang-(1-7) actions appear to be an early event in the betamethasone-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 brainstem of BMX sheep reveal an increase in Ang II and a decrease in Ang-(1-7) pathways mediating BRS at 6-weeks of age (69). We recently reported that expression of the Ang-(1-7) AT7/Mas receptor protein in the dorsal brainstem is 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 4th 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 et al. 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 ACE2 proteins that may be indicative of the formation of Ang-(1-7) (12).

CSF is secreted from the apical membrane of the choroid plexus and serves to protect and stabilize the brain. Components of the RAS previously described in the CSF include ACE, angiotensinogen, Ang I, 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 CSF-borne Ang II can target receptors within the brain to influence blood pressure (14). Therefore, we hypothesized 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.

Glossary of Terms

ACE = Angiotensin converting enzyme

ACE2 = Angiotensin converting enzyme 2

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}]

Aogen = Angiotensinogen

BBM = Brush border membrane

BM = Betamethasone

BMX = Betamethasone exposed

ChP = Choroid plexus

NEP = neprilysin

PCMB = para-chloromercuribenzoic acid

Materials and Methods

Animals.

Sheep were exposed to saline or betamethasone acetate: phosphate 1:1 mixture (IM, 2 doses of 0.17 mg/kg, 24 hours apart) at the 80th day of gestation via injections given to the pregnant ewe. After term delivery, animals were farm raised and weaned at 3-months of age. At 6-months of age, male offspring were brought to our Association for Assessment of Laboratory Animals Care (AAALAC) approved facility, where they were maintained on a normal diet, with free access to tap water and a 12-hour light/dark cycle (lights on 7 AM to 7 PM). Tissues and CSF for this study were collected from 6 month old sheep that were anesthetized with ketamine and isoflurane and euthanized by exsanguination. Choroid plexus from the 4th ventricle was removed and immediately covered in Clear Frozen Section Compound (VWR West Chester, PA) and stored at −80°C. Cerebrospinal fluid was extracted (~3 ml per animal), taking care to avoid contamination with blood, and tubes were stored at -80°C. The 6 month time point was chosen as we have previously shown that animals at this age exhibit lower nephron number, higher blood pressure, lower BRS and lower expression of the AT7/Mas receptor in the brain medulla of the betamethasone-exposed animals (41, 67, 82). All procedures were approved by the Wake Forest University School of Medicine ACUC for animal care.

Western blot analysis.

Isolated cytosolic fractions of ChP (35 μg) of 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 angiotensinogen (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 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 (25). Reactive proteins were detected with PerkinElmer ECL substrate (Waltham, MA) and exposed to Amersham Hyperfilm enhanced chemiluminescence (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.

Choroid plexus tissue preparation.

Approximately 40 mg of ChP4 tissue was added to 0.5 mL reaction buffer (25 mM HEPES, 125 mM NaCl, 10 μM $ZnCl_2$, pH= 7.4). Aliskiren and pepstatin (10 μM) were added to each sample. Samples were homogenized using a TissueLyser (Qiagen, Valencia, CA) for 90 seconds at 25 Hz and centrifuged at 28,000 g for 10 minutes. The supernatant was collected and frozen at -20°C for western blot analysis of soluble

components of the RAS. 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 minutes. Supernatant was collected and protein concentration measured using a Bradford protein assay (66).

Brush border membrane preparation.

Brush border membranes (BBM) 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 (100mM), KCl (100mM), and HEPES (10mM), pH 7.4 (MHK buffer) using a teflon tissue grinder and centrifuged at 300g for 15 minutes. Pellet was discarded, and a fraction of the supernatant saved for a nonenriched (NE) control. The remaining supernatant was precipitated with 10mM $CaCl_2$ for 20 minutes at 4°C. Samples were centrifuged at 2,500g for 10 minutes to precipitate nuclei. The supernatant was removed and centrifuged at 35,000g for 30 minutes. The resulting pellet, enriched for the BBM, was resuspended in a minimal volume of MHK buffer. Both the nonenriched fraction and the BBM enriched fraction were solubilized overnight with 0.5% Triton. The following day, samples were centrifuged at 28,000g for 5 minutes 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 (NE) 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 iodinated [^{125}I]-Ang I or [^{125}I]-Ang II, and 1 µM non-iodinated Ang I or Ang II (66). The reaction was stopped after 120

minutes by addition of ice-cold 1.0% phosphoric acid and centrifuged at 16,000g. The supernatant was immediately filtered for separation by reverse-phase high-performance liquid chromatography (HPLC). The [^{125}I]-products were monitored by a Bioscan flow-through γ detector as described (18). Products were identified by comparison of retention times to [^{125}I]-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, based on previous studies to distinguish ACE2 activity using Ang II as a substrate, comprised the inhibitor cocktail in the assay: amastatin (AM; 2 μM), bestatin (BS; 10 μM), chymostatin (CHYM; 10 μM), benzyl succinate (BSC; 10 μM), and para-chloromercuribenzoic acid (PCMB; 0.5 mM). We subsequently added lisinopril to block ACE activity, SCH39370 for neprilysin activity, or MLN4760 for ACE2 activity (all at 10 μM final concentration) (66).

CSF PCMB sensitive enzyme metabolism.

Metabolism of [^{125}I]-Ang-(1-7) in CSF was determined in the presence of no inhibitors, PCMB (0.5 mM), or PCMB and lisinopril (10 uM) following 30, 60, and 120 minute 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 MHK buffer and protein concentration was measured using a Bradford protein assay. Saturation curves were performed with [^{125}I]-Ang-(1-7) as a

substrate and increasing concentrations of either unlabeled Ang-(1-7) or Ang II (0-50 M) as previously described by Shaltout et al (66). Reaction velocities for Ang-(1-4) formation from Ang-(1-7) were expressed as nmol/min/mg protein. The assays included amastatin (AM; 2 μM), bestatin (BS; 10 μM), chymostatin (CHYM; 10 μM), benzyl succinate (BSC; 10 μM), and lisinopril (10 uM) to prevent the contribution of other peptidases in the CSF and preserve the Ang-(1-4) product. The inhibitory constant (K_i) of PCMB was determined by adding varying concentrations of PCMB (50 nM – 500 μM) to a pool of control CSF. Kinetic constants (Km, Vmax, K_i) 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) (3, 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), p-Chloromercuribenzoic acid (PCMB), and a renin inhibitor (3). 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. 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 the ChP4 and fmol/ml in the CSF. Total protein content was determined in the acid ethanol homogenate by Bradford protein assay with BSA as a standard.

Materials.

Angiotensin 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). SCH 39370, 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 mean ± SEM. 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 (GraphPad Software, 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 towards higher expression of AI-Aogen at 50 kDa in the exposed sheep ($p = 0.058$, Figure 1, panel A). There was no difference in either the 60 kDa band or the density of the combined bands (panels B and C, respectively). High molecular weight renin protein was detected as a single band at approximately 60 kDa, possibly indicating the presence of prorenin; however, there was no difference between the control and BMX groups (Figure 2).

ACE, ACE2, and NEP activities were determined by HPLC analysis of the hydrolysis of [^{125}I]-Ang I and [^{125}I]-Ang II (Figure 3A). There were no differences in enzyme activities between the control and BMX animals (Figure 3B). The combined activity data from BMX and control animals (N=10) revealed that ACE2 activity is significantly higher than either ACE or NEP (Figure 3C). Activity values expressed as fmol/min/mg protein averaged 9.0 ± 1.4 for ACE, 31.6 ± 3.4 for ACE2, and 13.0 ± 1.9 for NEP.

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 (NE) were compared to the solubilized BBM enriched fraction (Figure 4). All three enzyme activities were enriched 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 the upper and lower right panels of Figure 4, respectively. Note the larger peak of Ang-(1-7) from Ang II in the BBM fraction, indicating higher ACE2 activity (Figure 4, lower right panel). 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 (Figure 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 (Figure 5B).

CSF RAS

In addition to the ChP4 peptide content, we assessed angiotensin peptides in clear CSF samples from 6-month old male sheep (Figure 5C). In contrast to the choroid data, Ang-(1-7) exhibited the highest concentration of the three peptides (Figure 5D). Moreover, the CSF content of Ang-(1-7) was significantly lower in the BMX sheep as compared to the vehicle-treated group. AI-Aogen content of the control and BMX groups is shown in Western blots in Figure 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]-Ang-(1-7) metabolism in the CSF from both control and BMX animals over 120 minutes (Figure 7). The time course studies revealed a significantly higher rate of metabolism in the BMX animals at 120 minutes of reaction (Figure 7, right panel). The thiol peptidase inhibitor PCMB blocked the majority of Ang-(1-7) metabolism in CSF of either group. 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 (LIS) 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 as compared to controls (8.9 ± 0.5 vs. 6.6 ± 0.5 fmol/min/mg protein, $p = 0.02$). Since 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 panel A of Figure 8, the rate of metabolism of [^{125}I]-Ang-(1-7) to [^{125}I]-Ang-(1-4) appeared to plateau with increasing concentrations of unlabeled Ang-(1-7). Kinetic analysis of the activity curves revealed an apparent Km for Ang-(1-7) of 5.4 μM and a Vmax of 54.1nmol/min/mg in controls, and a Km of 4.1 μM and Vmax of 56.9 nmol/min/mg for BMX animals. In contrast, the rate of [^{125}I]-Ang-(1-7) metabolism did not appear to plateau with increasing concentrations of unlabeled Ang II in control CSF (Figure 8, panel B). These data suggest that over this concentration range; Ang II does not compete for the PCMB-sensitive peptidase to metabolize Ang-(1-7). Finally, based on the apparent Km for Ang-(1-7) of 5.4 μM in the pooled control CSF, we determined an inhibitory constant (K_i) for PCMB of 4.1 μM

Discussion

In the present study, we characterized components of the RAS in the ChP4 and CSF of control and BMX male sheep at 6-months of age. We report a trend towards

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 find that CSF ACE activity is 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-months 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 AT1 receptor antagonist candesartan into the NTS improved BRS in both control and BMX animals at 6-weeks of age (69). In contrast, Ang-(1-7) receptor blockade with the selective AT7 receptor/Mas antagonist D-Ala^7-Ang-(1-7) (D-Ala, A779) in the NTS inhibits 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 in 6-weeks, 6-months, or 1.8-year old control sheep only (67, 70, 71). Indeed, we recently show that protein expression of the AT7 receptor protein Mas is significantly

lower in the dorsal medullary region of the BMX sheep at 0.5- and 1.8-years of age (41). It now accepted that the functional actions of the Ang-(1-7)-ACE-Mas receptor axis of the RAS counteract or antagonize the actions of Ang II-ACE-AT1 receptor pathway (60, 61). In the current study, we expanded the characterization of the central RAS for a comprehensive analysis of the two axes in the ChP4 and CSF regarding their expression, compartmentalization, and regulation by BM exposure.

Tight junctions between ChP epithelial cells form the blood-CSF barrier (BCFSB). Unlike the capillaries that form the blood-brain barrier, the ChP endothelial cells are fenestrated, allowing for the free movement of molecules into the ChP epithelial cells (73). These epithelial cells are connected by tight junctions and tightly regulate the passage of molecules at the BCSFB (37, 56). ChP epithelial cells resemble renal proximal tubules, as both tissues act to transport fluids and ions across their epithelium and regulate the chemical composition of CSF or blood. The RAS is known to regulate epithelial transporters in both the kidney and choroid plexus and play a role in the development of certain types of hypertension (26, 31). However, our study shows no alterations in RAS peptide levels or enzyme activity; it is likely that antenatal BM has minimal programming effects on the ChP. Functional changes in MAP and BRS seen in this model of hypertension may be unrelated to the ChP RAS (24, 70, 76).

The ChP from the 4th 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 N-terminal sequence of Ang I (41). Two protein bands were evident in ChP tissue that likely represents multiple isoforms of Aogen. In nephrectomized sheep plasma, we find a 60 kDa band that is abolished upon exposure to renin indicating the specificity of the N-terminally directed antibody (25, 41). Since 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 towards greater Aogen expression in BMX animals which may reflect higher Aogen expression or altered processing of the protein mediated by active renin. However, 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 ten-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 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). While 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. While 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 (4, 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 (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 the ChP4 tissue were similar between BMX and control animals. Ang II was the predominant angiotensin peptide despite the fact that ACE2 activity was higher than ACE or NEP, likely reflecting the fact that ACE2 is located in the BBM facing the CSF rather than an intracellular location. Thus, the higher Ang II content in the ChP4 may reflect different processing compartments or pools within the ChP4. Indeed, it has been shown that the 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 the 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 compared to Ang I or Ang II, and we found lower levels of Ang-(1-7) in the BMX animals. Indeed, past studies by our group report a reduced Ang-(1-7) tone in the brain, circulation and kidney of BMX sheep (24,

41, 68-70). In this regard, ICV administration of Ang-(1-7) increased BRS in DOCA-salt and (mRen2)27 transgenic rats, as well as a heart failure model in rabbits (23, 35, 46). Conversely, the Ang-(1-7) antagonist D-Ala given ICV reduced BRS in normotensive Wistar and 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 a decrease in the peptide could contribute to alterations in pressure and BRS in the 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 towards 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 Ang I or Ang II in the CSF either alone or with PCMB and LIS to prevent Ang-(1-7) metabolism. It is likely that CSF Ang-(1-7) is formed by the 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 (2, 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 (2, 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 (2, 51). Alpha-secretase is present in the CSF of both healthy individuals and patients with

Alzheimer's disease (49), suggesting a similar sheddase that cleaves ACE may also be present in the CSF. While 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 approximately 23% of Ang-(1-7) in controls and 30% of Ang-(1-7) in BMX animals. ACE alone metabolized approximately 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 Vmax in BMX animals. ACE hydrolyzed the Ile^5-His^6 bond of Ang-(1-7) to form Ang-(1-5) while the thiol peptidase cleaved the Tyr^4-Ile^5 bond to generate Ang-(1-4). Kinetic analysis of the CSF peptidase revealed an apparent Km 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 the CSF or other tissue compartments in the sheep. Although there is a wealth of evidence on the enzymes that form Ang-(1-7) from Ang I or Ang II, there is little known on the enzymatic pathways that metabolize the peptide other than ACE (3). We previously reported that NEP can cleave the Tyr^4-Ile^5 bond of Ang-(1-7) in the BBM fraction from rat kidney (3); however, NEP is a metallopeptidase 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 the 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 (45, 63, 80). Moreover, we have not established whether the thiol-sensitive peptidase is released from the 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, as well as 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, as well as the potential changes in a betamethasone-induced model of fetal programming. As the brain and ChP are in direct contact with the CSF, it is likely that these tissues regulate CSF enzymes leading 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. While 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.

Conflict of Interest

The authors declare that there are no competing financial interests in the work described.

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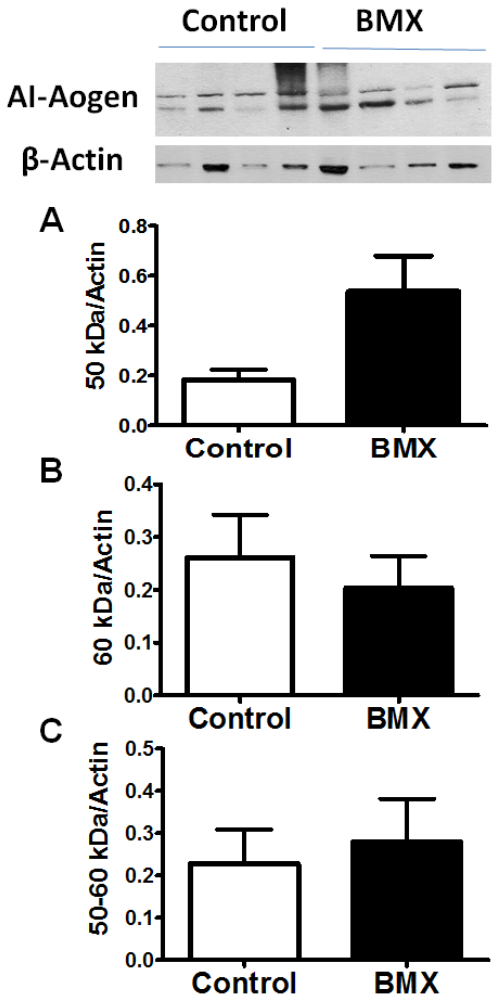

Figure 1: Protein expression of Ang I intact (AI)-angiotensinogen (Aogen) in cytosolic fractions from ChP4 tissue. Major bands were detected at 50 and 60 kDa of the Western blot. BMX animals have a trend towards higher AI-Aogen at 50 kDa ($p = 0.058$, panel A). There was no difference between control and BMX protein expression at 60 kDa (panel B). When both bands were quantified together, there was no difference between control and BMX animals (panel C). Proteins are normalized to β-Actin for quantification.

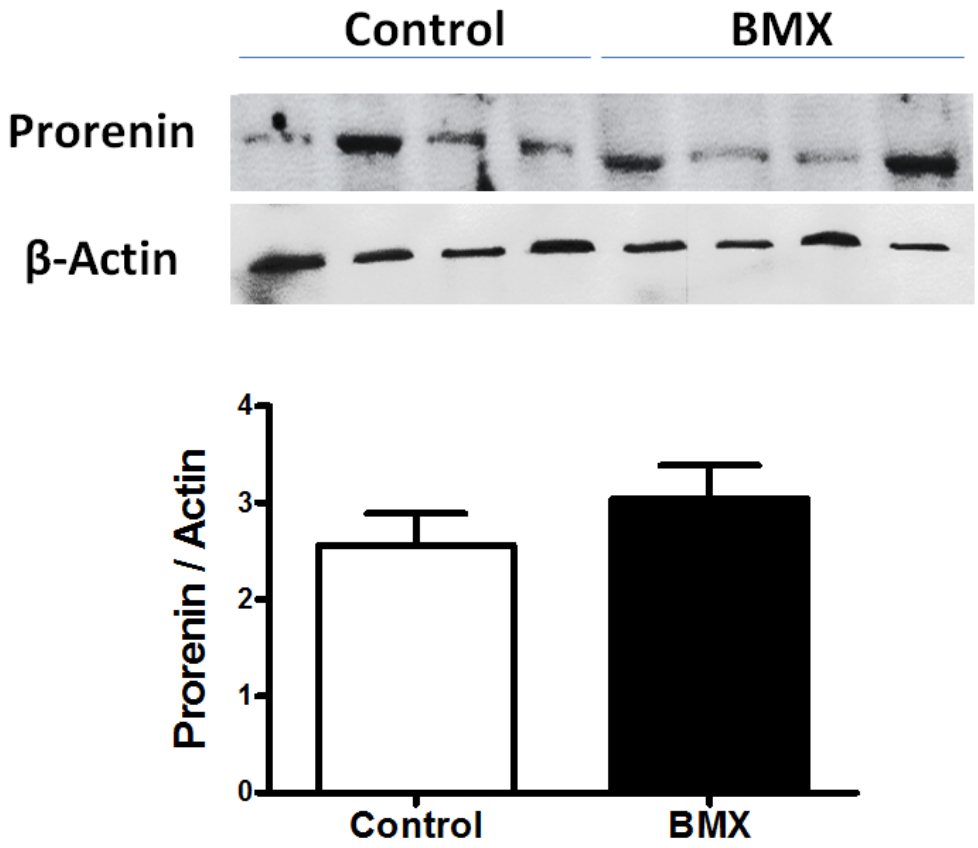

Figure 2: Renin protein expression in soluble ChP4 tissue. A prominent band was detected at 60 kDa in control and BMX animals of the Western blot. There was no difference in the expression of prorenin between groups. Protein expression was normalized to β-Actin.

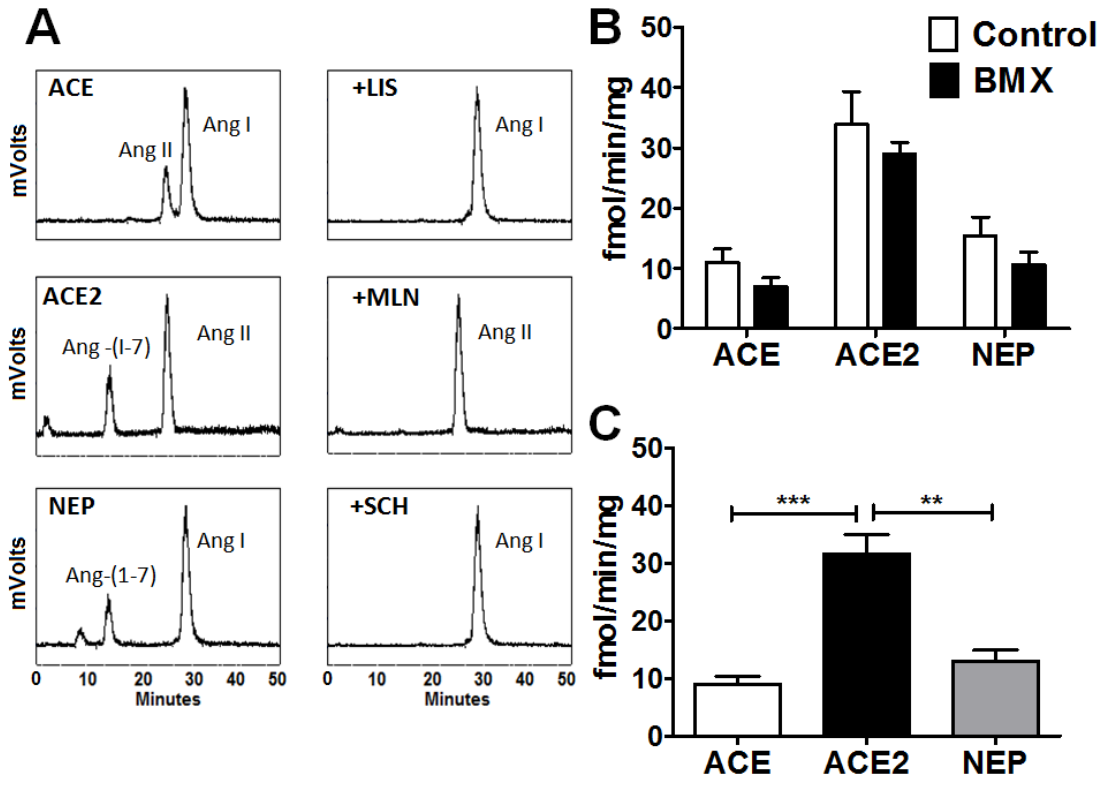

Figure 3: Peptidase activity in solubilized membrane fractions of ChP4 from control and BMX animals. Representative chromatographs for ACE, ACE2, and NEP activity are shown in panel A. Activity is abolished with specific inhibitors to ACE (lisinopril, +LIS); ACE2 (MLN4762, +MLN); and NEP (SCH37392, +SCH). There was no difference in peptidase activity between control and BMX animals (N=5, panel B). Enzyme activities of control and BMX animals were combined for comparison and ACE2 activity was significantly higher than either ACE or NEP (N= 10, panel C). **P < 0.01, ***P<0.001

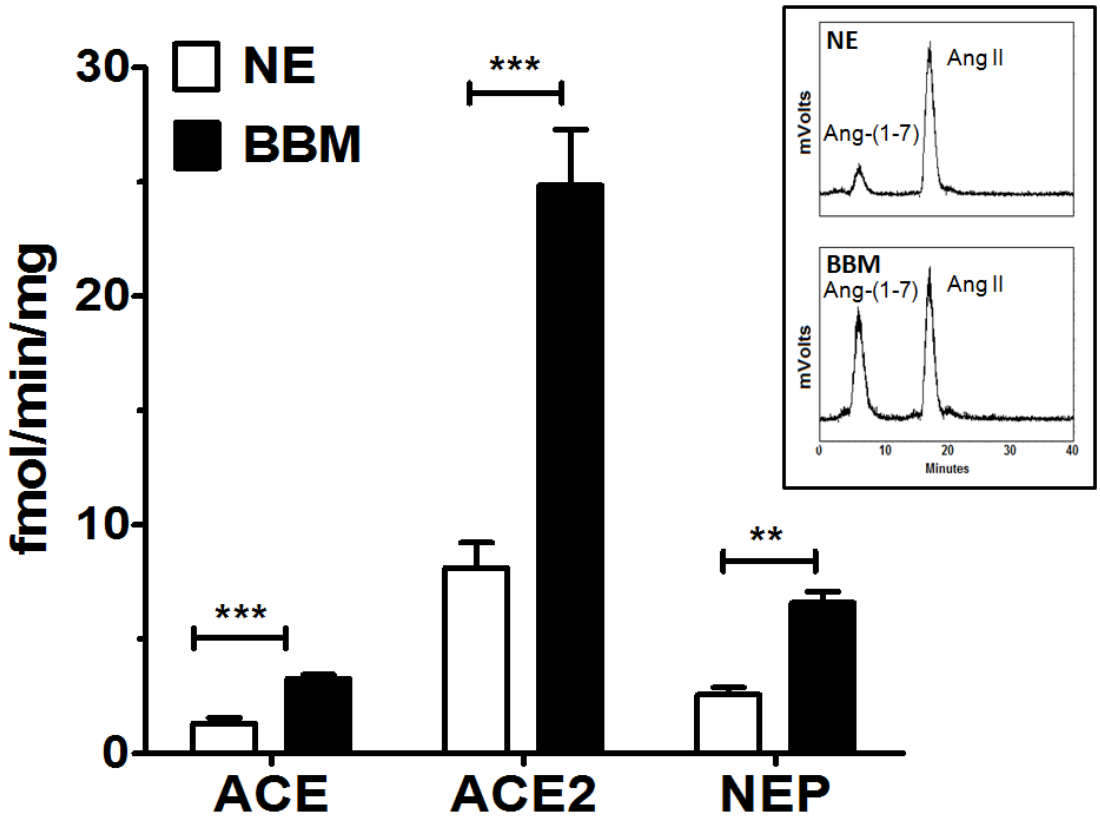

Figure 4: Peptidase activity in solubilized nonenriched (NE) and brush border membrane (BBM) enriched fractions. ACE, ACE2, and NEP activities are significantly higher in BBM fractions (left panel, N=5). Representative chromatographs are shown for ACE2 activity in the panel on the right from a nonenriched (top) and BBM fraction (bottom). $^{**}P < 0.01$, $^{***}P < 0.001$

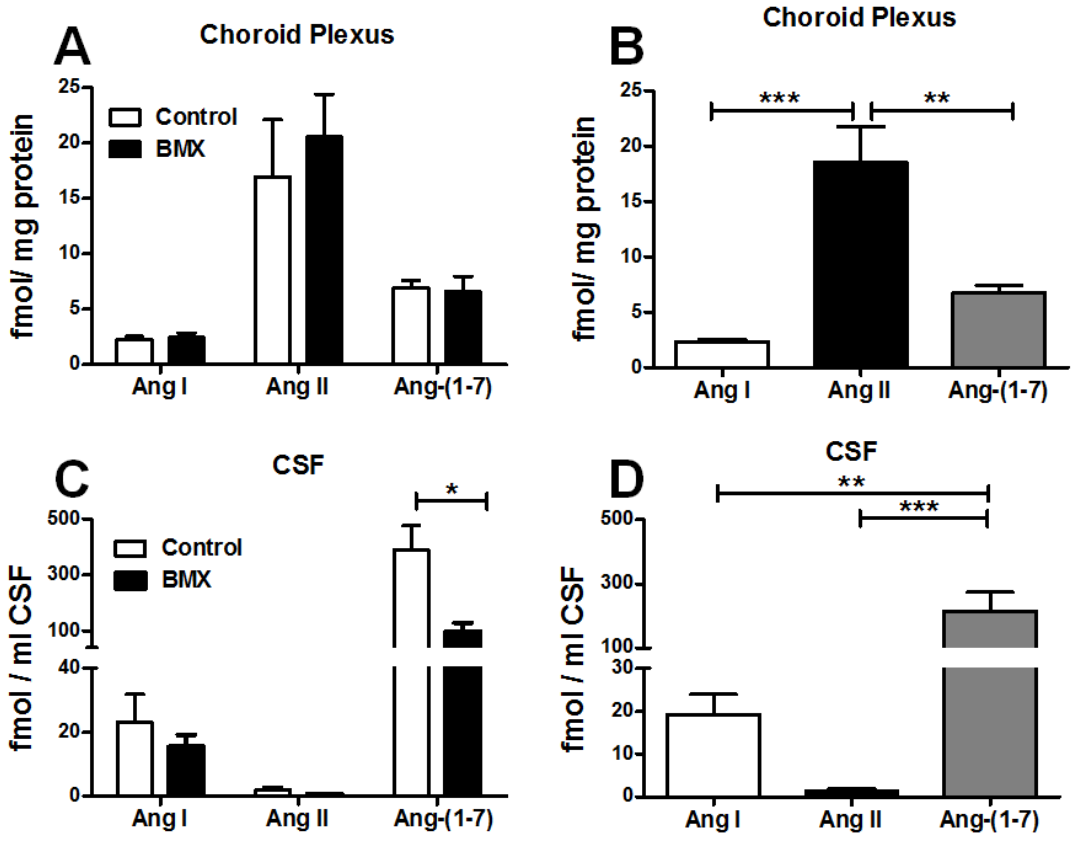

Figure 5: Radioimmunoassay detection of Ang I, Ang II, and Ang-(1-7) in choroid plexus and CSF samples. No differences in choroid plexus peptide levels exist between control and BMX animals for any of the three peptides (N = 5, panel A). Control and BMX peptide values were combined for analysis and Ang II levels are higher than Ang I or Ang-(1-7) in choroid plexus (N = 9, panel B). In the CSF, Ang-(1-7) is significantly lower in BMX animals (N = 5, panel C). Control and BMX peptide values were combined for analysis and Ang-(1-7) levels are significant higher than Ang I or Ang II in the CSF (N = 10, panel D). *P< 0.05, **P < 0.01, ***P< 0.001

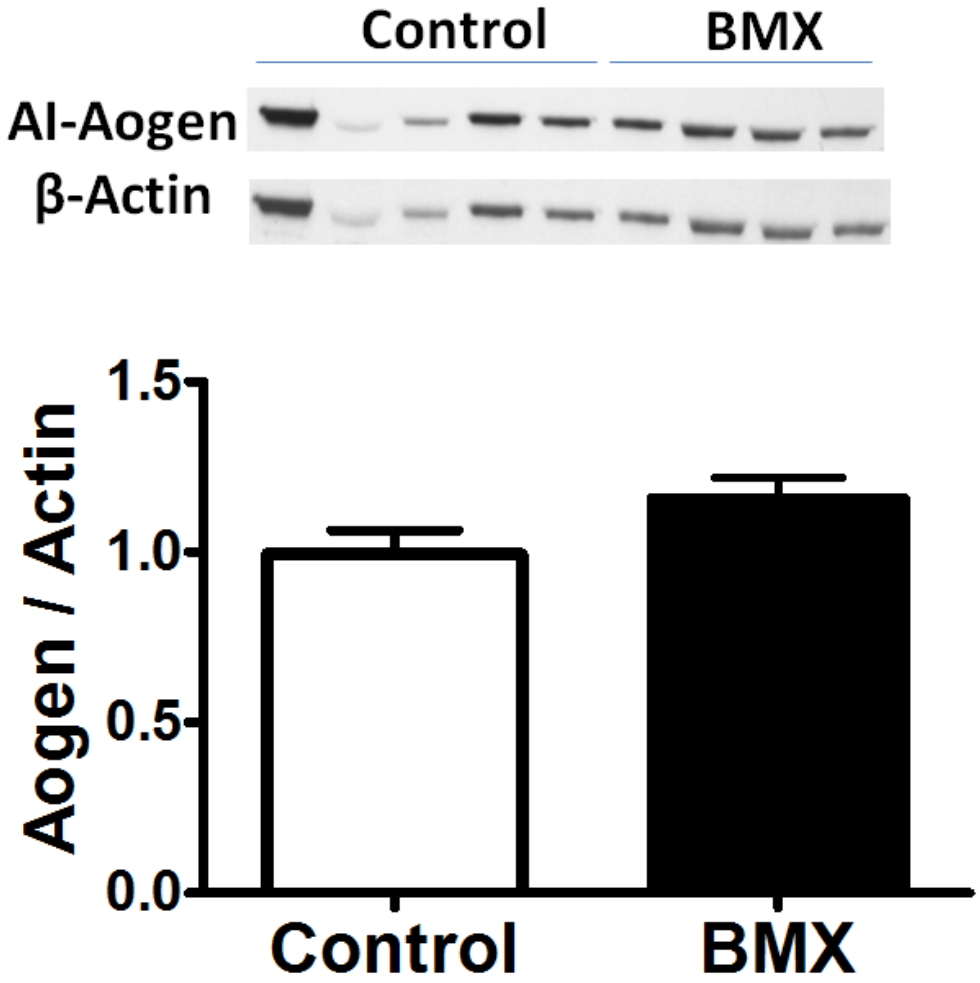

Figure 6: Ang I intact (AI) Aogen expression in CSF. A single 50 kDa band was detected in the CSF samples for both groups of the Western blot. There was no difference in protein expression between control and BMX animals (lower panel). Protein expression was normalized using β-Actin as a loading control.

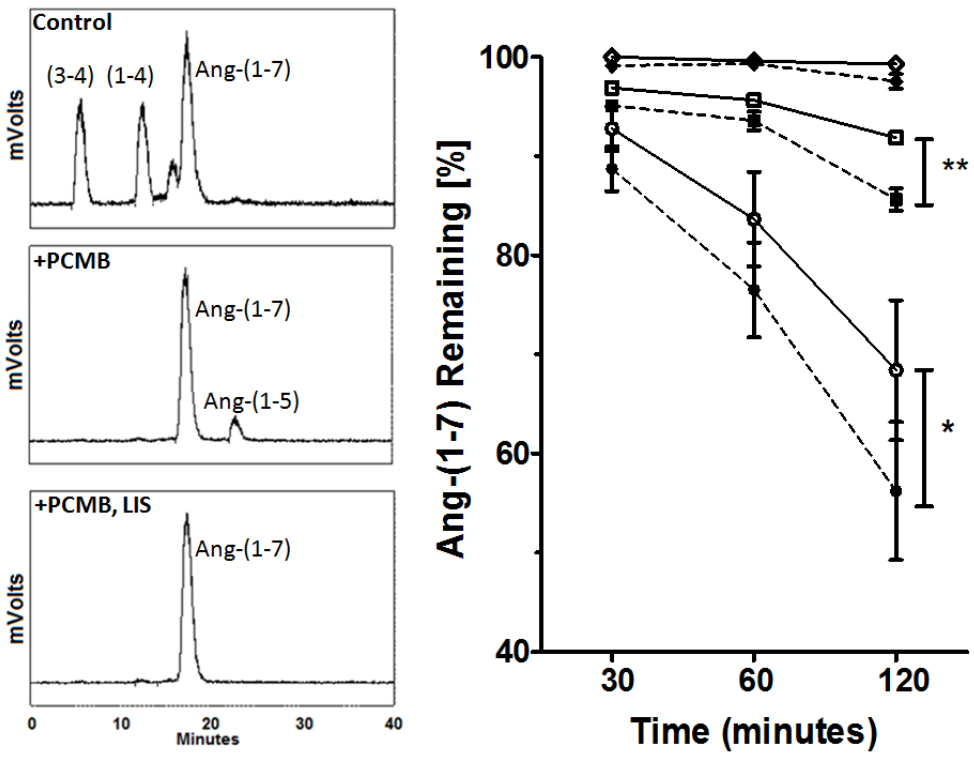

Figure 7: Analysis of Ang-(1-7) metabolism in the CSF. [^{125}I]-Ang-(1-7) was added to CSF from control or BMX animals in the presence of no inhibitors, 0.5 mM of the thiol agent PCMB, or 0.5 mM PCMB and 10 µM of the ACE inhibitor lisinopril (LIS). Representative chromatographs of Ang-(1-7) metabolism with no inhibitors, PCMB alone, or PCMB and LIS are show in the left panels. Reactions were terminated at 30, 60 or 120 minutes and the % of Ang-(1-7) remaining was determined. Legend: 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

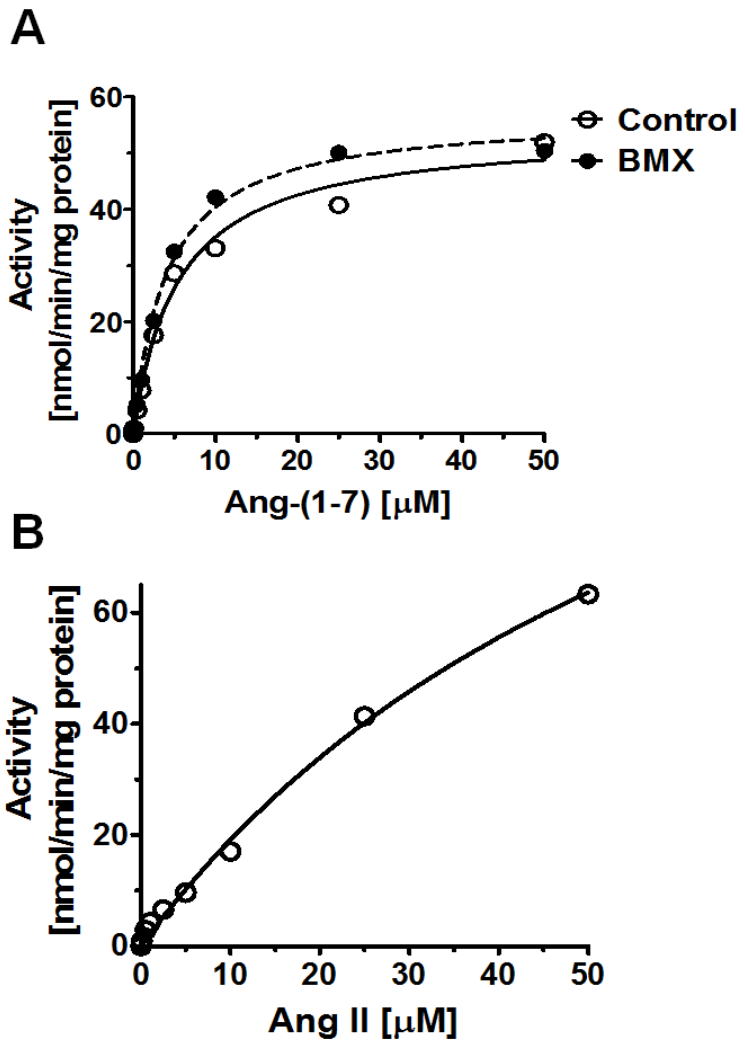

Figure 8: Saturation curves for Ang-(1-7) and Ang II metabolism by PCMB-sensitive enzyme in the CSF. Pooled CSF from control or BMX sheep was concentrated by a 50 kDa filtration tube. Legend: Control animals (○) with solid lines; BMX animals (●) with dashed lines. The Michaelis-Menten constant (Km) and maximal velocity (Vmax) for Ang-(1-7) metabolism were calculated by the GraphPad Prism 5 statistical program. Control animals: Km = 5.4 μM Ang-(1-7) and Vmax = 54.1 nmol/min/mg protein. BMX: Km = 4.1 μM Ang-(1-7) and Vmax = 56.9 nmol/min/mg protein.

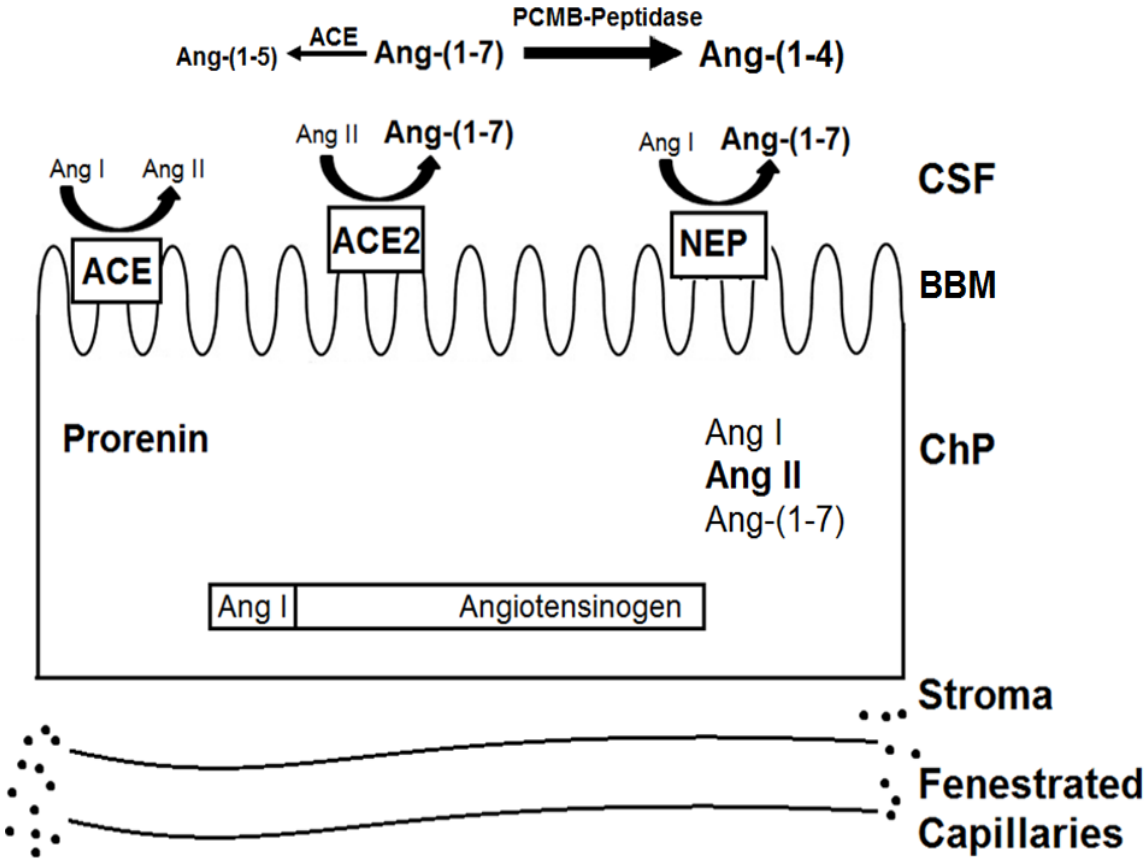

Figure 9: Diagram of potential ChP4 and CSF localization of RAS components. AI-Aogen, Ang peptides, and prorenin were detected in ChP tissue. ACE, ACE2, and NEP were detected in the BBM of the ChP. CSF peptide levels indicate potential BBM processing, as Ang-(1-7) levels were markedly higher than Ang II and Ang I. Metabolism of Ang-(1-7) to form Ang-(1-5) is mediated by ACE, while a PCMB-sensitive peptidase is the major activity that metabolizes Ang-(1-7) to Ang-(1-4).

CHAPTER 4

ENHANCED ACTIVITY OF AN ANGIOTENSIN-(1-7) METALLOPEPTIDASE IN GLUCOCORTICOID-INDUCED FETAL PROGRAMMING

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Abstract:

We previously identified angiotensin converting enzyme (ACE) and an endopeptidase activity that degraded angiotensin-(1-7) [Ang-(1-7)] to Ang-(1-5) and Ang-(1-4), respectively, in the cerebrospinal fluid (CSF) of 6-month old male sheep. The present study undertook a more comprehensive analysis of the CSF peptidase that converts Ang-(1-7) to Ang-(1-4) in control and in utero betamethasone exposed sheep (BMX). Characterization of the Ang-(1-7) peptidase revealed that the thiol agents 4-aminophenylmercuric acetate (APMA) and p-chloromercuribenzoic acid (PCMB), as well as the metallo-chelators o-phenanthroline and EDTA essentially abolished the enzyme activity. Additional inhibitors for serine, aspartyl, and cysteine proteases, as well as selective inhibitors against the endopeptidases neprilysin, neurolysin, prolyl and thimet oligopeptidases did not attenuate enzymatic activity. Competition studies against the peptidase revealed similar IC_{50}'s for Ang-(1-7)(5 μM) and Ang II (3 μM), but lower values for Ala^{1}-Ang-(1-7) and Ang-(2-7) of 1.8 and 2.0 μM, respectively. In contrast, bradykinin exhibited a 6-fold higher IC_{50} (32 μM) than Ang-(1-7) while neurotensin was a poor competitor. Mean arterial pressure (78 ± 1 vs. 94 ± 2 mmHg, N= 4-5, $P<0.01$) and Ang-(1-7) peptidase activity (14.2 ± 1 vs 32 ± 1.5 fmol/min/ml CSF, N = 5, $P<0.01$) were higher in the BMX group, and enzyme activity inversely correlated with Ang-(1-7) content in CSF. Lower Ang-(1-7) expression in brain is linked to baroreflex impairment in hypertension and aging, thus, increased activity of an Ang-(1-7) peptidase may contribute to lower CSF Ang-(1-7) levels, elevated blood pressure and impaired reflex function in this model of fetal programming.

Keywords: Renin Angiotensin System, Ang-(1-7), Cerebrospinal Fluid, Peptidase

Introduction

Evidence for the influence of early prenatal events in the fetus to induce a greater susceptibility to cardiovascular and metabolic pathologies is evident in both experimental models and in humans. Although the precise nature of fetal programming events is not known, altered expression of the biochemical components and functional actions of the renin-angiotensin system (RAS) may constitute an important underlying mechanism (9, 14, 15, 18, 19, 28, 30, 31). We utilize a sheep model of fetal programming in which pregnant ewes are administrated the glucocorticoid betamethasone (BM) at day 80 of gestation. This regimen parallels the dose and time that pregnant women are typically treated with glucocorticoids to enhance pulmonary function and reduce mortality of the fetus delivered preterm (1, 4, 12, 13, 24). Fetal exposure to glucocorticoids in sheep results in a significant reduction in the nephron number within the kidney, an increase in mean arterial pressure (MAP), attenuation of the baroreflex sensitivity (BRS) in the control of heart rate, and increased indices of metabolic dysfunction in adult animals (14, 26-28, 30, 31). Indeed, antenatal exposure to BM elicits decreased BRS by 6-weeks (27, 30), and elevated MAP by 6-months of age (14, 28, 31, 34).

Fetal programming events that have long-term consequences on the RAS may not solely reflect an influence on the angiotensin converting enzyme (ACE)-Ang II-AT1 receptor axis. Indeed, our studies suggest a shift away from the Ang-(1-7) axis and towards the Ang II pathway in the kidney, circulation, and brain tissue of BM exposed (BMX) sheep (15, 18, 28). Microinjections of the AT_1 and Ang-(1-7) receptor antagonists into the solitary tract nucleus (NTS) of the dorsal brainstem of BMX sheep reveal an increase in Ang II and a decrease in Ang-(1-7) pathways mediating BRS at 6-

weeks of age (29). We recently reported that Ang-(1-7) peptide levels were significantly lower in the CSF of male BMX sheep (19). We attributed the lower expression of Ang-(1-7) to increased metabolism of the peptide by both ACE and a soluble peptidase sensitive to the thiol inhibitor p-chloromercuribenzoic acid (PCMB). Since the CSF is in constant contact with the brain's extracellular fluid, this peptidase activity may be relevant to the regulation of Ang-(1-7) metabolism in the brain (5, 23). Therefore, the current study performed a comprehensive characterization of the Ang-(1-7) peptidase in regards to the sensitivity of various protease/endopeptidase inhibitors and selectivity among angiotensins and other peptides. Furthermore, we examined the expression of this peptidase in the CSF of control and BMX groups that directly exhibit differences in blood pressure and Ang-(1-7) content associated with fetal programming events.

Materials and Methods

Animals

Sheep received saline or betamethasone acetate: phosphate 1:1 mixture (IM, 2 doses of 0.17 mg/kg, 24 hours apart) at the 80th day of gestation. Mixed breed sheep were delivered at term, farm raised, and weaned at 3-months of age. At 6-months of age, male and female offspring were brought to our Association for Assessment and Accreditation of Laboratory Animals Care (AAALAC) approved facility, where they were maintained on a normal diet, with free access to tap water and a 12-hour light/dark cycle (lights on 7 AM to 7 PM). Sheep were anesthetized with ketamine and isoflurane and euthanized by exsanguination. Cerebrospinal fluid was extracted (~3 ml per animal), taking care to

avoid contamination with blood, and tubes were stored at -80°C. These procedures were approved by the Wake Forest University School of Medicine ACUC for animal care.

Blood Pressure Measurements

Sheep were anesthetized with ketamine and isoflurane followed by catheterization into the femoral artery and vein for blood pressure measurements. After at least 5 days recovery, MAP and was recorded in conscious animals and digitized with Acknowledge software (BIOPAC 3.8.1) (31).

Sample concentration

Cerebrospinal fluid (5 ml) was pooled from individual control and BMX animals and concentrated 1:5 using molecular weight filtration tubes to remove small proteins and endogenous angiotensin peptides (30 kDa, Millipore Bedford, MA). Concentrated CSF was resuspended in a final volume of 5 ml HEPES buffer (25 mM HEPES, 125 mM NaCl, 10 μM $ZnCl_2$, pH = 7.4) and protein concentration was measured using a Bradford protein assay.

pH Profile

Metabolism reactions were conducted with [^{125}I]-Ang-(1-7) [0.5 nM], 100 nM Ang-(1-7), and a cocktail of inhibitors (metabolism cocktail) containing the aminopeptidase inhibitors amastatin (AM, 2 μM) and bestatin (BS, 10 μM), the chymase inhibitor chymostatin (CHYM, 10 μM), the carboxypeptidase A inhibitor benzylsuccinate (BSC, 10 μM), and the ACE inhibitor lisinopril (LIS, 10 μM). CSF (25 μl) was added to buffers

ranging from pH 3-6: using 25 mM MES, 125 mM NaCl, and pH 6.5-9 using 25 mM HEPES, 125 mM NaCl).

Inhibitor profile

Various inhibitors were tested on Ang-(1-7) metabolism including PCMB, APMA, leupeptin, E-64, aprotinin, soybean trypsin inhibitor (SBTI), pepstatin A, EDTA, EGTA, o-phenanthroline, SCH 39370, 1-carboxy-3-phenyl-propyl (Ala-Ala-Phe-4-Abz-OH, CFP), Z-prolyl prolinal (ZPP), dithiothreitol (DTT), and the dipeptide Pro-Ile. Each reaction was conducted in the presence of [^{125}I]-Ang-(1-7) [0.5 nM], 100 nM Ang-(1-7), 25 µl CSF, and the inhibitor cocktail (AM, BS, CHYM, BSC, and LIS). All inhibitors were obtained from Sigma (St. Louis, MO) except SCH 39370 (gift from Schering Plow) and CFP (Bachem, King of Prussia, PA).

Peptidase kinetics

Kinetic or competition assays were performed with [^{125}I]-Ang-(1-7) as the substrate and increasing concentrations of either unlabeled Ang II, Ang-(1-7), Ala^{1}-Ang-(1-7) Ang-(2-7), D-Ala^{7}-Ang-(1-7), and D-Pro^{7}-Ang-(1-7) as previously described (19, 32). Reaction velocities for generation of [^{125}I]-Ang-(1-4) from [^{125}I]-Ang-(1-7) were expressed as pmol/min/mg protein. The assays for all peptides were performed in the inhibitor cocktail (AM, BS, CHYM, BSC, and LIS) to prevent the contribution of other peptidases in the CSF and preserve the Ang-(1-4) product. Kinetic constants (Km, Vmax, IC_{50}) were determined using Michaelis-Menten kinetics or non-linear regression one-site competition for IC_{50} with no constraints in the Prism 5 statistical program. All peptides

were obtained from Bachem (Torrance CA) or custom synthesized (Genscript, Piscataway, NJ).

HPLC separation

Metabolism reactions were conducted at 37°C in reaction buffer using concentrated CSF (25 µl; 35 µg) in a final volume of 250 µl. Each reaction included 0.5 nM iodinated [^{125}I]-Ang-(1-7) and 0.1 µM non-iodinated Ang-(1-7) (28). The reaction was stopped after 120 minutes by addition of ice-cold 1.0% phosphoric acid and centrifuged at 16,000xg. The supernatant was immediately filtered for separation by reverse-phase high-performance liquid chromatography (HPLC) on a Shimadzu equipped with an Aeris Peptide XB-C18 3.6 µm (2.1x100 mm, Phenomenex, Torrance CA). The [^{125}I]-products were monitored by a Bioscan flow-through γ detector as described (19). Products were identified by comparison of retention times to [^{125}I]-standard peptides and sensitivity to peptidase inhibitors. Peptides were iodinated by the chloramine T method and purified by HPLC to a specific activity >2,000 Ci/mmol (7).

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 $*P < 0.05$.

Results

We previously reported that ACE and a PCMB-sensitive soluble peptidase contributed to the metabolism of Ang-(1-7) in sheep CSF (19). ACE converted Ang-(1-7) to Ang-(1-5); however, the endopeptidase hydrolyzed Ang-(1-7) at the Tyr^4-Ile^5 bond to form the tetrapeptide Ang-(1-4). The current studies undertook a more extensive characterization of this peptidase in the CSF from both control and BMX sheep in which MAP and CSF levels of Ang-(1-7) were significantly altered (19). As shown in Figure 1A, the chromatograph reveals that the CSF activity hydrolyzed [^{125}I]-Ang-(1-7) to [^{125}I]-Ang-(1-4). The peak of Ang-(1-4) was abolished by the thiol inhibitor PCMB and the chelating agent o-phenanthroline (PHEN, Figure 1B-C). However, selective inhibitors against neprilysin (SCH39370, SCH), thimet oligopeptidase (c-Ala-Ala-Phe-pAB, CFP) and neurolysin (Pro-Ile) failed to attenuate the metabolism of Ang-(1-7) to Ang-(1-4) (Figure 1D-F). Figure 2 presents the results from an array of inhibitors on the hydrolysis of [^{125}I]-Ang-(1-7) to [^{125}I]-Ang-(1-4) in the CSF. Although both the mercuri-containing agents PCMB and APMA potently inhibited Ang-(1-7) to Ang-(1-4) conversion, the prototypic cysteine protease inhibitor E-64 and the lysosomal inhibitor leupeptin did not block activity. Moreover, the reducing agent DTT, which typically activates thiol proteases by the protection of critical cysteine or methionine residues, significantly inhibited activity by 73 ± 2%. Given the mixed effects among the thiol inhibitors, we tested several chelating agents to block Ang-(1-7) metabolism. EGTA, EDTA and o-phenanthroline inhibited 46 ± 3%, 79 ± 3% and 96 ± 0.3% of Ang-(1-4) formation, respectively (Figure 2). Inhibitors against other classes of enzymes including serine (aprotinin, SBTI) and aspartyl (pepstatin) did not alter activity (Table 1). The data in

Table 1 also revealed that selective inhibitors against neprilysin, thimet oligopeptidase, prolyl oligopeptidase and neurolysin did not attenuate the hydrolysis of Ang-(1-7). An optimal pH of 7.5 for [^{125}I]-Ang-(1-7) to [^{125}I]-Ang-(1-4) conversion was also demonstrated in both the control and BMX sheep; however the BMX pool exhibited higher activity than the control at pH 5 to 8.5 (Figure 3).

We also pooled and concentrated CSF samples from control and BMX sheep to compare the extent of inhibition (IC_{50}) of angiotensins and other peptides for the conversion of Ang-(1-7) to Ang-(1-4). As shown in Figure 4A, Ang-(1-7) and Ang II exhibited similar IC_{50} values of 5.3 and 3.0 μM, respectively, although Ang II competed for approximately 75% of enzyme activity over this concentration range. The Ang-(1-7) N-terminal analogs Ang-(2-7) and Ala^1-Ang-(1-7) exhibited IC_{50}'s of 1.8 and 2.0 μM, and completely inhibited activity (Figure 4B). The substituted C-terminal analogs [D-Pro^7]-Ang-(1-7) and [D-Ala^7]-Ang-(1-7) exhibited similar IC_{50} values of 2.0 and 1.1 μM and abolished activity as well (Figure 4C). Bradykinin exhibited an IC_{50} of 32 μM while neurotensin inhibited 15% of activity and an IC_{50} could not be derived over the concentration range of the peptide (Figure 4D).

We previously reported that a thiol-sensitive peptidase accounted for the majority of Ang-(1-7) metabolism in the CSF. A kinetic analysis revealed a tendency for higher activity in the BMX animals versus the controls but did not achieve a statistical difference in these groups (19). Since the CSF Ang-(1-7) content was over 2-fold lower in the BMX animals, we examined the peptidase activity in the identical cohort of BMX and control animals in which the peptide levels and blood pressures were obtained. As shown in Figure 5A, MAP was significantly higher in BMX animals as compared to

controls (78 ± 1 vs. 94 ± 2 mmHg, N = 4 control and 5 BMX, Figure 5A). The peptidase activity for [^{125}I]-Ang-(1-7) to [^{125}I]-Ang-(1-4) conversion was also significantly higher in the CSF of BMX animals (Figure 5B). Saturation studies on peptidase activity for Ang-(1-7) were then performed separately on pooled CSF from the same control and BMX animals. Kinetic analysis revealed an apparent Km (Km′) for Ang-(1-7) of 8.5 μM and a Vmax′ of 84 pmol/min/mg protein in controls, while the BMX pool exhibited a Km′ of 12.3 μM and Vmax′ of 223 pmol/min/mg protein (Figure 5C). The Km values were similar between the two groups based on overlapping 95% confidence intervals (Control: 7.4 - 9.5 μM; BMX: 7.9 - 17.7 μM). Finally, Ang-(1-7), but not Ang II or Ang I, peptide levels in CSF were inversely correlated to the peptidase activity of the individual samples [p=0.01; r of 0.81] based on the peptide content previously reported in the same control and BMX sheep (Figure 5D,E,F) (19).

Discussion

The present study identified a soluble metallopeptidase in the CSF from control and BMX sheep. The peptidase hydrolyzed [^{125}I]-Ang-(1-7) at the Tyr^{4}-Ile^{5} bond to form the tetrapeptide [^{125}I]-Ang-(1-4). The enzyme was highly sensitive to chelating agents (o-phenanthroline and EDTA), mercurial-based inhibitors (PCMB and APMA), and moderately sensitive to EGTA and DTT. By contrast, the peptidase was insensitive to serine, aspartyl and other cysteine protease inhibitors. The optimal pH of the Ang-(1-7) peptidase was approximately pH 7.5 and the activity was significantly higher in the CSF of BMX animals as compared to controls. Kinetic analysis confirmed that the apparent Vmax (Vmax′) of the peptidase for Ang-(1-7) was 2.5 fold higher in the BMX group

consistent with higher peptidase activity in the CSF of exposed animals. Moreover, the metabolizing activity in the CSF inversely correlated to Ang-(1-7) content suggesting that alterations in peptidase activity may contribute to the lower levels of peptide in the CSF of the BMX animals (19).

Previous studies in the betamethasone model of fetal programming in sheep focused on the peptidases ACE and ACE2 that comprise the main enzymatic components of the Ang II-AT_1 receptor and the Ang-(1-7)-Mas receptor pathways of the RAS (32). Indeed, we find that the adult offspring of glucocorticoid-exposed dams exhibit alterations in both enzymes that would contribute to an imbalance in the RAS axis (28). In the circulation, soluble ACE activity was increased while ACE2 activity decreased in the BMX sheep; the ratio of their specific activities highly correlated to blood pressure in the control and BMX groups (28). Although renal ACE activity was unchanged, ACE2 activity was lower in the renal cortex, isolated tubules and urine of the BMX sheep which would contribute to a higher ratio of Ang II to Ang-(1-7) (28). We have not assessed whether the expression of these peptidases are altered in the brain; however, we found that soluble ACE activity was higher in the CSF of the BMX sheep and that the peptide content of Ang-(1-7) was markedly lower (19). ACE is well-accepted as the primary pathway to form Ang II, but the enzyme plays a key role in the metabolism of Ang-(1-7) cleaving the Ile^5-His^6 bond to form the pentapeptide Ang-(1-5) (2). Indeed, ACE blockade markedly increased the half-life of Ang-(1-7) in the circulation which explains, in part, the increase in endogenous levels of the peptide following ACE inhibition (8). In addition to CSF ACE, we detected another soluble activity that metabolized Ang-(1-7) at the Tyr^4-Ile^5 bond to form Ang-(1-4). The conversion of Ang-(1-7) to Ang-(1-4) in CSF

was abolished by the thiol inhibitor PCMB but not the ACE inhibitor, and we had concluded that both ACE and a thiol-sensitive peptidase may metabolize Ang-(1-7) in the sheep CSF (19). These findings are summarized by the schematic in Figure 6. The CSF is in direct contact with both the medullary tissue and the choroid plexus (ChP) with expression of RAS components in all three compartments. Previously, we reported the peptide ratio of Ang II to Ang-(1-7) was higher in the medulla of BMX offspring compared to controls. Moreover, expression of the Ang-(1-7) receptor Mas was lower in medullary tissue of exposed animals, but protein level of the AT1 receptor were not different (18). The data indicate a shift in the central RAS towards the Ang II-ACE-AT1 receptor axis. The ChP may be a source of CSF peptides as it filters the blood and produces CSF. ACE, ACE2, and NEP were localized to the apical membrane of the ChP oriented to the CSF. Notably, ACE2 activity was 2.5-fold higher than ACE or NEP which may explain high levels of Ang-(1-7) in the CSF; however, these enzyme activities were similar between control and BMX animals (Figure 6). In this regard, the higher content of the neuropeptidase in BMX group may contribute to lower CSF level of Ang-(1-7).

The present study undertook a more comprehensive evaluation of the CSF peptidase regarding sensitivity to both general and specific protease inhibitors. Although the peptidase was sensitive to both the mercuri-containing inhibitors PCMB and APMA, the cysteine inhibitor E-64 and lysosomal agent leupeptin did not attenuate activity while the thiol reducing agent DTT significantly reduced the metabolism of Ang-(1-7). Further analysis revealed the metallo-chelating agents EDTA and o-phenanthroline effectively blocked activity, and inhibitors to other classes of peptidases were ineffective. These

data indicate that the Ang-(1-7) degrading activity is likely a metalloendopeptidase with pronounced sensitivity to mercuri-containing inhibitors such as PCMB and APMA. Subsequently, a review of known endopeptidases present in the brain and sensitive to both metallo-chelating agents and thiol inhibitors suggested the potential contribution of either thimet oligopeptidase [EC3.4.24.15, TOP] or neurolysin [EC3.4.24.16] (10, 11, 19, 33). However, the selective TOP inhibitor CFP (10 and 100 μM) did not attenuate activity (25). We utilized a 10-fold higher concentration CFP as well as the dipeptide Pro-Ile to inhibit neurolysin, but neither agent affected Ang-(1-7) conversion in the CSF. Ferro and colleagues recently investigated whether different splice variants that dictate the cellular trafficking of neurolysin influence either substrate or inhibitor specificity of the endopeptidase; however, both cytosolic and mitochondrial forms cleaved Ang I to Ang-(1-7) and were sensitive to Pro-Ile inhibition (20). Previous studies reported Ki's of 90 μM (Pro-Ile) and 4 μM (CFP) for neurolysin, and 180 nM (CFP) for TOP (3, 11). Selective inhibitors against other endopeptidases including neprilysin (SCH39370, Ki of 11 nM) and prolyl oligopeptidase (ZPP, Ki of 4 nM) did not attenuate the metabolism of Ang-(1-7) to Ang-(1-4). Indeed, our previous study found no evidence for neprilysin activity (conversion of Ang I to Ang-(1-7)) in the sheep CSF (19). The identity of the Ang-(1-7) peptidase is currently unknown; studies are in progress to obtain the purified enzyme for sequence analysis and determine whether this is a novel protein or reflects species variation of a known peptidase.

The extent of competition for the hydrolysis of [^{125}I]-Ang-(1-7) to [^{125}I]-Ang-(1-4) among angiotensins and other peptides was assessed in the CSF pool. Both Ang-(1-7) and Ang II exhibited similar IC_{50} values of 5 and 3 μM, respectively; however, the N-

terminal analogs including Ala^1-Ang-(1-7) and the des-Asp^1 form [Ang-(2-7)] exhibited lower IC_{50} values of 2 μM. The D-Pro^7 and D-Ala^7 analogs of Ang-(1-7) also exhibited lower IC_{50} values than Ang-(1-7) or Ang II. In contrast, both bradykinin and neurotensin competed to a lesser degree than Ang-(1-7); the IC_{50} for bradykinin was 6-fold higher than Ang-(1-7) while the IC_{50} for neurotensin could not be obtained likely reflecting the minimal competition of the peptide. It should be noted that neurotensin, a 13-residue peptide, contains the same dipeptide sequence Tyr-Ile of Ang-(1-7) and Ang II, but at positions 11 and 12 of the peptide. Ala^1-Ang-(1-7) was recently described as an endogenous peptide that may arise from decarboxylation of the aspartic residue of Ang-(1-7) or the direct conversion of Ala^1-Ang II by ACE2 (17). Ala^1-Ang-(1-7) recognizes the Mas-related receptor D and the present data suggest that Ala^1-Ang-(1-7) may be a potential substrate for the CSF peptidase based on its lower IC_{50} than Ang-(1-7) (17). Ala^1-Ang-(1-7) and Ang-(2-7) both exhibit a low IC_{50}, suggesting that alterations to the N-terminus of the peptide do not significantly influence recognition by the peptidase. Although both the D-Ala^7 and D-Pro^7 forms of Ang-(1-7) are not endogenous peptides, these antagonists are frequently used *in vivo* and the kinetic results suggest they may interact or compete with Ang-(1-7) for the peptidase. We did not attempt to identify the extent and site of hydrolysis of Ala^1-Ang-(1-7), Ang II or the other peptides apart from Ang-(1-7; however, the relatively low Km for Ang-(1-7) and that peptidase activity inversely correlated to Ang-(1-7) levels in the CSF support an endogenous role of the peptidase in the Ang-(1-7)-Mas receptor arm of the RAS.

Conclusion

Glucocorticoid exposure in utero is associated with reduced Ang-(1-7) "tone" in the kidney and in the brain medulla which may contribute to long-term changes in blood pressure and baroreflex activity (15, 18, 19, 29). In this regard, the immunoreactive expression of the Mas receptor was significantly lower in the brain medulla of the BMX versus the control animals while the density of the AT_1 receptor was unchanged (18). We determined that the ratio of Ang II to Ang-(1-7) in the brain medulla and CSF was significantly higher in BMX animals; thus, alterations in the metabolism of Ang-(1-7) may lead to lower tissue content of the peptide and reduced activation of the Mas receptor pathway following BM exposure (18, 19). There is increasing evidence of a role for brain medullary Ang-(1-7) in blood pressure regulation and autonomic function in fetal programming (29), hypertension (16), and aging (21). Thus, attenuated Ang-(1-7) activity in pathological conditions may reveal a unique therapeutic role to target the peptidase and maintain levels of Ang-(1-7) in the brain (6, 22, 30).

Glossary

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]

Ang-(1-4) - [Asp^1-Arg^2-Val^3-Tyr^4]

ACE - Angiotensin converting enzyme

BM- Betamethasone

BMX- Betamethasone Exposed

CSF - Cerebrospinal fluid

RAS - renin-angiotensin system

Conflict of Interest

The authors declare that there are no competing financial interests in the work described.

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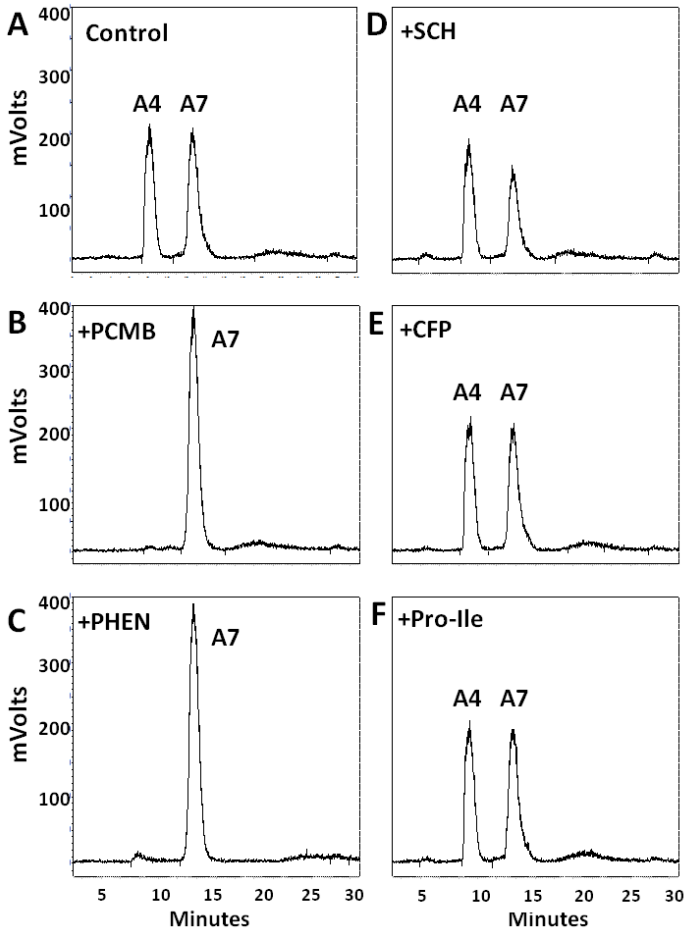

Figure 1: PCMB and o-phenanthroline abolish [^{125}I]-Ang-(1-7) metabolism. HPLC chromatographs reveal that both PCMB (10 μM, Panel B) and o-phenanthroline (PHEN, 1 mM, Panel C) abolished conversion of ^{125}I-Ang-(1-7) [A7] to ^{125}I-Ang-(1-4) [A4] as compared to control (Panel A). Addition of the neprilysin inhibitor SCH39370 (SCH, 10 μM, Panel D), thimet oligopeptidase/neurolysin inhibitor CFP (100 μM, Panel E), or the neurolysin dipeptide Pro-Ile (1 mM, Panel F) did attenuate the processing of A7 to A4. Activity was assayed from pooled control and BMX CSF (N=10) in the presence of 100 nM Ang-(1-7) and an inhibitor cocktail (AM, BS, CHYM, BSC, LIS) for 120 minutes at 37°C.

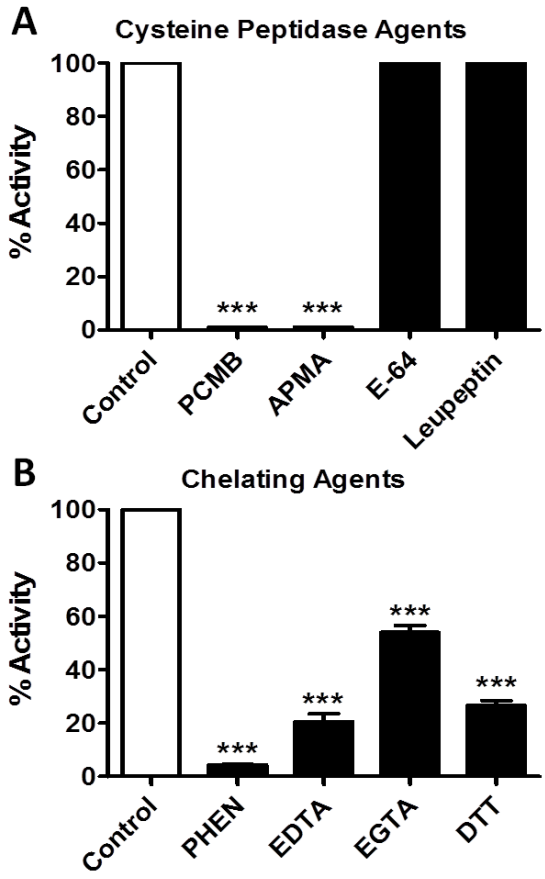

Figure 2: Cysteine peptidase inhibitors and chelating agents inhibit enzyme activity. The mercuri-containing peptidase agents PCMB (10 μM) and AMPA (10 μM) abolish activity while E-64 (10 μM) and leupeptin (100 μM) did not alter [^{125}I]-Ang-(1-4) formation (Panel A). The chelating agents PHEN (o-phenanthroline, 1 mM), EDTA (5 mM), EGTA (5 mM), and DTT (5 mM) inhibit ^{125}I-Ang-(1-4) formation from [^{125}I]-Ang-(1-7) (Panel B). Extent of inhibition was determined as percent (%) [^{125}I]-Ang-(1-4) formation compared to controls. Activity was assayed from pooled CSF of control and BMX animals (N=10) in the presence of 100 nM Ang-(1-7) and an inhibitor cocktail (AM, BS, CHYM, BSC, LIS) for 120 minutes at 37°C. N = 3, ***P<0.0001 vs. control

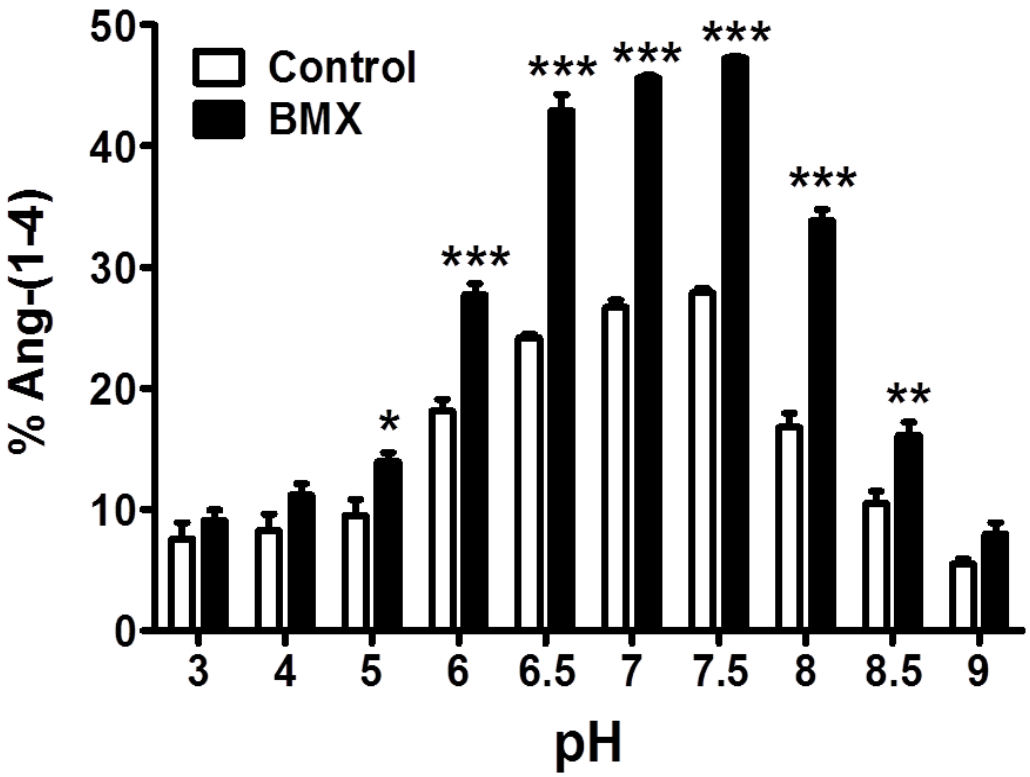

Figure 3: Optimal pH of CSF enzyme is 7.5 in BMX and control animals. Metabolism reactions were run separately for BMX and control animals in buffers pH 3-9 and the % Ang-(1-4) formed was quantified. Buffers: pH 3-6 25 mM MES, 125 mM NaCl, and pH 6.5-9 25 mM HEPES, 125 mM NaCl. BMX animals had significantly higher metabolism than controls at pH 5-8.5. (N = 3, *P<0.05, **P<0.01, ***P<0.001)

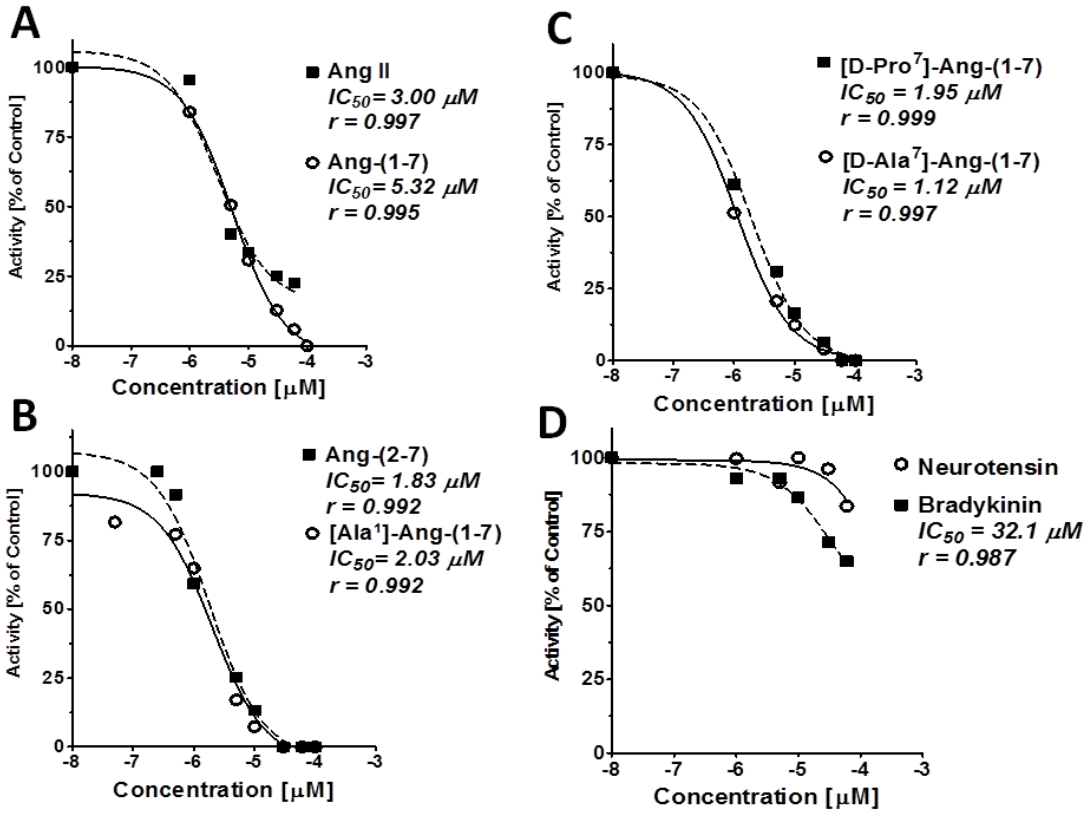

Figure 4: Analysis of the competition of angiotensin peptides for the conversion of [^{125}I]-Ang-(1-7) to [^{125}I]-Ang-(1-4) in pooled CSF. Activity was assayed in a CSF pool of control and BMX animals (N=10) and an inhibitor cocktail (AM, BS, CHYM, BSC, LIS) for 120 minutes at 37°C.

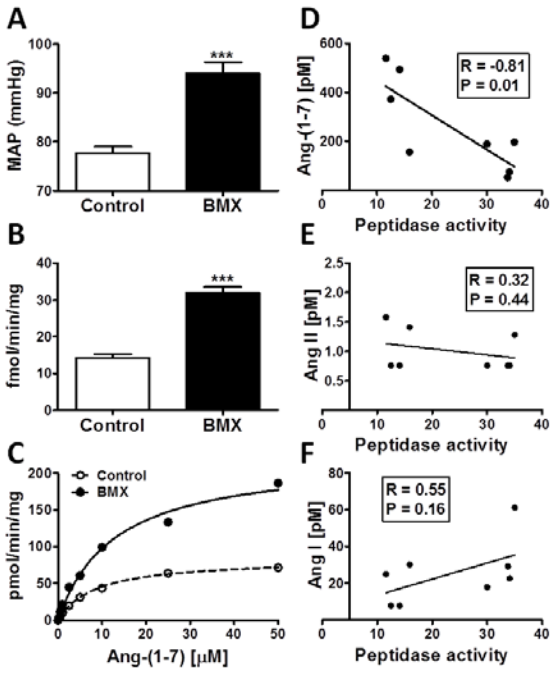

Figure 5: Betamethasone-exposed sheep exhibit higher mean arterial pressure (MAP) and Ang-(1-7) endopeptidase activity than controls. **A-** Mean arterial pressure (MAP) was significantly higher in BMX animals than controls (N=4 control, N = 5 BMX). **B-** Peptidase activity was significantly higher in BMX animals (N = 5 per group). Activity was assayed in the CSF of control and BMX animals in the presence of 100 nM Ang-(1-7) and an inhibitor cocktail (AM, BS, CHYM, BSC, LIS) for 120 minutes at 37°C (*P < 0.05 vs. control; N=5 per group). **C-** The apparent Km (Km′) and maximal velocity (Vmax′) for the metabolism of ^{125}I-Ang-(1-7) to ^{125}I-Ang-(1-4). Control: Km′ = 8.5 μM and Vmax′ = 84 pmol/min/mg; BMX: Km′ = 13 μM and Vmax′ = 223 pmol/min/mg for BMX animals. **D-** Correlation of Ang-(1-7) peptide levels and peptidase activity in CSF of control and BMX animals (r = -0.81). **E-** Ang II and **F-** Ang I peptide levels do not correlate with peptidase activity. *P < 0.05, ***P < 0.0001.

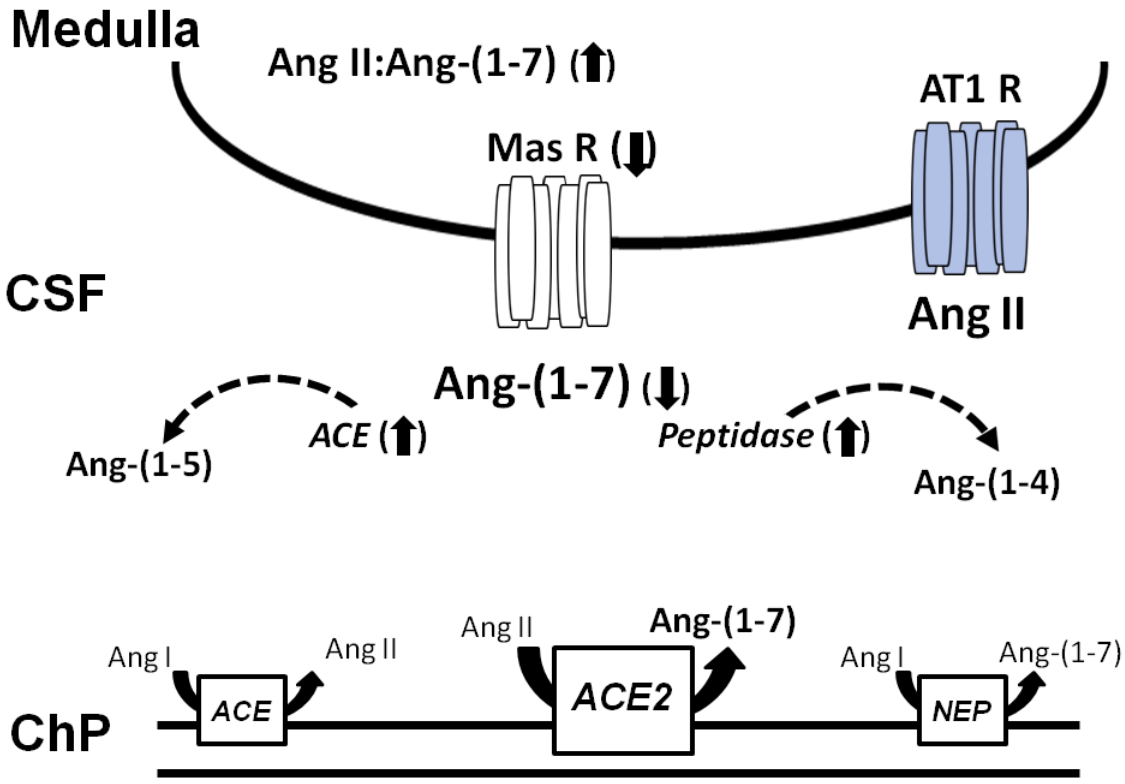

Figure 6: Proposed scheme for the role of the neuropeptidase in RAS processing pathways in the brain medulla, CSF, and choroid plexus (ChP). In the brain medulla, the tissue ratio of Ang II: Ang-(1-7) increased (↑) and Mas receptor expression is reduced (↓) in BMX offspring. CSF Ang-(1-7) peptide levels are lower and ACE and Ang-(1-7) peptidase activities are higher in exposed animals. ACE, ACE2, and neprilysin (NEP) are expressed on the apical membrane of the ChP. ACE2 activity is predominant among the three membrane-bound enzymes; however; these activities are similar between the BMX and control offspring.

Inhibitor Class	*Inhibitor*	*% Remaining Activity (N = 3)*
Neprilysin	**SCH (10 µM)**	**100**
Thimet oligopeptidase	**CFP (10 µM)**	**102 ± 3**
Thimet oligopeptidase	**CFP (100 µM)**	**97 ± 2**
Neurolysin	**Pro-Ile (1 mM)**	**101 ± 2**
Prolyl endopeptidase	**ZPP (10 µM)**	**96 ± 2**
Serine	**Aprotinin (80 µM)**	**100**
Serine	**SBTI (100 µM)**	**101 ± 2**
Aspartic acid	**Pepstatin A (100 µM)**	**97 ± 1**

Table 1: Specific inhibitors do not inhibit enzyme activity. Inhibitors SCH39370 (SCH), 1-carboxy-3-phenyl-propyl (CFP), Pro-Ile, Z-prolyl prolinal (ZPP), aprotinin, soybean trypsin inhibitor (SBTI) and pepstatin A did not inhibit [^{125}I]-Ang-(1-4) formation compared to control. Activity was assayed in a CSF pool of control and BMX animals (N=10) in the presence of 100 nM Ang-(1-7) and an inhibitor cocktail (AM, BS, CHYM, BSC, LIS) for 120 minutes at 37°C. Assays were repeated three times.

CHAPTER FIVE

EVIDENCE FOR AN ANGIOTENSIN-(1-7) NEUROPEPTIDASE EXPRESSED IN THE BRAIN MEDULLA AND CSF OF SHEEP

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The following manuscript is published in *The Journal of Neurochemistry*, and represents the efforts of the first author. Differences in formatting and organization reflect requirements of the journal.

Abstract:

Angiotensin-(1-7) [Ang-(1-7)] is an alternative product of the brain renin-angiotensin system (RAS) that exhibits central actions to lower blood pressure and improve baroreflex sensitivity. We previously identified a peptidase that metabolizes Ang-(1-7) to the inactive metabolite product Ang-(1-4) in CSF of adult sheep. The current study purified the peptidase 1445-fold from sheep brain medulla and characterized this activity. The peptidase was sensitive to the chelating agents o-phenanthroline and EDTA, as well as the mercury compound p-chloromercuribenzoic acid (PCMB). Selective inhibitors to angiotensin-converting enzyme, neprilysin, neurolysin, and thimet oligopeptidase did not attenuate activity; however, the metallopeptidase agent JMV-390 was a potent inhibitor of Ang-(1-7) hydrolysis (Ki = 0.8 nM). Kinetic studies using ^{125}I-labeled Ang-(1-7), Ang II, and Ang I revealed comparable apparent Km values (2.6, 2.8 and 4.3 μM, respectively), but a higher apparent Vmax for Ang-(1-7) (72 vs. 30 and 6 nmol/min/mg, respectively; $P<0.01$). HPLC analysis of the activity confirmed the processing of unlabeled Ang-(1-7) to Ang-(1-4) by the peptidase, but revealed <5% hydrolysis of Ang II or Ang I, and no hydrolysis of neurotensin, bradykinin or apelin-13. The unique characteristics of the purified neuropeptidase may portend a novel pathway to influence actions of Ang-(1-7) within the brain.

Introduction

Angiotensin-(1-7) [Ang-(1-7)] is a bioactive hormone of the renin-angiotensin system (RAS) that we originally identified in the brain and circulation of the rat twenty–five years ago (9). Since that time, accumulating scientific evidence suggests that Ang-(1-7) opposes the actions of Ang II and may constitute an endogenous buffering system to the ACE-Ang II-Ang II type 1 (AT_1) receptor axis in the regulation of autonomic function, blood pressure, and blood pressure-independent metabolic pathologies (8, 10, 41). Indeed, administration of the Ang-(1-7) antagonist D-Ala^7-Ang-(1-7) (A779) decreases baroreflex sensitivity (4, 22) and increases inflammation (7), implying a significant degree of endogenous Ang-(1-7) tone in the brain. Moreover, the Ang-(1-7) receptor antagonist reduces the actions of either angiotensin-converting enzyme (ACE, EC 3.4.15.1) or AT_1 receptor inhibition, further suggesting a key role of the peptide in the therapeutic benefits of RAS blockade (8).

Ang II is primarily formed through the direct processing of Ang I by the dipeptidyl carboxypeptidase ACE, although other peptidases including chymase (EC 3.4.21.39) and cathepsin G (EC 3.4.21.20) may contribute under pathological conditions (30, 31). In contrast, Ang-(1-7) can be processed directly from Ang I by several endopeptidase including neprilysin (EC 3.4.24.11), thimet oligopeptidase (TOP, EC 3.4.24.15), and prolyl endopeptidase (PE, EC 3.4.21.26) (2, 3, 6, 39). Additionally, Ang-(1-7) is efficiency generated directly from Ang II by the mono-carboxypeptidases ACE2 (EC 3.4.15.1) and prolyl carboxypeptidase (EC 3.4.16.2) (17, 28). Although ACE constitutes the major Ang II-forming pathway, the peptidase also degrades a number of biological active peptides including Ang-(1-7) (11). The impact of ACE inhibitors to

increase circulating levels of Ang-(1-7) reflects the marked reduction in Ang-(1-7) metabolism to Ang-(1-5), as well as the alternative processing of Ang I by the metallopeptidase neprilysin (6, 11, 42). We recently observed that ACE activity was higher in CSF from adult offspring of betamethasone-exposed pregnant ewes, a model of glucocorticoid-induced fetal programming characterized by higher blood pressure and reduced baroreflex function (25). Although the hydrolysis of Ang-(1-7) in sheep CSF *ex vivo* was attenuated by ACE inhibition, the majority of the peptide-degrading activity in the CSF was due to a thiol-sensitive endopeptidase that cleaved Ang-(1-7) to Ang-(1-4) (25). The Ang-(1-7) peptidase activity was significantly higher in the CSF of the betamethasone-exposed group and the CSF content of Ang-(1-7) was inversely correlated to peptidase activity (25). Selective inhibitors against the endopeptidases neprilysin, TOP, and neurolysin (EC 3.4.24.16) did not attenuate the hydrolysis of Ang-(1-7) to Ang-(1-4) in the CSF possibly suggesting a unique Ang-(1-7)-degrading activity in brain. To address this possibility, the present study sought to obtain a sufficient amount of the purified activity from brain medullary tissue to achieve a more complete characterization of the peptidase. A 1445-fold enrichment of the peptidase was achieved from the brain medulla of sheep and the purified activity evaluated against angiotensins and other neuropeptide substrates. We report that the medullary peptidase appears similar to the activity in the CSF to metabolize Ang-(1-7) to Ang-(1-4), exhibits marked sensitivity to mercury-based inhibitors, chelating agents, and metalloendopeptidase agent JMV-390, and hydrolyzes Ang-(1-7) to a greater extent than Ang II and Ang I, while other bioactive peptides including bradykinin, neurotensin and apelin-13 were not cleaved.

Methods

Animals

Mixed breed sheep were delivered at term, farm raised, and weaned at 3-months of age. At 10-12-months of age, male offspring were brought to our Association for Assessment and Accreditation of Laboratory Animals Care (AAALAC) approved facility, where they were maintained on a normal diet with free access to tap water and a 12-hour light/dark cycle (lights on 7 AM to 7 PM). Sheep were anesthetized with ketamine and isoflurane and euthanized by exsanguination. Brain medullae were removed and immediately covered with optimal cryosection media and stored at -80°C. CSF (~3 ml per animal) was extracted, taking care to avoid contamination with blood, and tubes were stored at -80°C. All procedures were approved by the Wake Forest University School of Medicine ACUC for animal care.

Homogenization of Sheep Brain

Brain medullae 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. The dorsal medullae from two animals were pooled (2.5 g) for each purification and homogenized in HEPES buffer (25 mM Na+ free HEPES, 10 μM ZnCl2, 0.05% Triton, pH 7.0) using a Power Gen 1000 tissue grinder (Fisher Scientific) on setting 5 for 60 sec and centrifuged at 25,000xg for 30 min at 4°C. Supernatant was retained for the subsequent purification steps.

Cerebrospinal Fluid Concentration

CSF (5 ml) was pooled and concentrated from five animals 1:5 using molecular weight filtration tubes to remove small proteins and endogenous angiotensin peptides (30

kDa, Millipore Bedford, MA). Concentrated CSF was resuspended in a final volume of 5 ml HEPES buffer (25 mM HEPES, 125 mM NaCl, 10 μM $ZnCl_2$, pH = 7.4) and protein concentration was measured using a Bradford protein assay.

DEAE-Cellulose Chromatography

Diethylaminoethyl Sepharose (DEAE, Sigma-Aldrich, St Louis MO) was equilibrated with HEPES buffer (25 mM Na+ free HEPES, 10 μM $ZnCl_2$, 0.05% Triton, pH 7.0) and incubated with the medullary supernatant for 60 min at room temperature (21°C). The DEAE gel was pelleted at 1,000xg for 60 sec, and supernatant was removed. The gel was subsequently washed in HEPES buffer with an increasing step gradient of NaCl (100, 250, 500 mM, 1M for 30 mins) and the gel pelleted at 1,000xg. The eluted material from the gradient was assayed for protein content and enzyme activity.

Cibacron Blue Chromatography

Fractions containing the highest activity from the DEAE gel were combined (250 and 500 mM NaCl), concentrated on 50 kDa molecular weight concentrations tubes (Millipore Bedford, MA), washed with Na+ free HEPES buffer, and incubated with equilibrated Cibacron Blue Sepharose Fast Flow (GE Healthcare BioSciences, Pittsburg, PA, USA) for 60 min. The flow-through fractions from the Cibacron gel contained the majority of activity and were applied directly to the Q-Sepharose column.

Q-Sepharose Chromatography

Q-Sepharose Fast Flow (Sigma-Aldrich, St Louis, MO) was equilibrated with Na-free HEPES buffer in a 2.5 x 10 cm column. The unbound Cibracron fraction was applied directly to the Q-Sepharose column and washed with a step-gradient of increasing

NaCl (0, 100, 200, 400, 600 mM and 1 M NaCl). Fractions were collected and assayed for protein content and enzyme activity.

Gel Electrophoresis

Purified protein eluted from the Q-Sepharose column was diluted 1:1 in Laemmli buffer containing β-mercaptoethanol and boiled for 5 mins to induce denaturing conditions. Proteins were separated on 10% Mini-PROTEAN TGX gels for 80 min at 120 V in Tris-glycine buffer. Gels were stained with Imperial™ Blue Stain (Thermo Fisher Scientific, Rockford, IL) or silver stain (PlusOne™ Silver Stain Kit, GE Healthcare BioSciences, Pittsburg, PA) according to manufacturer's instructions.

Inhibitor and pH Profile

Using aliquots of partially purified peptidase, various inhibitors were tested for their ability to inhibit ^{125}I-Ang-(1-7) metabolism including PCMB, E-64, EDTA, o-phenanthroline, N-[N-[1-(S)-carboxyl-3-phenylpropyl]-(S)-phenyl-alanyl]-(S)-isoserine e (SCH), N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-p-aminobenzoate (CFP), Z-prolyl prolinal (ZPP), dithiothreitol (DTT), the dipeptide Pro-Ile, phenylmethylsulfonyl fluoride (PMSF), aprotinin, soybean trypsin inhibitor (SBTI), and the metallopeptidase inhibitor *N*-[3-[(hydroxyamino)carbonyl]-1-oxo-2(*R*)-benzylpropyl]-L-leucine (JMV-390). Each reaction was conducted in the presence of 0.5 nM ^{125}I-Ang-(1-7), 100 nM Ang-(1-7), 92 ng of the Q-Sepharose fraction or 25 µl of CSF, and a cocktail of inhibitors (metabolism cocktail) containing the aminopeptidase inhibitors amastatin (AM, 2 µM) and bestatin (BS, 10 µM), the chymase inhibitor chymostatin (CHYM, 10 µM), the carboxypeptidase A inhibitor benzylsuccinate (BSC 10 µM), and the ACE inhibitor

lisinopril (LIS, 10 μM). All inhibitors were obtained from Sigma (St. Louis, MO) except SCH 39370 (gift from Schering Plow), CFP (Bachem, King of Prussia, PA), and JMV-390 (Tocris bioscience, UK). A pH profile was conducted as previously described (26). Purified peptidase (10 μl) was added to buffers ranging from pH 3.5 to 5.5 using 25 mM MES, 125 mM NaCl, and pH 7.5-9.5 using 25 mM HEPES, 125 mM NaCl. Iodinated products generated by the incubation of peptidase and ^{125}I-Ang-(1-7) were separated using HPLC.

Peptidase kinetics

Kinetic or competition assays were performed with ^{125}I-Ang-(1-7), -Ang II or -Ang I as substrates, and increasing concentrations of corresponding unlabeled peptides as previously described (25, 32). Reaction velocities for generation of ^{125}I-Ang-(1-4) from the radiolabeled peptides were expressed as pmol/min/mg protein. The kinetic assays were performed with the metabolism cocktail to prevent the contribution of other peptidases and preserve the Ang-(1-4) product. Apparent kinetic constants (Km', Vmax') were determined using Michaelis-Menten kinetics in the GraphPad Prism 5 (San Diego, CA, USA) statistical program. Peptides were obtained from Bachem (Torrance, CA, USA) or custom synthesized (Genscript, Piscataway, NJ, USA).

HPLC Separation

Metabolism reactions were conducted at 37°C in reaction buffer using 1445-fold purified peptidase (92 ng in a final volume of 250 μl) or 25 μl CSF. Each reaction included 0.5 nM ^{125}I-Ang-(1-7) and 0.1 μM non-iodinated Ang-(1-7) (32). The reactions were stopped after 60 min by addition of ice-cold 1.0% phosphoric acid and centrifuged

at 16,000xg. The supernatant was immediately filtered for separation by reverse-phase HPLC on a Shimadzu equipped with an Aeris Peptide XB-C18 3.6 μm (2.1x100 mm, Phenomenex, Torrance CA). The ^{125}I-products were monitored by a Bioscan flow-through γ detector as described (25). Products were identified by comparison of their retention times to ^{125}I-standard peptides and sensitivity to peptidase inhibitors. Peptides were iodinated by the chloramine T method and purified by HPLC to a specific activity >2,000 Ci/mmol (9).

UV HPLC Separation

Metabolism reactions were conducted at 37°C in reaction buffer using 1445-fold enriched peptidase (185 ng) and 50 nmol of unlabeled Ang I, Ang II, Ang-(1-7), Ang-(1-6) or Ang-(1-5), neurotensin, bradykinin, bradykinin-(1-7), bradykinin-(1-5), or apelin-13 in a final volume of 125 μl (final concentration of 0.4 mM). The reactions were stopped after 24 hours by addition of 1.0% phosphoric acid and separated by HPLC using a 0.1% phosphoric acid solvent system performed under a gradient of 0-15-25% phase B (80% acetonitrile) at a flow rate of 0.35 ml/min at ambient temperature. Peptides were monitored at 220 nm and the products identified by the retention time of standard peptides.

Statistics

Data are expressed as mean ± SEM. Unpaired t tests and one-way repeated measures ANOVA with Bonferroni posttests were used for the statistical analysis of the data with GraphPad Prism. The criterion for statistical significance was set at $*P < 0.05$.

Results

Comparison of CSF and medullary metabolism of Ang-(1-7)

We initially compared the metabolism of ^{125}I-Ang-(1-7) in the cytosolic fraction of the brain medulla to metabolism in the CSF. As shown in Figure 1, ^{125}I-Ang-(1-7) was metabolized primarily to ^{125}I-Ang-(1-4) with a minor peak corresponding to ^{125}I-Ang-(3-4) in the medullary supernatant fraction; the ^{125}I-Ang-(1-4) peak was abolished by addition of the thiol inhibitor PCMB (10 μM). Comparison of the Ang-(1-7) saturation curves revealed similar apparent Km (Km') values for the CSF and brain medulla activities, but a 6-fold higher apparent Vmax (Vmax') for the medullary peptidase (Figure 1, right panel).

Purification of Ang-(1-7) peptidase activity from sheep medulla

In lieu of the higher tissue activity, the soluble fraction of the brain medulla was used as the initial source for the purification of peptidase activity. Soluble activity from 2.5 g of dorsal brain medulla was subjected to consecutive steps on DEAE, Cibacron Blue, and Q-Sepharose chromatography (Table 1). Peptidase activity was monitored by the conversion of ^{125}I-Ang-(1-7) to ^{125}I-Ang-(1-4) and sensitivity to the metalloendopeptidase inhibitor JMV-390 (see inhibitor sensitivity, below). The peptidase activity eluted in 250 mM NaCl on DEAE, was not retained on the Cibacron Blue gel, and eluted at 200 mM NaCl on Q-Sepharose following extensive washing with the 100 mM NaCl buffer. Fractions were collected in 15 ml increments from the 200 mM NaCl elution on the Q-Sepharose column; fraction 3 had the highest specific activity and was used to calculate purity and yield. We attained a 1445-fold purification with a 25% yield of the active peptidase using this protocol (Table 1). Peptidase activity

apparently increased following the Cibacron Blue column (89 to 128%) which may reflect the separation of inhibitory substances that compete for the metabolism of Ang-(1-7). A second purification of the medullary soluble fraction obtained from the medullary tissue of different animals resulted in a similar degree of enrichment (1970-fold) and enzyme yield (31%) following the Q-Sepharose fractionation (preparation 2). SDS gels of the Q-Sepharose purified fraction stained with Imperial™ Protein Stain or the silver stain revealed approximately five protein bands in the 50 to 80 kDa range indicating that complete purification of the Ang-(1-7) peptidase was not achieved. Since the denatured gels abolished activity, it remains unclear what band corresponds to the peptidase.

Inhibitor Sensitivity

The sensitivity of the enriched peptidase from the Q-Sepharose column or concentrated CSF to various inhibitors was assessed by the conversion of ^{125}I-Ang-(1-7) to ^{125}I-Ang-(1-4). Similar to the CSF activity, the medullary peptidase was insensitive to specific inhibitors against neprilysin (10 μM, SCH 39370), TOP (10 μM, CFP), neurolysin (1 mM, Pro-Ile), the general cysteine peptidase inhibitor E-64 (10 μM), and the serine protease inhibitors PMSF (100 μM), aprotinin (80 μM), and SBTI (100 μM) (Figure 2A, Table 2). PCMB (10 μM) and o-phenanthroline (1 mM) abolished peptidase activity while DTT (5 mM) and EDTA (5 mM) exhibited partial inhibition (Figure 2B). The optimal pH for the peptidase purified from medullary tissue was pH 7.5 (Figure 2C). As compared to control conditions (Figure 3A), 1 nM JMV-390 reduced the Ang-(1-4) peak and 100 nM JMV-390 essentially abolished activity (Figure 3B and 3C, respectively). A dose-response curve for JMV-390 inhibition revealed an IC_{50} of 0.8 ± 0.2 nM with a R value of 0.996 for a one-site competition curve (Figure 3D). This was

not significantly different than the IC_{50} for JMV-390 of the CSF activity (1.1 ± 0.4 nM) (Table 2).

Kinetic Properties

Saturation studies on the purified peptidase activity were performed using ^{125}I-labelled Ang-(1-7), Ang II and Ang I to compare the apparent kinetic constants among the three peptides. The kinetic analysis revealed apparent Km (Km′) and Vmax′ values of 2.6 ± 0.3 μM and 72 ± 2.2 nmol/min/mg for Ang-(1-7), 2.8 ± 0.7 μM and 30 ± 3 nmol/min/mg for Ang II, and 4.3 ± 0.7 μM and 6 ± 0.3 nmol/min/mg for Ang I (Figure 4A-C, respectively). Although the Km' values were similar for all 3 peptides, Ang-(1-7) exhibited the highest Vmax' as compared to Ang II or Ang I ($p<0.01$ vs. Ang I or Ang II, N=3). Apparent kinetic values for ^{125}I-labelled Ang-(1-7), Ang II and Ang I were also derived from the second batch of purification. While the Km' values for Ang-(1-7) (2.0 μM), Ang II (7.4 μM) and Ang I (1.7 μM) were similar to the first purification, the Vmax' values were >2-fold higher (174, 69, and 20 nmol/min/mg protein for Ang-(1-7), Ang II, and Ang I, respectively) and likely reflect the greater degree of purity in preparation 2 that yields a higher Vmax'.

Substrate Specificity

We compared the metabolism of ^{125}I-Ang-(1-7) in the purified medullary fraction to the initial tissue supernatant in the absence of any peptidase or protease inhibitors. Essentially complete metabolism of ^{125}I-Ang-(1-7) to ^{125}I-Ang-(3-4) was evident in the medullary supernatant fraction that likely reflects dipeptidyl aminopeptidase activity (Figure 5A). In contrast, ^{125}I-Ang-(1-4) was the primary product following a 60 or 120 minute incubation with the purified peptidase (Figure 5B and C, respectively). Addition

of the inhibitor JMV-390 essentially abolished the ^{125}I-Ang-(1-4) peak, and did not reveal additional metabolites suggesting the absence of other activities that metabolize Ang-(1-7) (Figure 5D). Given the apparent lack of other peptidase activities in the purified preparation, we determined its specificity against various unlabeled angiotensins, as well as the bioactive peptides neurotensin, bradykinin, and apelin-13 (Figures 6 and 7). Ang-(1-7) was exclusively hydrolyzed to Ang-(1-4) and addition of JMV-390 abolished the peak of Ang-(1-4) (Figure 6A and 6B, respectively). Ang II and Ang I were metabolized to Ang-(1-4); however, the extent of metabolism was markedly less than that of Ang-(1-7) (Figure 6C and 6D). Ang-(1-6) and Ang-(1-5) were also metabolized to Ang-(1-4), but at lower rates than Ang-(1-7) (Table 3). Neurotensin, bradykinin, and the bradykinin metabolites bradykinin-(1-7) and bradykinin-(1-5) were not hydrolyzed by the peptidase as reflected by the absence of intermediate peaks distinct from the parent peptides (Figure 7 A-D). Based on the extent of hydrolysis of the unlabeled peptides, we calculated the rate of metabolism for each peptide substrate (Table 3). Ang-(1-7) was hydrolyzed to a greater extent than Ang II (11-fold) or Ang I (27-fold). The rate of Ang-(1-7) metabolism was 2-fold higher than Ang-(1-6) and 26-fold greater than the pentapeptide Ang-(1-5). There was no apparent metabolism of neurotensin, bradykinin or bradykinin metabolites, or apelin-13 (chromatograph not shown) following the 24 hour incubation conditions.

Discussion

In the present study, we isolated and characterized an Ang-(1-7) metabolizing activity in the brain medulla of sheep that extends the original identification of the

peptidase activity in the CSF (26). With respect to the similar kinetic parameters, substrate specificity, inhibitor sensitivity, and optimal pH, it is likely that the medullary peptidase and the CSF peptidase activities are identical (Table 2). Using a combination of DEAE Sepharose, Cibacron Blue, and Q-Sepharose chromatography, the peptidase was purified 1445- and 1950-fold from supernatant fraction of the brain medulla in two preparations using tissues pooled from multiple animals. The isolated activity is optimally active at pH 7.5 and sensitive to the mercury compounds PCMB and aminophenylmercuric acetate (APMA), and chelating agents o-phenanthroline, EDTA, and DTT; however peptidase activity is not reduced by selective inhibitors against the metalloendopeptidases neprilysin, neurolysin, and TOP nor the serine protease inhibitors PMSF, aprotinin, or SBTI. The peptidase appears to preferentially cleave the Tyr^4-Ile^5 bond of Ang-(1-7) in comparison to other angiotensin peptides or the bioactive peptides neurotensin, bradykinin, and apelin-13. Kinetic analyses using [^{125}I]-labeled angiotensins revealed similar Km' values (2.6 ± 0.3, 2.8 ± 0.7, and 4.3 ± 0.7 μM, for Ang-(1-7), Ang II, and Ang I, respectively), but a significantly higher Vmax' for Ang-(1-7) (72 ± 2.2 nmol/min/mg) versus Ang II (30 ± 3 nmol/min/mg) or Ang I (6 ± 0.3 nmol/min/mg). These data likely represent a novel pathway for specific regulation of central Ang-(1-7) levels.

Metallopeptidases are the major class of enzymes involved in extracellular peptide metabolism (34). ACE, ACE2, neprilysin, TOP, and neurolysin belong to the same class of thermolysin-like zinc-dependent peptidases that hydrolyze peptide substrates (<40 amino acids) and are maximally active at a neutral pH (29, 34). The characterization of a soluble metalloendopeptidase in the CSF and brain medulla is

consistent with this class of peptidases, yet appears to be more restricted regarding the substrate length and the site of hydrolysis. For example, angiotensin peptides larger than Ang-(1-7) such as Ang II and Ang I exhibited an 11- and 27-fold lower rate of hydrolysis to generate Ang-(1-4). Interestingly, neurotensin contains a Tyr-Ile site at positions 12 and 13 of the peptide but was not cleaved by the peptidase; this may reveal a more constrained effect of peptides greater than 10 residues in length. In this regard, both neurolysin and TOP exhibit restriction on the length of their peptide substrates that may reflect the limited access to the active sites of these peptidases (33). Peptides that lack the Tyr-Ile site such as bradykinin and apelin-13 were not cleaved by this peptidase. Moreover, the seven and five amino acid metabolites of bradykinin were not hydrolyzed by the medullary peptidase. The shorter fragments of Ang-(1-7) including Ang-(1-6) and Ang-(1-5) that are generally considered biologically inactive were hydrolyzed at a lower rate (2- and 25-fold, respectively) than Ang-(1-7), again suggesting an optimal length for recognition by the peptidase. Finally, we note that while the velocity rates for the hydrolysis of ^{125}I-Ang-(1-7) and Ang-(1-7) were similar (72 versus 54 nmol/min/mg), the velocity rate for ^{125}I-Ang II was 6-fold higher than that of Ang II (30 versus 5 nmol/min/mg) and the rate for ^{125}I-Ang I was 3 fold higher than Ang I (6 versus 2 nmol/min/mg). These data suggest an apparent influence of the addition of the ^{125}I-group on the Tyr^4 residue, particularly for Ang II that may enhance the rate of hydrolysis of the peptide. Although additional kinetic studies are required to elucidate the mechanism for this effect on the Tyr residue, the use of ^{125}I-labeled peptides other than Ang-(1-7) may not accurately reflect the substrate characteristics for the peptidase.

The Ang-(1-7) peptidase was isolated from the supernatant fraction of the medullary tissue, consistent with the soluble activity detected in the CSF (26). Metallopeptidases such as ACE, ACE2, neprilysin and neurolysin are predominantly membrane-associated, thus they are situated to hydrolyze bioactive peptides in the extracellular space. In contrast, TOP is a soluble enzyme that is primarily intracellular with no hydrophobic membrane-spanning domain (1, 21, 35, 40). Several studies suggest that TOP localizes to the nucleus, consistent with the identification of a nuclear localization sequence for the peptidase (3, 19, 27, 37). TOP reportedly undergoes both stimulated secretion and constitutive release from neuronal cells and is known to associate with lipid rafts in the plasma membrane (16, 20, 21). Once in the extracellular space, TOP participates in the extracellular metabolism of neuropeptides such as gonadotropin-releasing hormone, bradykinin, and neurotensin (16). We have not established the intracellular distribution of the Ang-(1-7) peptidase nor the specific cell type (ie. neuronal vs. glia) that express the peptidase within the medulla. Increasing evidence supports an intracellular RAS including the expression of intracellular Ang II and Ang-(1-7), as well as their respective receptors (10, 15, 18, 36). Indeed, we quantified the peptide content of Ang-(1-7), Ang II and Ang I in the brain medulla of sheep (24). Krob et al demonstrated intense Ang-(1-7) immunostaining in hypothalamic neurons of the mRen2(27) transgenic rats suggesting the intracellular localization of the peptide (23). Furthermore, Gironacci and colleagues reported intracellular Ang-(1-7) expression in primary neuronal cells cultured from the hypothalamic-brainstem areas (38). In this regard, the peptidase may potentially influence the local processing of Ang-(1-7) within the cells. Alternatively, the peptidase may be secreted or released from

medullary tissue to degrade extracellular Ang-(1-7) or perhaps other substrates that are unidentified at this time. Indeed, the presence of the soluble peptidase in the CSF may reflect the secretion of the enzyme into this compartment. Additional studies are necessary to identify the cell types that contain the peptidase and address whether the enzyme activity is actively secreted from medullary tissues.

The current chromatographic approach was not intended to achieve a preparation of pure enzyme, but to obtain sufficient activity to characterize the peptidase; thus, we cannot establish the identity of the protein with this preparation at the present time. Nevertheless, the studies of the enriched enzyme suggest a metalloendopeptidase-like activity to convert Ang-(1-7) to Ang-(1-4). Although the peptidase activity was not blocked by selective inhibitors to neprilysin, ACE, neurolysin or TOP, the enzyme exhibits marked sensitivity to the inhibitor JMV-390 (IC_{50} = 0.80 nM). In contrast, the reported IC_{50} values for JMV-390 against neprilysin, neurolysin, TOP, and leucine aminopeptidase (EC 3.4.11.1) are 30 to 60 nM, and a far higher value of 70 μM against ACE (14). JMV-390 was originally synthesized to inhibit metalloendopeptidase activity thereby extending the functional benefits of neurotensin and neuromedin in brain (14). The molecular design of JMV-390 was based on the recognition of the leucine-leucine sequence that corresponds to neurotensin, and the amino-terminal hydroxy amino group for an interaction with the zinc cofactor in the active site (14).

ACE constitutes a prominent pathway for the metabolism of Ang-(1-7) in the circulation (8). ACE inhibition significantly increases the half-life of Ang-(1-7) in the circulation by blocking the hydrolysis of Ang-(1-7) to Ang-(1-5) (11, 42). The marked increase in circulating Ang-(1-7) following administration of ACE inhibitors also reflects

an increase in neprilysin-dependent processing of Ang I to Ang-(1-7) (6, 11). We reported that soluble ACE contributes to Ang-(1-7) metabolism in sheep CSF *ex vivo*; however, the majority of the Ang-(1-7)-degrading activity was ACE independent and this activity inversely correlated to Ang-(1-7) content in the CSF (26). The presence of an Ang-(1-7) peptidase in the CSF and brain medulla may represent an alternative pathway to metabolize the peptide, particularly if the local expression of ACE is low or there is restricted access to ACE. Additional studies are necessary to compare the relative expression of ACE and the Ang-(1-7) peptidase in other areas of the brain, as well as the presence of the metallopeptidase in the circulation and peripheral tissues such as the heart and kidney that express Ang-(1-7).

Perspective and Significance

Central Ang-(1-7) is critically involved in blood pressure control and autonomic regulation. Low levels of Ang-(1-7) are associated with hypertension, reduced baroreflex sensitivity for control of heart rate, inflammation, cell proliferation, and oxidative stress (5, 12, 13). The peptidase characterized in this study may potentially represent a novel Ang-(1-7) metabolizing pathway involved in the pathological reduction of central Ang-(1-7) levels (26). Moreover, we identified JMV-390 as a potent inhibitor of the Ang-(1-7) peptidase that exhibits an IC_{50} value far below that reported for neprilysin, neurolysin, TOP, leucine aminopeptidase, and ACE. JMV-390, or more selective analogs, may constitute a novel selective inhibitor against the Ang-(1-7) peptidase to prevent metabolism of Ang-(1-7) in brain. Enhanced peptidase activity in CSF of animals exposed *in utero* to glucocorticoids, where Ang-(1-7) levels were significantly lower than

control animals, further suggests that the peptidase participates in physiological regulation of the brain RAS.

Acknowledgements / Conflict of interest disclosure

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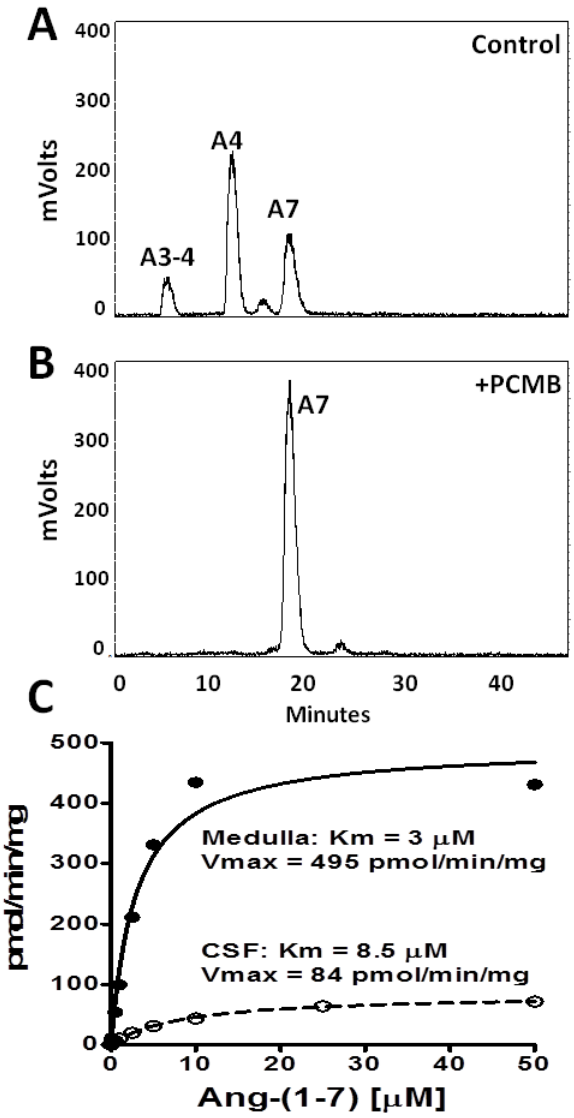


Figure 1: Ang-(1-7) metabolizing peptidase activity in the brain medulla and CSF. **A:** ^{125}I-Ang-(1-7) [A7] is metabolized to ^{125}I-Ang-(1-4) [A4] and ^{125}I-Ang-(3-4) [A3-4]. **B:** A7 metabolism is abolished with the addition of PCMB (10 μM). **C:** Comparison of apparent kinetics of ^{125}I-Ang-(1-7) metabolizing peptidase in brain medulla and CSF. Reactions were run in the presence of an inhibitor cocktail (AM, BS, CHYM, BSC, LIS) for 60 minutes at 37°C.

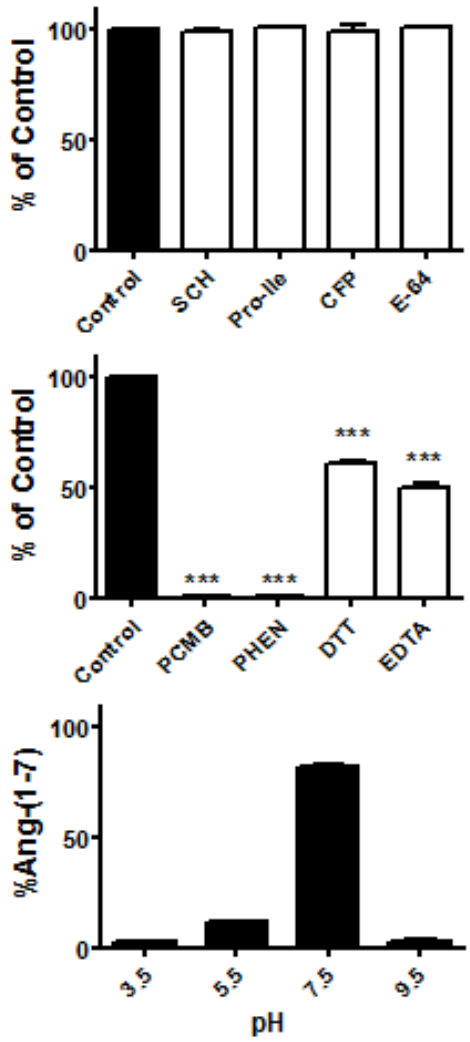


Figure 2: Inhibitor and pH profiles of the enriched Ang-(1-7) brain medullary peptidase. **A:** Specific inhibitors SCH 39370 (10 µM, SCH), Pro-Ile (1 mM), CFP (10 µM), E-64 (10 µM), or PMSF (100 µM) did not inhibit activity. **B:** Peptidase was sensitive to the chelating agents PCMB (10µM), o-phenanthroline (1 mM, PHEN), dithiothreitol (5 mM, DTT), and EDTA (5 mM). **C:** pH profile reveals an optimal pH of 7.5 for peptidase activity. Reactions were carried out in the presence of an inhibitor cocktail (AM, BS, CHYM, BSC, LIS) for 60 minutes at 37°C. N=3 separate determinations from the Q-Sepharose purified fraction of preparation 1, ***P<0.0001 vs. control

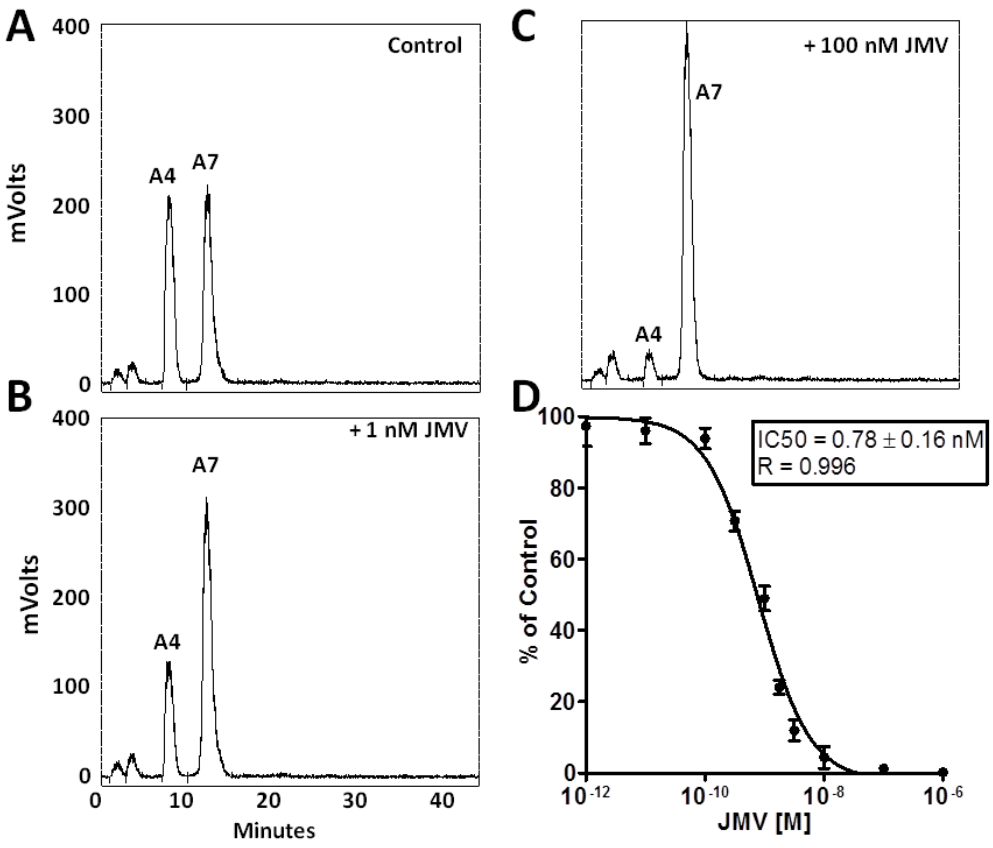


Figure 3: JMV-390 inhibits the Ang-(1-7) peptidase activity. **A-C:** ^{125}I-Ang-(1-7) was incubated with purified enzyme in the presence of 0, 1 nM, or 100 nM JMV-390. **D:** A dose-response curve was conducted to determine the IC_{50} for JMV-390. Reactions were carried out in the presence of an inhibitor cocktail (AM, BS, CHYM, BSC, LIS). N=3 separate determinations from the Q-Sepharose purified fraction of preparation 1.

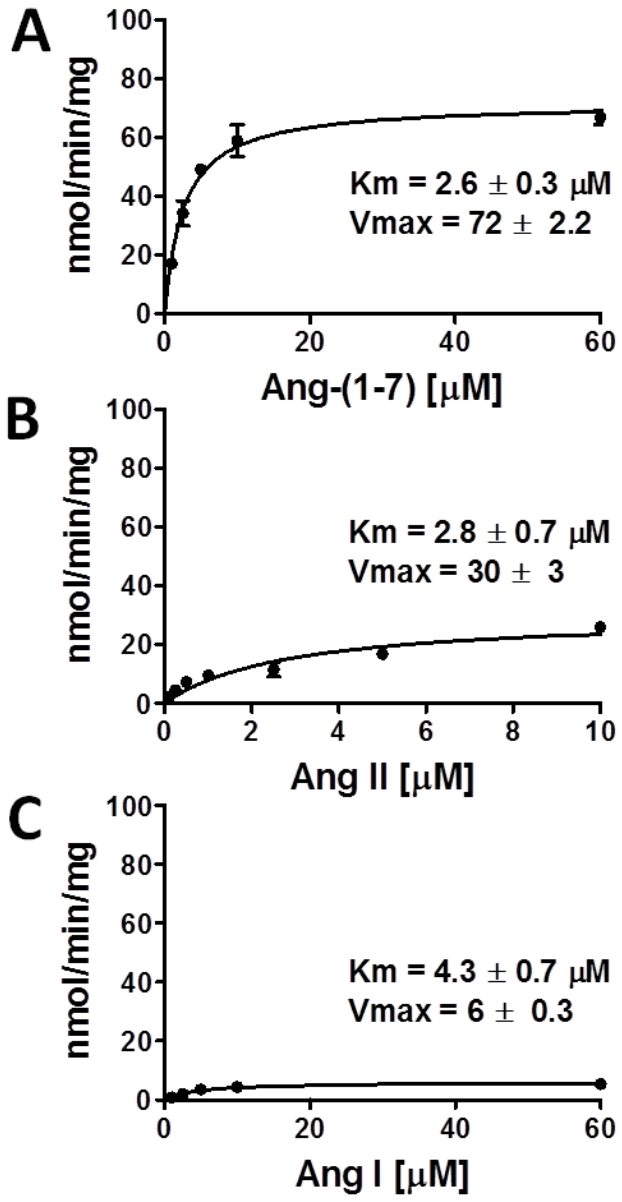


Figure 4: ^{125}I-Ang-(1-7) exhibits the highest apparent Vmax (nmol/min/mg) for the brain medullary peptidase. **A-C:** Apparent kinetic values were determined for ^{125}I-Ang-(1-7), ^{125}I-Ang II, and ^{125}I-Ang I. Kinetic values were determined based on the conversion of radiolabeled peptide to ^{125}I-Ang-(1-4) in the presence of an inhibitor cocktail (AM, BS, CHYM, BSC, LIS). N=3 separate determinations from the Q-Sepharose purified fraction of preparation 1.

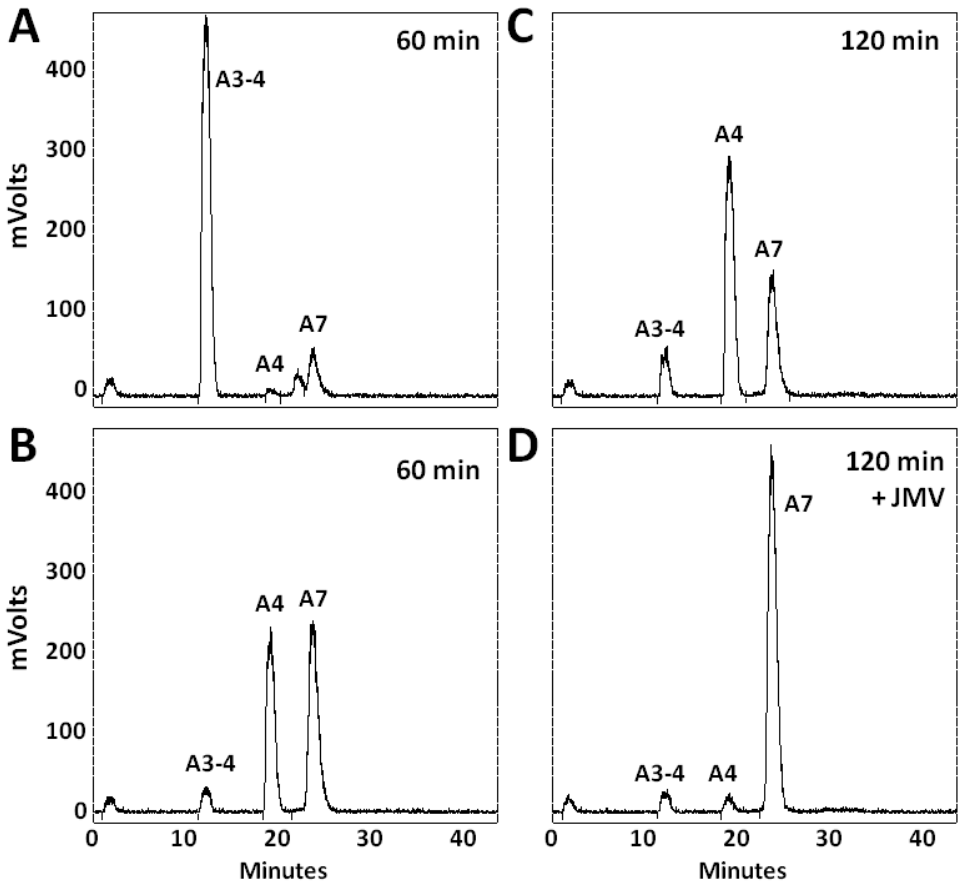


Figure 5: Purified peptidase lacks other Ang-(1-7) metabolizing enzyme activity. **A:** Medullary supernatant incubated with ^{125}I-Ang-(1-7) and no inhibitors for 60 min. **B:** Q-Sepharose purified fraction incubated with ^{125}I-Ang-(1-7) and no inhibitors for 60 min. **C:** Q-Sepharose purified fraction incubated with ^{125}I-Ang-(1-7) and no inhibitors for 120 min. **D:** Q-Sepharose purified fraction incubated with ^{125}I-Ang-(1-7) and 1 nM JMV for 120 min.

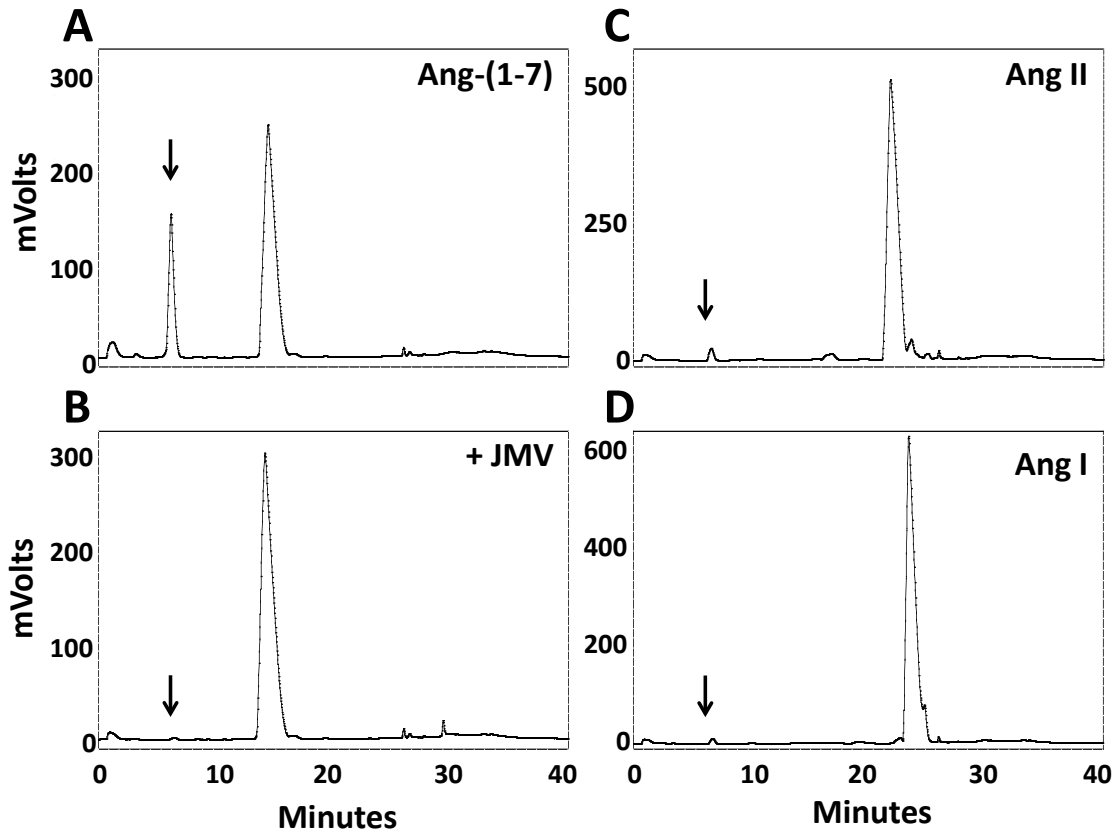


Figure 6: Purified medullary peptidase preferentially hydrolyzes Ang-(1-7) to Ang-(1-4). Purified peptidase was incubated with 50 nmol (final concentration of 400 μM) of **A:** Ang-(1-7), **B:** Ang-(1-7) + 10 μM JMV-390, **C:** Ang II, **D:** Ang I for 24 hours at 37°C in the presence of no inhibitors.

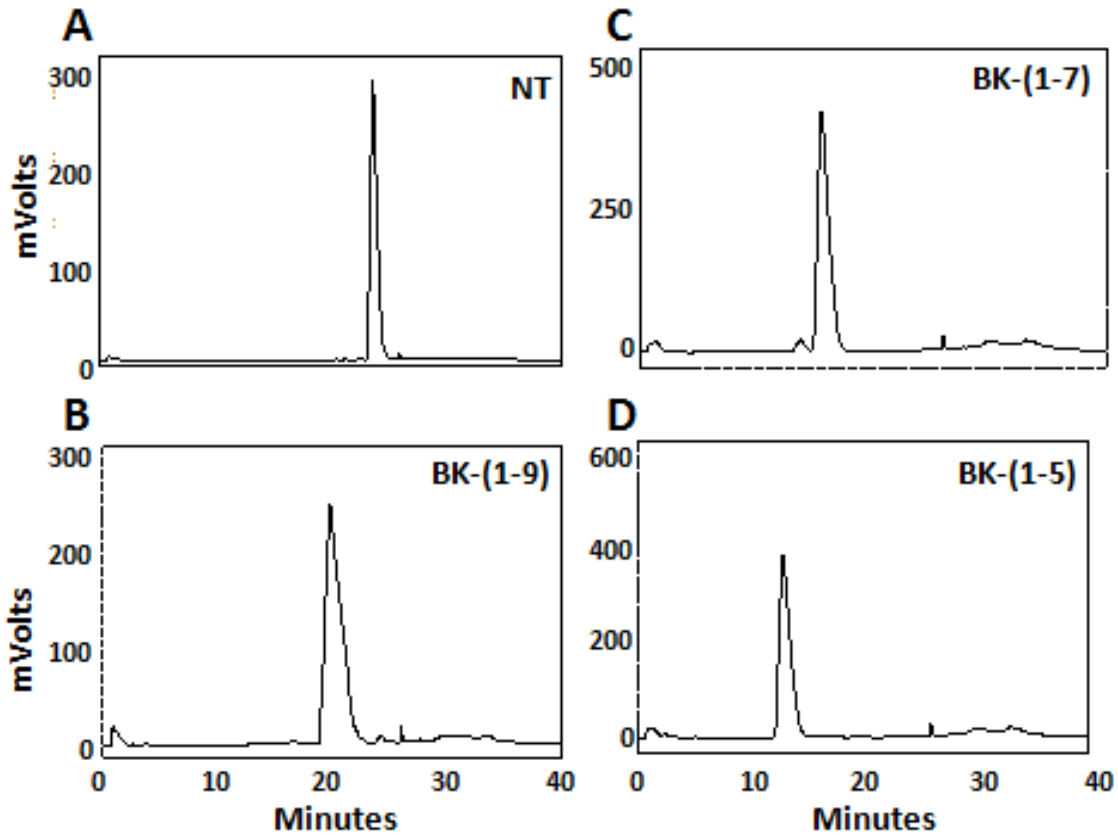


Figure 7: Purified medullary peptidase does not hydrolyze other selected neuropeptides. Purified peptidase was incubated with 50 nmol (final concentration of 400 µM) of **A:** Neurotensin (NT), **B:** Bradykinin (BK)-(1-9), **C:** BK-(1-7), or **D:** BK-(1-5) for 24 hours at 37°C in the presence of no inhibitors.

Stage	Activity (pmol/ml)	Protein (mg/ml)	Specific Activity (pmol/mg)	Yield (%)	Purification Factor (fold)
Supernatant (25,000xg)	142	244	0.58	100%	1
DEAE	127	17	7.5	89%	13
Cibacron Blue	183	4	44.5	128%	77
Q-Sepharose	40	.047	831.9	25%	1445

Table 1: Four-step purification of brain peptidase. A combination of centrifugation, DEAE, Cibacron Blue, and Q-Sepharose chromatography were used to achieve 1445-fold purification over starting material.

	CSF	Medullary Tissue
Ang-(1-7) Affinity		
Km' (μM)	8.5 ± 0.5	3.0 ± 0.6
Vmax' (pmol/min/mg)	84 ± 1.5	495 ± 29
Inhibitor Sensitivity (% of control)		
JMV-390	0 (IC_{50} = 1.1 ± 0.3 nM)	0 (IC_{50} = 0.8 ± 0.2 nM)
PCMB (10 μM)	0	0
APMA (10 μM)	0	0
EDTA (5 mM)	61 ± 2	79 ± 5
DTT (5 mM)	50 ± 4	73 ± 3
o-phenanthroline (1 mM)	1 ± 2	4 ± 0.3
E-64 (10 μM)	100 ± 1	100
SCH (10 μM)	100	99 ± 0.6
CFP (10 μM)	102 ± 3	98 ± 3
Pro-Ile (1 mM)	101 ± 2	101 ± 0.5
Aprotinin (80 μM)	100	103 ± 0.9
SBTI (100 μM)	101 ± 2	101 ± 0.3
Lisinopril (10 μM)	100	100
Optimal pH	7.5	7.5

Table 2: Comparison of CSF and the purified medullary peptidase activities for hydrolysis of ^{125}I-Ang-(1-7). Values for each inhibitor represent percent of control. N=3 separate determinations from the Q-Sepharose purified fraction.

Peptide	Amino Acid Sequence	Velocity (nmol/min/mg)
Ang I	DRVYIHPFHL	2.0
Ang II	DRVYIHPF	4.9
Ang-(1-7)	DRVYIHP	54
Ang-(1-6)	DRVYIH	27
Ang-(1-5)	DRVYI	2.1
Neurotensin	ELYENLPRRPYIL	NC
Bradykinin-(1-9)	RPPRFSPFR	NC
Bradykinin-(1-7)	RPPRFSP	NC
Bradykinin-(1-5)	RPPRF	NC
Apelin 13	KFRRQRPRLSHKG	NC

Table 3: Comparison of the metabolism velocities for angiotensins and other peptides. Velocity is an approximation of Vmax, as reactions are carried out in the presence of excess peptide substrate. NC = not cleaved within 24 hours of digestion.

CHAPTER SIX

SUMMARY AND CONCLUSIONS

1. Summary of Findings

Antenatal betamethasone (BM) exposure is associated with decreased autonomic function and increased blood pressure in adult offspring as a result of long term programming induced by excess exposure to steroids during a critical time point of gestation (29, 67). Reductions in baroreflex sensitivity, a measure of autonomic function, precede and may contribute to the elevation in blood pressure observed in this model (76). Although the link between *in utero* BM exposure and decreased cardiovascular health is well established in animal models, the underlying mechanisms for these functional alterations are not completely understood. In order to correct the programmed alterations in offspring exposed *in utero* to BM, it is necessary to understand the timing and localization of these changes. Previous studies show the RAS is a target of BM-induced alterations, both in the kidney and circulation (35, 66). While the brain is critically involved in blood pressure regulation and contains all RAS components, its role in BM induced fetal programming is not well studied. To this end, the present study identifies components of the central RAS that are altered in offspring exposed *in utero* to BM. These findings clearly identify a shift in the brain medullary and CSF RAS towards the pro-hypertensive Ang II axis of the RAS in BM-exposed (BMX) animals. As thousands of preterm infants are exposed to BM each year, the long term health of these

individuals is dependent on understanding the programming effects of *in utero* exposure to synthetic glucocorticoids.

The studies presented in Chapter Two examine the balance between the ACE-Ang II- AT_1 receptor axis and the ACE2-Ang-(1-7)-Mas receptor axis in the dorsal brain medulla of control and BMX sheep. These findings demonstrate that BMX animals lose "Ang-(1-7) tone" as reflected by lower expression of the Mas receptor and a higher Ang II/Ang-(1-7) peptide ratio by 6-months of age. In addition to alterated angiotensin peptide ratios, levels of Ang I intact-angiotensinogen are lower in BMX animals at 6-months of age while total levels of angiotensinogen are constant among the two groups. This indicates that BMX animals have increased renin dependent processing of Ang I from the precursor peptide. As a result, BMX animals may also have higher levels of Ang II and Ang-(1-7), as they are derived from the shared Ang I precursor. While we identify peptides and receptors in the medullary tissue, activity of the processing enzymes ACE, ACE2, and neprilysin are below the detection limits for our methods. Therefore, we investigated the ChP as a more enriched source of processing enzyme activities in the studies described in Chapter Three. The ChP is affixed directly to brain tissue and filters blood to produce CSF. Components of the RAS are not altered in the ChP between control and BMX offspring; however, we present new data on the localization and activities of ACE, ACE2, and neprilysin in the ChP. While all three peptidases are localized to the apical, or BBM, membrane facing the CSF, ACE2 activity is 2.5-fold higher than ACE or neprilysin. Peptide levels within the ChP are inconsistent with the corresponding processing activities on the BBM, indicating differential regulation of

peptides between ChP tissue and the CSF. These data signify a more complex role for peptidases that form and metabolize the Ang peptides both in the ChP and the CSF.

In the CSF compartment, Ang-(1-7) content is markedly higher compared to Ang I or Ang II. Furthermore, Ang-(1-7) levels are lower in BMX animals, again suggesting a shift in the central RAS towards the Ang II axis. ACE2 activity is indeed higher than ACE or neprilysin in the ChP BBM, consistent with the high levels of Ang-(1-7) peptides in the CSF; however, there is no difference in ChP ACE2 activity between control and BMX animals. These data contrast with our previous findings in the circulation and kidney showing BMX exposure was associated with lower ACE2 activity (66). We further investigated Ang-(1-7) metabolism in the CSF, a compartment in contact with the apical surface of the ChP. Indeed, we find higher metabolism of Ang-(1-7) in the CSF of BMX animals. A small fraction of this metabolism is blocked with the potent and selective ACE inhibitor lisinopril. The majority of Ang-(1-7) metabolism is inhibited by the mercuri-compound p-chloromercuribenzoic acid (PCMB). As a result, we conclude that the lower levels of Ang-(1-7) in BMX animals may result from a higher rate of Ang-(1-7) metabolism both by ACE and an unknown PCMB-sensitive peptidase.

The studies in Chapter Four entail a more complete characterization of the Ang-(1-7) peptidase in the CSF of control and BMX animals. We find the peptidase insensitive to selective inhibitors against ACE, neprilysin, neurolysin, thimet oligopeptidase, and prolyl endopeptidase. However, the activity is sensitive to chelating agents such as EDTA, DTT, and o-phenanthroline, as well as the mercuri-compounds PCMB and APMA. Kinetic analysis of the peptidase revealed a higher maximal velocity

and an overall higher rate of metabolism in BMX animals. Accordingly, peptidase activity inversely correlates with Ang-(1-7), but not Ang I or Ang II peptide levels.

Finally, we identify the brain medulla as a source of the Ang-(1-7) peptidase activity in the studies described in Chapter Five. The inhibitor sensitivity and pH profile are identical between the CSF and medullary peptidase; therefore, it is likely that the medulla acts as a sorce of soluble peptidase in the CSF. We purified the peptidase from brain dorsal medullary tissue using a combination of chromatography techniques. Using a 2,000-fold purified fraction, we determined peptidase specificity for Ang-(1-7) versus Ang I, Ang II, Ang-(2-7), Ang-(3-7), apelin-13, bradykinin, bradykinin-(1-7), bradykinin-(1-5), and neurotensin. This peptidase may represent a novel Ang-(1-7) metabolizing pathway that is physiologically involved in regulation of the central RAS.

Together, these data identify specific alterations in the brain RAS that result from *in utero* exposure to BM. The identification of a novel Ang-(1-7) specific peptidase may be particularly relevant as it potentially represents a new target for therapies designed to increase endogenous Ang-(1-7) peptide levels.

2. Fetal Programming and RAS Involvement

Fetal exposure to a programming event during a critical window in gestation is associated with augmented cardiovascular morbidities including increased mean arterial pressure and decreased baroreflex function for control of heart rate later in life. By understanding the mechanisms by which long term development is impaired, interventions may be designed to treat these diseases. It is well accepted that low birth weight is correlated with metabolic conditions such as diabetes mellitus, cardiovascular

disease, and hypertension (77). *In utero* stressors that result in impaired growth include hypoxia, deficiencies in vitamins or protein, caloric restriction, or excess glucocorticoids (11, 32).

The development of these chronic diseases cannot be accounted for by genomic heridability alone; accordingly, epigenetic regulation has emerged as a potentially important regulator of expression patterns in genes in response to cellular insults during a critical developmental period (49). Mechanisms of epigenetic regulation include DNA methylation, post-translational histone modification, and microRNA-mediated repression and activation (77). Numerous studies have uncovered a role for epigenetic modifications in models of fetal programming (4, 75, 83). These data suggest DNA methylation may be an important regulator of phenotype expression in response to adverse *in utero* environments. Further evidence for the role of epigenetics in fetal programming comes from multigenerational studies. Drake and colleagues demonstrate altered methylation patterns in imprinted genes from an F2 generation whose parents were exposed *in utero* to the glucocorticoid dexamethasone (26, 27). While not all fetal insults induce epigenetic changes, epigenetic regulation is certainly one mechanism by which fetal programming may occur.

In addition to epigenetic modifications, expression of the glucocorticoid receptor may directly contribute to fetal programming events. The glucocorticoid receptor is ubiquitously expressed and has pleiotropic effects involved in the stress response, metabolism, inflammation, and development. After ligand binding, the glucocorticoid receptor translocates to the nucleus and exerts actions either through direct binding to DNA or by protein-protein interactions with other transcription factors (48, 60). Fetal

overexposure to glucocorticoids, in conditions such as maternal stress or adminstration of antenatal BM, alters glucocorticoid receptor expression in organs including the brain and liver (46, 54). Increased expression of the receptor may occur due to decreased fetal cortisol production and downregulation of the endogenous hypothalamic-pituitary-adrenal (HPA) axis. Interestingly, glucocorticoid receptor expression is under epigenetic regulation. Maternal protein restriction during gestation leads to decreased methylation of the glucocorticoid receptor promotor, resulting in higher expression of the receptor, an augmented stress response, and altered glucose utilization (47).

The RAS is another hormone system susceptible to fetal programming induced alterations. As hypertension is often a long term consequence of fetal programming events, the RAS is a clearly one target of these alterations. In an early study, Langley-Evans and colleagues demonstrated an imbalance in the RAS of rats exposed to a low protein diet *in utero* (44). Treatment with captopril, an ACE inhibitor, had no effect on the blood pressure of animals exposed to a normal protein diet. However, captopril treatment significantly lowered blood pressure of hypertensive low-protein exposed rats (44). The ACE inhibitor may act by decreasing circulating Ang II and increasing Ang-(1-7) peptide levels (19). In the sheep model, organ specific RASs are identified as sites of programming. The kidney is the key regulator of long term blood pressure control and contains a local tissue RAS. Administration of antenatal glucocorticoids during late gestation coincides with a key point in kidney development and significantly reduces nephron number by approximately 25% (71, 80). While reduced nephron number does not necessarily lead to hypertension (15), additional alterations in glucocorticoid exposed sheep kidneys are observed, including upregulation of angiotensinogen and increased

expression of Ang II receptors (80). Decreased nephron number, together with higher angiotensinogen or AT_1 receptor expression, contributes to the development of hypertension in these animals.

Recent studies in our laboratory focus on understanding the contribution of organ specific RAS components on blood pressure and baroreflex function in BMX and control sheep. Gwathmey and colleagues found higher expression of AT_1 receptors and lower expression of AT_2 and Mas receptors in plasma and nuclear membranes isolated from the renal cortex of BMX sheep compared to controls (35). Shaltout et al. reported BMX sheep exhibit lower ACE2 activity and expression in the circulation. Indeed, the ratio of serum ACE to ACE2 activity positively correlated to blood pressure in control and BMX sheep.

RAS components are also localized in brain areas that regulate sympathetic and parasympathetic outflow for control of autonomic function and may be a target of fetal programming events (24). AT_1 receptors are densely expressed within the solitary tract nucleus, a region known to modulate baroreflex function. While Ang II stimulation of the AT_1 receptor inhibits baroreflex sensitivity, Ang-(1-7) enhances sensitivity at the level of the solitary tract nucleus. Shaltout and colleagues found a decreased role for Ang-(1-7) in the control of baroreflex sensitivity in the solitary tract nucleus of BMX sheep (68). Alterations in the balance of Ang II and Ang-(1-7) may contribute to the loss of baroreflex function in this model of fetal programming. In Chapter Two, we hypothesized that alterations in angiotensin receptors may contribute to altered solitary tract nucleus regulation of baroreflex control and blood pressure. While we do not find increased expression of the AT_1 receptor in BMX sheep using protein immunoblot

analysis, Mas receptor expression is significantly lower in BMX animals. Additionally, Ang-(1-7) peptide levels correlate with Mas receptor protein expression (50). Thus, we hypothesize that low levels of Ang-(1-7) may decrease Mas protein expression while high levels may upregulate receptor expression. Conversely, we find no change in AT_1 receptor expression but a trend towards an inverse correlation between Ang II peptide levels and receptor expression. This is consistent with previous reports that high levels of Ang II reduce expression of the AT_1 receptor (13, 62). These findings are also consistent with the observations of Shaltout et al., in which microinjection of the AT_1 receptor antagonist losartan enhances baroreflex function to an equal degree in control and BMX animals, but microinjection of the Mas receptor antagonist A779 depresses baroreflex function only in control animals (68). Because we find a positive relationship between Ang-(1-7) levels and Mas receptor expression, pharmacological therapies that increase Ang-(1-7) including ACE inhibitors or direct administration of the peptide may be important in restoring Mas receptor expression to control levels following BM exposure. To that end, higher Ang-(1-7) and Mas receptor levels could contribute to higher parasympathetic nervous system activity and help restore baroreflex function to normal levels. Future studies focus on processing enzymes involved in regulating levels of angiotensin peptides in and around the dorsal brainstem.

3. Localization and Specificity of ChP RAS Alterations

Under normal conditions, the ChP modulates the transport of specific ions and molecules from the blood into the CSF. The protein content of CSF is approximately 0.4% of that in plasma, providing evidence for the selectivity of ChP transporters. In certain models of hypertension, such as the Dahl salt-sensitive rat, increased activity of

sodium transporters in the ChP contributes to higher concentrations of sodium in the CSF (5). Finkielman and colleagues found an Ang I-like pressor peptide in the CSF that was significantly elevated in patients with essential hypertension (30). Thus, it is possible that the ChP contributes to the development of hypertension by altering transport of ions and RAS components in the CSF. The data in our studies indicate that the ChP is not a site of programming-induced changes in the RAS. RAS peptide levels and processing enzyme activities were not different between BMX and control animals. This provides important insight into tissues that are vulnerable or protected against long term programming effects. While we do not investigate the mechanisms of programming, such as epigenetic modifications or altered expression of glucocorticoid receptors, certain tissues appear to be protected from these programming effects. It is possible that because the ChP is critical in regulating CSF content, this compartment is not susceptible to RAS related programming alterations.

The three major RAS peptidases (ACE, ACE2, and neprilysin) are localized to the BBM of the ChP. Other studies have reported ACE activity in the BBM, and detected protein for all three peptidases in the tissue (14, 59, 79); however, we established that ACE2 activity is significantly higher than both ACE and neprilysin. We propose that these peptidases are involved in CSF peptide metabolism due to their apical localization and the extracellular orientation of their active (processing) sites. RAS peptides are detectable in the CSF, but renin is reported to be either low or not present (64). It is therefore likely that a nearby tissue acts as a source of CSF peptides or processing enzymes. Analysis of CSF peptides revealed high peptide levels of Ang-(1-7), consistent with high ACE2 activity in the BBM of the ChP, and low levels of Ang I and Ang II.

Furthermore, Ang-(1-7) content is significantly lower in BMX animals as compared to controls. Indeed, previous findings reveal that BMX animals have decreased Ang-(1-7) tone at the peptide and receptor level in the brain (50, 69). The functional role of Ang-(1-7) in the CSF is currently unknown. It is possible that Ang-(1-7), moreso than Ang II, is involved in normal physiological homeostasis in the CSF. While we are not aware of any studies investigating Ang-(1-7) signaling in the CSF, high CSF Ang II is associated with pathological conditions such as multiple sclerosis and Alzheimer's Disease (22, 42, 84). The elevated Ang II CSF levels in these conditions may stimulate AT_1 receptors in the brain tissue and activate NADPH oxidase production of reactive oxygen species and NF-κB signaling pathways (40, 82). Under nonpathological conditions, basal levels of Ang-(1-7) may be higher than Ang II to counterbalance AT_1 receptor signaling. Further studies must be conducted to investigate the localization of AT_1 and Mas receptors on the ChP. High Mas receptor expression on the ChP BBM would suggest Ang-(1-7) signaling from the CSF. On the other hand, localization of the angiotensin receptors to the basolateral membrane of the ChP would suggest receptor stimulation from blood-borne angiotensin peptides. While we did not investigate the functional actions of Ang-(1-7) in the CSF compartment, production and metabolism pathways of the peptide were characterized.

4. Ang-(1-7) Metabolism in the CSF

Unlike the ChP, we find clear differences in CSF RAS components between control and BMX sheep. Ang-(1-7) levels are significantly lower in BMX sheep. Since this is not due to differential production of Ang-(1-7) by ACE2 in the ChP, we investigate the potential for differential metabolism of the peptide within the CSF. While numerous

peptidases are known to form Ang-(1-7) from Ang I [neprilysin, prolyl endopeptidase, thimet oligopeptidase, and neurolysin] or Ang II [ACE2, prolyl endopeptidase, prolyl oligopeptidase] less is known about Ang-(1-7) metabolism (3, 19, 57, 78). Allred and colleagues demonstrate ACE and neprilysin cleavage of Ang-(1-7) at the Ile^5-His^6 and Tyr^4-Ile^5 bonds in renal and pulmonary tissues, respectively (3). Indeed, we find that ACE contributes to a portion of CSF Ang-(1-7) metabolism. However, the majority of Ang-(1-7) metabolism occurs through cleavage at the Tyr^4-Ile^5 bond to form Ang-(1-4) and is due to a peptidase sensitive to the mercuri-compound PCMB. Peptidase activity is increased in BMX animals, and correlates negatively with both Ang-(1-7) peptide levels and mean arterial pressure in the sheep. The findings in Chapter Three provide evidence for fetal programming alterations of the CSF RAS, as well as an initial characterization on a novel Ang-(1-7) peptidase in the CSF.

Chapter Four further characterizes this Ang-(1-7) peptidase in the CSF of control and BMX animals. From these data, we conclude the enzyme activity is a metallopeptidase based on its sensitivity to chelating agents. We did not identify the divalent cation responsible for peptidase activity; however, zinc is the most common metal ion coordinated to metallopeptidases, and is the cation required for ACE, ACE2, and neprilysin activity (73). Future studies can determine the identity of the divalent cation by chelating the metal ion with EDTA or o-phenanthroline and adding increasing concentrations of zinc, cobalt, magnesium, or mercury. The metal that restores peptidase activity at the lowest concentration is likely the cation required for activity (58). An alternative approach uses the inclusion of different divalent cations directly to the

peptidase activity. The metal that inhibits activity at the lowest concentration likely represents the cation necessary for peptidase activity.

Selective inhibitors against the metallopeptidases neprilysin, thimet oligopeptidase, neurolysin, and ACE did not block the peptidase activity. Additionally, the prototypic cysteine protease inhibitors E-64 and leupeptin did not attenuate the Ang-(1-7) peptidase, suggesting the enzyme does not belong to the class of cysteine proteases that include cathepsins. Furthermore, we investigated the possibility that this peptidase is involved in lysosomal degradation by establishing the pH profile of optimal activity. Cathepsins are ubiquitous proteases involved in intracellular protein degradation and exhibit acidic optimal pHs due to their lysosomal localization. Indeed, the serine peptidase cathepsin G is involved in angiotensin metabolism that may contribute to the hydrolysis of Ang II following internalization of the Ang II-AT_1 receptor complex in endosomes (74). However, the pH profile revealed a physiological optimal pH of 7.5. This, together with the fact that this peptidase is soluble, suggests a role for the peptidase to selectively reduce Ang-(1-7) signaling rather than as a general metabolism pathway.

Substrate specificity was assessed using non-radiolabeled Ang-(1-7) analogues to compete with radiolabeled Ang-(1-7) for peptidase activity. Dose response curves were constructed, and the concentration of cold peptide required to inhibit half the conversion of radiolabeled Ang-(1-7) to Ang-(1-4), or the IC_{50}, was calculated. Interestingly the amino-terminal analogues of Ang-(1-7), Ang-(2-7) and [Ala^1]-Ang-(1-7), as well as the carboxy-terminal analogues, Ang II, [D-Pro^7]-Ang-(1-7) and [D-Ala^7]-Ang-(1-7) (A779), inhibit the reaction at similar concentrations to Ang-(1-7). In addition to being an amino-terminal analogue of Ang-(1-7), [Ala^1]-Ang-(1-7) was recently shown to be an

endogenous peptide that acts on a Mas related G protein coupled receptor with actions similar to Ang-(1-7) (45). This peptide may represent another endogenous substrate for the peptidase. However, the non-angiotensin brain peptides neurotensin, apelin-13 and bradykinin did not inhibit the reaction. While these data provide evidence that the peptidase recognizes unlabeled Ang-(1-7) and its analogues, it does not indicate whether these peptides are utilized as substrates for the peptidase or simply act as inhibitors. To establish if the peptides are substrates or inhibitors, further studies using ^{125}I-labeled or unlabeled peptides coupled to HPLC separation and either gamma or ultra violet (UV) detection is necessary.

In the future, experiments can be done to determine the role of this peptidase in the CSF by conducting intracerebral ventricular infusions of Ang-(1-7) in BMX animals. Following intracerebral ventricular infusion of Ang-(1-7), a normalization of blood pressure or baroreflex sensitivity would indicate a physiologically relevant role for reduced CSF Ang-(1-7) and highlight the importance of low Ang-(1-7) in the CSF of BMX animals. If the dorsal medullary Mas receptor expression increased following infusion of Ang-(1-7), it is likely that CSF peptides act on receptors located in brain tissue. A separate study could use specific inhibitors against ACE and the CSF peptidase to augment Ang-(1-7) peptide levels in BMX animals back to control values. Any resulting changes in blood pressure, autonomic function, or inflammatory markers would be the result of higher CSF Ang-(1-7). Experiments involving chronic intracerebroventricular infusion of Ang-(1-7) or peptidase inhibitors into the CSF of BMX sheep would reveal the importance of CSF Ang-(1-7) levels. Any functional changes induced by central infusion of Ang-(1-7) should be blocked with coinfusion of

Ang-(1-7) and the Mas receptor antagonist A779. Central A779 infusion in control and BMX animals would assess endogenous Ang-(1-7) tone. Shaltout et al. report BMX animals have redued Ang-(1-7) tone in the solitary tract nucleus by measuring changes in baroreflex sensitivity following microinjection of A779 (68). Similarly, central infusion of A779 may depress functional responses more in control animals, indicating BMX animals have reduced tone.

Overall, Chapters Three and Four identify and characterize a potentially novel Ang-(1-7) metabolizing peptidase in the CSF. As the CSF is in constant contact with brain tissue and the apical membrane of the ChP, it is likely that the peptidase originated from one of these two sources. We proceeded to investigate the brain medulla as a possible source of peptidase activity.

5. Brain Medullary Ang-(1-7) Peptidase Activity

Guminski studied several glycolytic enzymatic activities in the brain, CSF, and blood and established that the origin of the CSF enzymes was exclusively from cerebral tissue cells (33). To test the hypothesis that the brain medulla is the source of a CSF Ang-(1-7) peptidase, we initially assayed a soluble portion of the brain medulla for Ang-(1-7) metabolizing activities. Indeed, an Ang-(1-7) metabolizing peptidase is present in medullary tissue and exhibits a six-fold higher rate of metabolism than CSF (495 vs 84 pmol/min/mg). We then employed three chromatography steps to enrich the peptidase activity while removing other proteins and peptidases from the soluble preparation. Due to the identical inhibitor and pH profiles, and similar apparent Kms for Ang I, Ang II, and Ang-(1-7) we conclude that an identical peptidase is present in the CSF and medulla.

Using the peptidase purified from medullary tissue, the metallopeptidase agent JMV-390 was tested as an inhibitor of Ang-(1-7) hydrolysis. Originally developed as inhibitor against peptidases that metabolize neurotensin, it was designed to endogenously preserve the analgesic effects of the peptide (25). As a result, JMV-390 is extremely potent and inhibits sensitive peptidases at nM concentrations. Accordingly, JMV-390 inhibits the metabolism of neurotensin by thimet oligopeptidase hydrolysis of the Arg^{8}-Arg^{9} bond (K_i of 31 nM), neurolysin cleavage at the Pro^{10}-Tyr^{11} bond (K_i of 58 nM) and neprilysin hydrolysis of both the Pro^{10}-Tyr^{11} and Tyr^{11}-Ile^{12} bonds (K_i of 40 nM) (25). Ingram and colleagues subsequently reported sensitivity of TNF-alpha converting enzyme (TACE) to JMV-390 (K_i of 65 nM) (37). We find the medullary Ang-(1-7) peptidase sensitive to JMV-390, but at a concentration far below those characterized by Doulut for other metallopeptidases (K_i of 0.8 nM) (25). Sensitivity to JMV-390 at a subnanomolar range not only indicates its ability to be used as an endogenous inhibitor, but clearly distinguishes JMV-390's actions on the Ang-(1-7) peptidase from neurolysin, thimet oligopeptidase, and neprilysin. Chronic intracerebroventricular administration of JMV-390 may augment CSF Ang-(1-7) levels and reverse some of the long term functional alterations associated with this model of fetal programming.

Further studies regarding peptidase sensitivity attest to the specificity of the peptidase for Ang-(1-7). While radiolabeled kinetic analysis yielded Ang II metabolism at approximately half the rate of Ang-(1-7), UV HPLC revealed very little production of Ang-(1-4) from Ang II compared to that from Ang-(1-7) (4.9 vs 54 nmol/min/mg). This discrepancy between radiolabeled and nonradiolabeled HPLC detection may be due to the location of the radiolabeled iodine. Because the peptidase cleaves at the Tyr^{4}-Ile^{5}

bond, and the hydroxy group of Tyr^4 is substituted with ^{125}I, it is possible that this alteration enhances the hydrolysis of this bond in Ang II, thus conveying better catalytic properties of the peptide. Apart from the influence of the ^{125}I-Tyr group, the reaction velocity was markedly reduced for peptides that are either shorter or longer than the Ang-(1-7) sequence. Notably, the seven amino acid peptide bradykinin-(1-7) and the Tyr-Ile containing peptide neurotensin are not cleaved by the peptidase after 24 hours of incubation. An early study by Schechter and Berger emphasized the size limitations of peptidase active sites (63). Based on our characterization of the peptidase, we hypothesize the active site of the Ang-(1-7) peptidase may be optimal for peptides of seven amino acids. In order to further test active site specificity, kinetic analysis should be performed using unlabeled Ang-(1-7) analogues in which animo acids from the sequence are substituted with similar or different amino acids. Of particular importance is the Tyr^4-Ile^5 bond, substitution of Try^4 or Ile^5 to their D-conformation stereoisomers or non-hydrophobic residues would provide important information on the selectivity of the enzyme's active site.

Chapter Five identifies the medulla as a rich source of peptidase activity, describes the enrichment of the activity approximately 2,000-fold, and characterizes the specificity and purity of the peptidase; however, we do not investigate BM induced alterations in enzyme activity. Peptidase activity is approximately two-fold higher in the CSF of BMX animals compared to controls (31.98 ± 1.5 vs 14.22 ± 1.0 fmol/min/mg). Because we propose the medulla is a source of CSF peptidase activity, the medulla of BMX animals must release a greater amount of peptidase than control animals. Additional studies are

necessary to test whether the medullary content or release of the peptidase is altered in BMX exposed tissues.

6. Subcellular Localization and Peptidase Release

Numerous proteases and peptidases are released from tissues into circulation. Transmembrane proteins such as ACE, ACE2, and the Alzheimer's disease amyloid precursor protein (APP) undergo proteolytic shedding at the juxtamembrane stalk by constitutively active metalloproteases (2, 43). While proteases and peptidases frequently contain a membrane-spanning domain for localization and regulation, there are notable exceptions. Thimet oligopeptidase, was purified in 1989 from rat testis and the majority of enzyme activity (>90%) is found in a soluble fraction (55). Examination of the cDNA encoding thimet oligopeptidase revealed a lack of a signal sequence or membrane-spanning domain, indicating the enzyme is unlikely to act as a membrane-bound ectoenzyme (2). However, thimet oligopeptidase is known to associate with plasma membrane lipid rafts and undergo basal and inducible secretion into the extracellular space (28, 38). Unlike thimet oligopeptidase, neurolysin is predominantly membrane bound. Checler and colleagues originally purified neurolysin from rat synaptic membranes, and later identified the peptidase in the rat ileum and kidney (10, 21). Additional studies find 10-20% of neurolysin activity associated with membrane fractions in rat and pig brain and kidney (9, 20), and several studies identify localization and targeting of the peptidase to the mitochondria (41). Immunocytochemical studies confirm cytoplasmic and membrane associated neurotensin in rat neurons (31, 81).

The peptidase identified and characterized in Chapters Three, Four, and Five appears to be a soluble peptidase with no membrane localization or association. In the CSF, the peptidase is clearly soluble. However, peptidases such as ACE and ACE2 are found in the CSF as a result of proteolytic cleavage from brain tissue (33, 65). Differential centrifugation of brain tissue indicates the peptidase is soluble, as activity is recovered in the soluble supernatant (100,000 *x* g centrifugation) but not in fractions including nuclei, plasma membranes, mitochondria, or synaptic vesicles. We did not confirm the localization of the peptidase by checking the purity of each fraction using markers of nuclei, mitochondria, and other organelles. However, this can be investigated in the future. If we are able to identify the peptidase through sequence homology to a known protein, an antibody against that protein can be obtained to confirm subcellular localization as well as the cell type in the brain. This will be of particular importance, because at this point we are unable to definitively identify the peptidase as intracellular or extracellular. Thimet oligopeptidase is an example of a soluble peptidase with both intracellular and extracellular localization that localizes to lipid rafts on neuronal plasma membranes and undergoes basal and stimulated release into the extracellular space (28, 38). Importantly, these studies provide evidence for regulated secretion of a soluble peptidase from the intracellular to extracellular space. Indeed, this may constitute one mechanism for the release or secretion of the Ang-(1-7) peptidase into the CSF compartment.

In order to study stimulated and basal release of this peptidase into the extracellular space, future studies could isolate synaptosomes from dorsal medullary tissue and monitor release of the peptidase into the media. Agents such as corticotropin-releasing

hormone or a calcium ionophore are reported to induce peptidase release from the intracellular space (28). Induction and inhibition of peptidase release by these agents would facilitate our understanding of the mechanisms that regulate peptidase activity. Recently, Gwathmey and colleagues reviewed the intracellular localization of the RAS (34). Renal nuclei isolated from proximal tubule cells contain many components of the RAS including ACE, ACE2, neprilysin, Ang-(1-7) and the Mas receptor (36). Nuclear Mas receptors are functionally linked to nitric oxide formation, and are sensitive to Ang-(1-7) stimulation (36). The intracellular localization of the Ang-(1-7) peptidase would contribute to deactivation of Ang-(1-7) within the cell and may influence nuclear signaling and the nitric oxide response (34, 53). Extracellular localization or secretion may indicate actions of the peptidase on extracellular Ang-(1-7) peptide levels and consequent signaling through the Mas receptor. It is possible that products of Ang-(1-7) metabolism may have some functional actions distinct from Ang-(1-7); however, it is generally considered that Ang-(1-4) is biologically inactive.

7. General Limitations of Studies

Animals

The studies described in this dissertation are performed on frozen tissue collected from adult sheep. The sheep are of mixed breed background, and exhibit genetic diversity. Unlike genetically identical strains of rats or mice typically used in experimental studies, the sheep have an intrinsic genetic diversity, possibly contributing to a certain level of RAS variability. We were limited by the availability of fresh tissue, and accordingly utilized tissue stored in optimal cryosectioning media at -80°C. In

general, frozen tissues are an accepted source to quantify expression of angiotensin peptides and receptors (12, 39). Frozen tissues were selected with regard to treatment group, sex of the animal, and year of sacrifice. We considered the amount of time each tissue was maintained at -80°C to ensure a similar period of storage time among the animals. Tissues collected more than eight years prior were not used in these studies. Frozen tissues were also used to quantify enzyme activity and purify the Ang-(1-7) peptidase; once the tissue was thawed it was kept on ice to prevent enzyme degradation or conformational changes induced by multiple freeze-thaw cycles.

A second limitation or consideration of this study is the use of only male offspring. Females are typically thought to be protected against cardiovascular pathologies because of the beneficial role of estradiol. Numerous studies have investigated the role of estrogen on the ACE-Ang II-AT_1 receptor axis and report an inhibitory effect on Ang II and AT_1 receptor expression (7, 16). In regard to tissue expression of Ang II and Ang-(1-7), Pendergrass and colleagues reported Ang II expression was two-fold higher and Ang-(1-7) expression three-fold lower in the kidneys of hypertensive mRen2.Lewis males compared to female rats (56). The hypertensive male mRen2.Lewis rats exhibit higher blood pressure and a greater degree of renal injury than their female littermates. In the sheep model of BM induced fetal programming, sex differences are also apparent. Acute infusion of Ang-(1-7) induces a greater natriuretic response in BMX females than males (72). The mechanism for the sex-dependent effects of Ang-(1-7) on sodium excretion in BMX animals is not known; however, differences in Mas receptor expression and signaling may contribute to these differences. Because we did not investigate female offspring, we are not able to speculate on sex differences in the central RAS following

BM exposure. Hormone levels may differ between female sheep and differentially influence RAS components, thus, making comparison between individuals difficult to interpret (61). For these reasons we focused only on male offspring in this study. However, the possibility of sex differences in BMX offspring remains to be investigated.

Enzyme Kinetics

While the use of HPLC to measure peptide metabolism is both sensitive and accurate, there are two major considerations with regard to enzyme kinetics. The first consideration involves the assumption that the addition of an ^{125}I-tag does not alter the enzyme-peptide interaction. HPLC coupled to a flow through gamma counter to measure radioactivity is an extremely sensitive method for separating and quantifying radiolabeled peptides (18). For the current studies, an angiotensin peptide labeled with ^{125}I on Tyr^4 is exposed to a peptidase, separated using HPLC, and the peptide products labeled with ^{125}I-Tyr are detected using a gamma counter. This method effectively focuses the study of metabolism on the ^{125}I-angiotensin peptide even though many other endogenous peptides may be present, particulary in crude samples preparations. Accordingly, the derivation of the kinetic constants is based on the assumption that the labeled and unlabeled peptide substrates are equally recognized and hydrolyzed by the enzyme. This may not be the case for peptides other than Ang-(1-7) since we noted a marked difference in the maximal velocity for ^{125}I-Ang II and ^{125}I-Ang I as compared to the maximal rate for the unlabeled peptides.

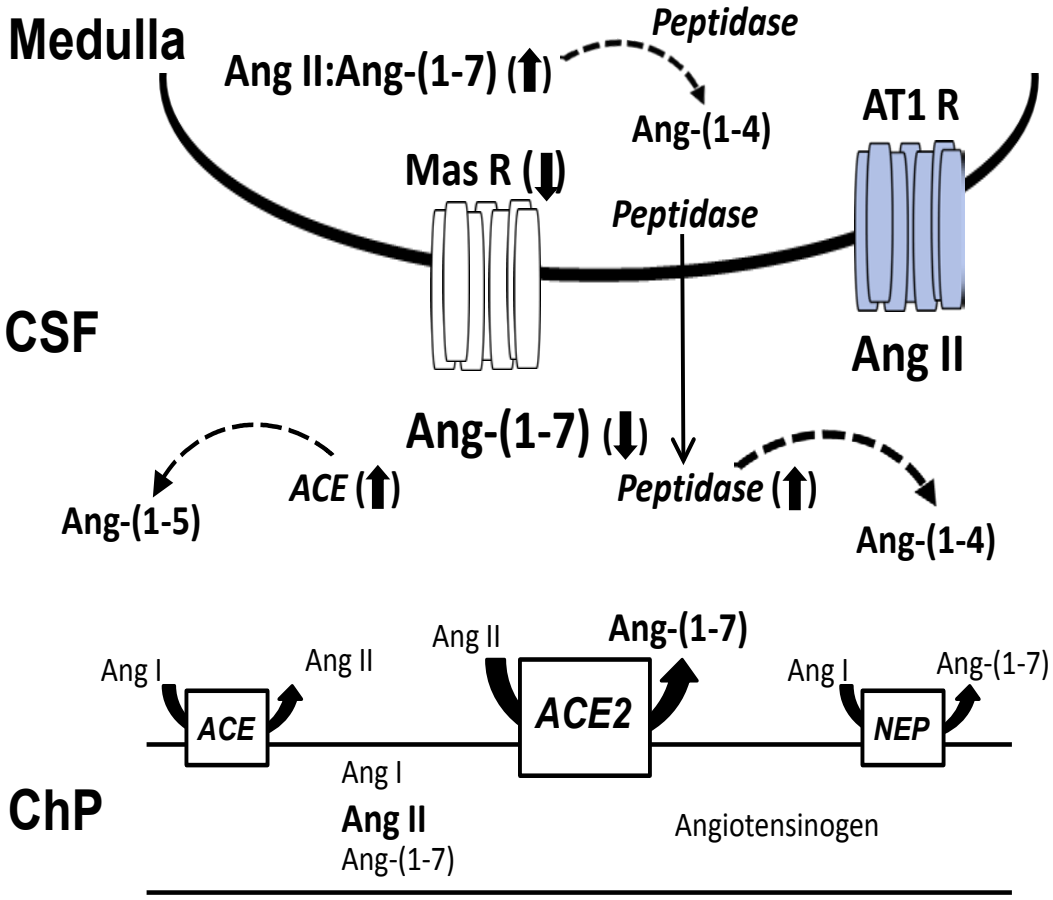
The second consideration is that true kinetic constants are calculated using pure enzyme, where the entirety of the protein in each sample is from the enzyme (19). The

preparations used in these experiments use CSF or soluble brain medulla enriched for the peptidase. Only a small portion of the protein in these samples corresponds to pure peptidase, and as a result the maximal velocity is greatly underestimated. Although our calculations of Km and Vmax are apparent, we are able to directly compare the derived kinetic constants in order to identify ideal substrates under identical assay conditions.

Similarly, HPLC coupled to a UV detector has both advantages and disadvantages. The UV HPLC does not require radiolabeled peptides, and therefore eliminates the need to assume equal enzyme affinity and catalytic properties between radiolabeled and nonradiolabeled peptides. Physiological samples are directly separated and detected using UV-HPLC and all products are detected, provided there are sufficient amounts of the products (approximately 0.1 to 1 nmol). To its disadvantage, UV-HPLC is approximately 10^5 to 10^6-fold less sensitive than HPLC coupled to a gamma detector. Moreover, peptide products exhibit different sensitivities by UV analyses that reflect the peptide size and the number of side chains for each residue that will absorb light. Therefore, each peptide product must be compared to its standard peptide to quantify the amount of product formed. This is not the case with ^{125}I-labeled peptides, since each peptide exhibits the same amount of the ^{125}I-Tyr. However, peptide fragments that no longer contain the ^{125}I-Tyr will be not be detected by the HPLC-gamma detector. For UV-HPLC analysis, larger amounts of sample must be applied to the HPLC and the ensuing chromatographs may be too complex to analyze given the potential number of interfering substances that absorb in the UV ranges. This is not an issue with the use of radiolabeled substrate since only the products with the ^{125}I-tag are detected.

8. Concluding Statements

Adminstration of synthetic glucocorticoids to a woman entering into preterm labor has immediate positive outcomes including decreased morbidity and mortality of the fetus (1, 8). However, animal studies suggest deleterious cardiovascular consequences in offspring exposed *in utero* to glucocorticoids in the form of epigenetic changes, increased glucocorticoid receptor expression, altered autonomic function, and changes in circulating and organ specific RAS components (23, 26, 50-52, 66, 69, 70). The findings presented in this thesis support the hypothesis that the central RAS is altered following fetal exposure to BM. Moreover, recent studies suggest that hypertension and cardiovascular dysfunction are associated with alterations in Ang-(1-7) tone both in the brain and periphery. Ang-(1-7) tone is important in the solitary tract nucleus for baroreflex function (6, 69). In fetal programming, we find Ang-(1-7) peptides, receptors, and metabolism enzymes altered in the central nervous system contributing to reduced Ang-(1-7) tone (50-52). Our findings are summarized on the following page.



Although the exact mechanism underlying the development of increased blood pressure and decreased autonomic function in offspring exposed *in utero* to BM is unknown, data in this dissertation provide evidence for the early involvement of the central RAS. Decreased levels of Ang-(1-7) contribute to autonomic imbalance, oxidative stress, inflammation, and angiogenesis (17). We identify the central nervous system, particularly the CSF, as a site of BM induced RAS imbalance. Future studies investigating the supplementation or preservation of Ang-(1-7) in the CSF may help correct clinically relevant pathologies in offspring exposed to BM.

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2014	Co-chair of symposium "Fetal Programming of Adult Cardiovascular Disease," Experimental Biology
2014	First place in Integrative Systems, Translational and Clinical Pharmacology Division Young Investigator Oral Session, Experimental Biology

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Cardiovascular Division Trainee Committee

American Heart Association
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MANUSCRIPTS

Marshall AC, Shaltout HA, Nautiyal M, Rose JC, Chappell MC, Diz DI. Fetal betamethasone exposure attenuates angiotensin-(1-7)-Mas receptor expression in the dorsal medulla of adult sheep. *Peptides*. 2013; 44: 25-31. PMID 23538211

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