TARGETING THE ACE2/ANG-(1-7)/MAS AXIS FOR CEREBROPROTECTION DURING ISCHEMIC STROKE

By

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To Marcia

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Recent progress in cardiovascular therapy suggests that stimulation of Angiotensin Converting Enzyme 2 (ACE2), production of Angiotensin-(1-7) [Ang-(1-7)], and activation of the Ang-(1-7) receptor, Mas, are viable targets for disease prevention and treatment. The ACE2/Ang-(1-7)/Mas axis has been shown to counteract many of the physiological effects of the Angiotensin II (Ang II) Type 1 Receptor (AT1R), including vasoconstrictor and proliferative actions. In addition, activation of the ACE2/Ang-(1-7)/Mas axis also attenuates many of the pathophysiological states that involve increased production of Ang II by Angiotensin Converting Enzyme (ACE), and subsequent activation of the AT1R (ACE/Ang II/AT1R axis). For example, many studies targeting the ACE2/Ang-(1-7)/Mas axis have revealed its broad therapeutic potential for the treatment of hypertension, hypertension-related pathology, myocardial infarction, and heart failure. Furthermore, ACE2 can form endogenous Ang-(1-7) from Ang II and has recently been a target for cardiovascular disease therapy. In fact, several small molecule ACE2 activators have been identified that selectively increase ACE2 activity without having an effect on ACE activity. One of these molecules, Diminazene

aceturate (DIZE) has been shown to decrease blood pressure dramatically and dose dependently when administered acutely. In addition, a modest decrease in blood pressure and associated reductions in end organ damage are observed with chronic administration of DIZE to spontaneously hypertensive rats. Therefore, small molecule ACE2 activators are promising compounds for ACE2/Ang-(1-7)/Mas axis activation and treatment of cardiovascular disease. Based on previous studies indicating the presence of ACE2 and Mas in the brain as well as a protective role for ACE2/Ang-(1-7)/Mas axis during cardiovascular disease, we have developed the general hypothesis that activation of the brain ACE2/Ang-(1-7)/Mas axis will have cerebroprotective action during ischemic stroke.

Specific Aim 1 was designed to evaluate the endothelin-1 (ET-1) induced middle cerebral artery occlusion (MCAO) model for investigations of the rennin angiotensin system (RAS) during stroke. Following ET-1 induced MCAO, rats had significant neurological impairment that correlated with the size of infarction. Systemic pre-treatment with AT1R blocker (ARB), candesartan, for 7 days attenuated both the infarct size and neurological deficits caused by ET-1 induced MCAO without altering blood pressure. The effect of candesartan pre-treatment on ET-1 induced vasoconstriction of the MCA was also evaluated by visualization of the middle cerebral artery (MCA) through a cranial window. It was determined that candesartan pre-treatment did not alter ET-1 induced constriction of the MCA, which validates the use of this stroke model during ARB pharmacotherapy. This study solidifies the cerebroprotective properties of ARBs during ischemic stroke and validates the ET-1 induced MCAO model for examination of the brain RAS's role in ischemic stroke.

Specific Aim 2 was designed to test whether central administration of Ang-(1-7) *via* lateral ventricular cannula would provide cerebroprotection during ET-1 induced MCAO. Sprague Dawley rats were treated *via* the intracerebroventricular route with Ang-(1-7) or artificial cerebrospinal fluid (aCSF) prior to ET-1 induced MCAO. Ang-(1-7) treatment reduced the cerebral infarct size, neuronal damage and neurological deficits measured 72 h after MCAO induction. Ang-(1-7) treatment also reduced the neurological deficits produced by ET-1-induced ischemic stroke, as indicated by a battery of neurological tests including the Bederson Exam, Garcia Exam, and the sunflower seed eating task. These protective actions of Ang-(1-7) were reversed by blockade of the Ang-(1-7) receptor, Mas, with A-779. In addition, the effect of Ang-(1-7) pre-treatment on ET-1 induced vasoconstriction of the MCA was also evaluated by visualization of the MCA through a cranial window. It was determined that central Ang-(1-7) pre-treatment did not alter ET-1 induced constriction of the MCA, which validates the use of this stroke model during Ang-(1-7) pharmacotherapy. In order to investigate alterations in cerebral blood flow (CBF) as a mechanism of Ang-(1-7) induced cerebroprotection, we measured CBF in the penumbra during ET-1 induced MCAO. Ang-(1-7) did not affect the reduction of CBF in the penumbra which ruled out the possibility of a protective mechanism of Ang-(1-7) mediated through improved CBF during MCAO. This is the first demonstration of cerebroprotective properties of Ang-(1-7) during ischemic stroke.

Specific Aim 3 was designed to test whether central pre-treatment with DIZE will provide cerebroprotection in a rat model of ET-1 induced MCAO. Adult male Sprague Dawley Rats were pre-treated with intracerebroventricular DIZE or H_2O for 7 days prior

to ET-1 induced MCAO. DIZE treatment reduced neurological deficits and infarct size measured 72 h after MCAO induction. Additionally, neurological deficits were reduced in DIZE treated rats as determined by the Bederson Exam, Garcia Exam, and the sunflower seed eating task. Furthermore, the histological and neurological benefits of pre-treatment with DIZE were attenuated when DIZE was co-administered with the Ang-(1-7) receptor antagonist, A-779. In order to investigate alterations in CBF as a mechanism of DIZE induced cerebroprotection, we measured CBF in the penumbra during ET-1 induced MCAO. DIZE did not affect the reduction of CBF in the penumbra which ruled out the possibility of a protective mechanism of DIZE mediated through improved CBF during MCAO. This data indicates that central administration of DIZE prior to stroke is cerebroprotective and extends the known cardiovascular protective effects elicited by stimulation of the ACE2/Ang-(1-7/)Mas axis.

CHAPTER 1
THE RENIN ANGIOTENSYSTEM AND ITS ROLE IN ISCHEMIC STROKE

Stroke Epidemiology

There are two forms of stroke: ischemic stroke, where blood supply to the brain is restricted by a blocked vessel, and hemorrhagic stroke, which occurs when a blood vessel within the brain ruptures.[1] During stroke the lack of oxygen and nutrients, and/or sudden bleeding into the brain elicits neuronal death and a host of consequent behavioral and motor symptoms. Stroke is the third leading cause of death in the United States and is a major cause of serious, long-term disability.[2] Each year 795,000 people experience a primary or secondary stroke accounting for 1 of every 17 deaths. 610,000 are primary strokes and 185,000 are secondary strokes. Stroke prevalence in adults is 2.7% for men and 2.5% for women. Someone suffers a stroke every 45 seconds and someone dies from a stroke every 3 minutes.[2] The estimated cost attributed to treating stroke in 2009 was $68.9 billion. Risk factors for stroke include hypertension, atrial fibrillation, diabetes, smoking, advancing age, low levels of physical activity, dyslipidemias, and hormone replacement therapy. Women also have an increased risk of stroke during pregnancy and the postpartum period.[1] Patients who have a primary stroke or transient ischemic attack are also at an increase risk of having a recurrent stroke.[3] In addition to the patients who present with stroke symptoms, there are also a number of strokes discovered incidentally by brain imaging studies temporally dissociated from the ischemic event. In fact, the prevalence of silent cerebral infarction increases dramatically with age (Figure 1-1).[2] When applied to the 1998 United States population estimates, these statistics indicate that about 13 million people had a

prevalent silent stroke. This is clear evidence supporting investigations into both preventative and acute treatment strategies for stroke.

Of all reported strokes, 87% are ischemic, 10% are an intracerebral hemorrhage, and 3% are a subarachnoid hemorrhage. Thus, ischemic strokes are the most prevalent and are treated with attempts to restore cerebral blood flow to the effected vascular territory. This is most commonly attempted through the use of systemic administration of recombinant tissue Plasminogen Activator (rtPA). Despite the existence of rtPA treatment protocols, only between 4 and 8 percent of patients with a stroke are eligible for thrombolytic therapy.[2] This is due to the high risk of ischemic transformation and a limited window of time for rtPA treatment after stroke onset. In summary, stroke is a highly prevalent disease and there is a paucity of effective stroke prevention and acute treatment strategies. Thus, understanding the mechanisms that underlie stroke and the development of new therapeutic strategies are crucial.

Pathophysiology of Ischemic Stroke

Stroke etiology can be classified into several relatively homogenous categories and this has helped in studies evaluating stroke pathophysiology and treatment.[4] These classifications include cardioembolic stroke, large-artery atherosclerosis, small-artery occlusion, stroke of other determined causes, and stroke of undetermined causes.[5] Many of these strokes are thromboembolic and most affect the middle cerebral artery.[6] The majority of patients with an ischemic stroke have some degree of reperfusion either spontaneously or as a result of thrombolytic therapy.[6, 7] This sequence of occlusion and ischemia followed by reperfusion is associated with a cascade of pathological events. These events include cerebral hypoperfusion, excitotoxicity, oxidative stress, blood brain barrier dysfunction, microvascular injury, hemostatic activation, post-ischemic

inflammation, and cell death (Figure 1-2).[8] In areas of severe cerebral hypoperfusion, the cascade of ischemic pathology occurs quickly resulting in irreversible cell death of the ischemic core. In contrast an area of tissue on the periphery of the ischemic core undergoes a less dramatic reduction of blood flow and remains viable despite functional impairment. This region is called the ischemic penumbra and can be thought of as the front line in a battle for tissue survival. Flattening of EEG occurs in animals and humans associated with a specific decrease in cerebral blood flow indicating a loss of neuronal function. This loss of function in the absence of cell death correlates with the ischemic penumbra, whereas a more dramatic reduction in cerebral blood flow occurs in association with both EEG flattening and cell death in the ischemic core.[9-12] Therefore, depending on the duration and degree of ischemia, tissue in the ischemic penumbra can progress to irreversible injury, which leads to expansion of the ischemic core.[8]

Acute Ischemic Cell Death

Stroke leads to an energetic depletion and collapse of ionic gradients that causes swelling and disruption of cellular and organelle membranes. This process of cell death is known as necrosis and takes place primarily in the ischemic core. During stroke, the lack of oxygen and glucose delivery to the brain results in a rapid decrease in ATP concentration due to disruption of oxidative phosphorylation and continued consumption of ATP.[13, 14] In addition to this reduction in ATP, oxygen depletion results in electron leakage from the electron transport chain and production of reactive oxygen species.[15] Finally, glycolysis in the absence of oxidative phosphorylation leads to the accumulation of protons and lactate.[14] Together, these events lead to a cellular environment that consists of ATP depletion, acidification, and free radical formation. Depletion of ATP inhibits the Na+-K+ ATPase and leads to cell depolarization, as well as the release of

neurotransmitters, including glutamate, from neurons and astrocytes.[16-18] Released glutamate activates ionotropic glutamate receptors on neurons and astrocytes that results in further release of glutamate.[19] This positive feedback loop ultimately causes dramatic increases in intracellular calcium and ends in excitotoxic cell death. Terminal depolarization describes this deleterious cascade of events that is triggered in ischemic core after dramatic reductions in blood flow. Cells outside of the ischemic core in the penumbra have less severe reductions in blood flow and undergo transient ischemic depolarization. The transient depolarization of cells in the ischemic penumbra can ultimately evolve into terminal depolarization and expansion of the infarct.[20]

Programmed Cell Death

Alternatively, released neurotransmitters and oxidative stress can activate pathways of programmed cell death. Although, programmed cell death can occur in the ischemic core, it is the predominant type of cell death that occurs in the penumbra. Several programmed cell death pathways are activated in the ischemic penumbra during stroke.[21] Apoptosis is one of these pathways which consist of activation of cellular processes that require metabolic expenditure.[8] Activation of apoptotic cascades causes cell death with minimal inflammatory response. Cytochrome c release from the mitochondria and activation of apoptotic cascades ultimately activates caspase 3, which is a common effector molecule in apoptotic pathways. Parthanatos is another type of programmed cell death that is independent of caspase activity. During ischemic reperfusion injury, poly(ADP-ribose) polymerase-1 (PARP1) causes an increase in Poly(ADP-ribose) (PAR) polymer.[22] PAR polymer translocation from the nucleus to the cytoplasm and mitochondria leads to the release of apoptosis inducing factor (AIF) from mitochondria.[23] Poly(ADP-ribose) glycohydrolase (PARG) is involved in degrading PAR

polymer and therefore inhibits this system. In a mouse model of focal cerebral ischemia reperfusion injury, reduction of PARG caused an increase in infarct volume. In addition, over-expression of PARG decreased infarct volume in the same model.[24] Together, these studies provide evidence for the involvement Parthanatos during ischemic penumbral injury. In addition, there are several pathways that lead to AIF release from the mitochondria that may be independent of parthanatos. For example, calpain I, a calcium-dependent protease, can cleave an N-terminal fragment of AIF that causes its dissociation from HSP10 in the mitochondria. The involvement of calpain I and AIF in programmed cell death have been substantiated by experiments showing that both inhibition of calpain activity and knockdown of AIF are neuroprotective during cerebral ischemia.[24] Furthermore, cyclophilin A can interact with AIF after ischemic injury and facilitate AIF translocation to the nucleus.[25] Another process of programmed cell death is autophagy. Autophagy is a process that can be triggered by endoplasmic reticulum stress and oxidative stress. Normally, autophagy is involved in the turnover of organelles and proteins through a lysosomal pathway.[26] Autophagy-promoting proteins such as Beclin 1 and microtubule-associated protein 1 light chain 3 are upregulated in the ischemic penumbra following stroke and inhibition of these pathways can decrease cell death due to ischemia.[27-29]

Reperfusion Injury

Ultimately, reperfusion can occur after several hours of ischemia either spontaneously or due to thrombolytic therapy. Reperfusion injury refers to a paradoxical injury of tissue despite improved cerebral blood flow that can occur. Production of reactive oxygen species by the NADPH oxidase (NOX) may contribute to this injury.[15] In addition, reperfusion leads to proinflammatory cytokine release and

activation of inflammatory cascades. Breakdown of the blood brain barrier by matrix matalloproteinases, as well of microvascular injury can lead to brain swelling and hemorrhagic transformation.[8, 30] Despite these mechanisms of reperfusion injury, early reperfusion is associated with improved final outcomes compared to delayed reperfusion.[6]

Inflammation

Stroke is associated with proinflammatory cytokine release by astrocytes, microglia, smooth muscle cells, and endothelial cells.[8] These cytokines, including TNF-α, IL- 1β and IL-6, are associated with an increase in inducible nitric oxide synthase (iNOS), as well as early invasion of neutrophils and transmigration of adhesion molecules. Inflammatory interactions that occur at the blood-endothelium interface, involving cytokines, adhesion molecules, chemokines and leukocytes, are critical to the pathogenesis of tissue damage in cerebral infarction.[31] For example, after middle cerebral artery occlusion TNF-α, IL-1ß, IL-6 and iNOS, as well as phosphorylated ERK1/2 are increased in smooth muscle cells of the middle cerebral artery and in associated intracerebral microvessels. An inhibition of ERK phosphorlyation decreases the tissue damage, as well as the cytokine and iNOS production after cerebral ischemia.[32] Despite this evidence for inflammatory injury after stroke, there is also evidence that inflammation is a protective process that attempts to limit damage and restore tissue architecture by eliminating damaged cells and repairing extracellular matrix.[33]

Renin Angiotensin System Components

The Renin Angiotensin System (RAS) was initially discovered as a circulating endocrine system with the components produced in various organs and secreted into the blood.

For example, Renin was discovered when the injection of kidney homogenates were shown to increase blood pressure.[34] Later, it was discovered that Renin is an enzyme that converts Angiotensinogen (ATG) to Angiotensin I (Ang I) and that Ang I is converted to Angiotensin II (Ang II) by the enzyme Angiotensin Converting Enzyme (ACE) in the pulmonary endothelium.[35] It is now known that Ang II is one of the primary mediators of RAS activity and is involved in blood pressure (BP), fluid, and electrolyte homeostasis.[34, 36, 37] Later, it was discovered that several Ang II receptor types existed.[34, 38-40] The Ang II Type 1 Receptor (AT1R) mediates most of the classical physiological effects attributed to Ang II and the Ang II Type 2 Receptor primarily opposes AT1R action.[34, 41] The obvious physiological role of the RAS in BP regulation led to drugs which targeted this system for the treatment of hypertension. Currently, ACE inhibitors (ACEi) and AT1R blockers (ARBs) are among the first line drugs to treat hypertension.[42] More recently, the concept of the RAS has evolved from that of a circulating endocrine system to a more ubiquitous system consisting of both circulating endocrine and tissue specific paracrine signaling systems. Tissue specific RASs have been identified in the brain, heart, kidneys, pancreas, skin, intestines, and many other organs. [43]

In addition to the important physiological actions of Ang II in regulating fluid homeostasis and blood pressure, it is clear that this peptide is of primary importance in the promotion of cardiovascular diseases and stroke. In both the circulation and in specific organs, the disease promoting arm of the RAS is mediated by increased Ang II stimulation of AT1R to increase BP and promote organ damage. Interestingly, this AT1R arm is opposed by Ang II stimulation of AT2R, which counteracts many of the

deleterious actions of AT1R stimulation.[44] The cardiovascular protective arm of the RAS has expanded with the discovery of Angiotensin Converting Enzyme 2 (ACE2) in 2000 (Figure 1-3).[45, 46] ACE2 converts Ang II to Ang-(1-7) with high efficiency. Ang-(1-7) effects counteract many of the physiological and pathological processes of AT1R activation.[47] In 2003, the Ang-(1-7) receptor was discovered to be a G protein-coupled receptor, Mas, which was initially identified as an orphan protooncogene.[48]

Several pathways for Ang-(1-7) production exist. The pathways leading to Ang-(1-7) can be either ACE dependent or ACE independent. ACE dependent pathways include conversion of Ang I to Ang II by ACE and subsequent conversion of Ang II to Ang-(1-7) by ACE2.[45] In addition, ACE2 is capable of converting Ang I to Ang-(1-9) which can then be converted to Ang-(1-7) by ACE.[46] This second ACE dependent route involving Ang-(1-9) production as an intermediate step is likely less relevant because ACE2 is 400 times more efficient at catalyzing the conversion of Ang II to Ang-(1-7) than Ang I to Ang-(1-9).[49] In addition to the ACE dependent pathways for Ang-(1-7) production, various enzymes contribute to the production of Ang-(1-7) *via* ACE independent mechanisms. These enzymes include Neprilysin, proyl-endopeptidase, proyl-carboxypeptidase, Chymase, and Cathepsin A.[50-53] The contributions of ACE independent pathways likely become much more relevant during ACE inhibition. However, these enzymes lack specificity for angiotensin peptide metabolism and they are unlikely to be major contributors to Ang-(1-7) synthesis under physiological conditions. Although, multiple pathways leading to Ang-(1-7) production exist, it is clear that ACE2 is a key regulator of this angiotensin peptide.

The complexity of the RAS is increasing with the discovery of new components and pathways. For example, studies show that circulating Ang II levels do not decrease after chronic treatment with an ACEi. This phenomenon has been termed angiotensin escape and the enzyme Chymase is thought to contribute *via* is high efficiency for converting Ang I to Ang II.[54] Additionally, the pro(renin) receptor ((P)RR)was discovered in 2002 and is a receptor for both renin and its precursor prorenin. (P)RR binding to its ligand can increase prorenin activity and also directly activate an independent intracellular signaling cascade.[55] Finally, it is established that various other RAS components including Ang III and Ang IV, as well as the enzymes that produce them, aminopeptidase A and aminopeptidase N, elicit physiological and pathological actions.[56] Clearly, the numerous components of the RAS collectively provide a system of checks and balances during cardiovascular health and disease. These components and their pathways are, therefore, good targets for the treatment of cardiovascular disease.

Angiotensin-(1-7) Opposes AT1R Signaling

Activation of AT1R is known to contribute to the pathophysiology of many cardiovascular diseases including hypertension, cardiac hypertrophy, cardiac fibrosis, atherosclerosis, myocardial infarction, and many others.[47] Events mediated by Ang II stimulation of the AT1R include contraction growth, migration, endothelial dysfunction, expression of proinflammatory cytokines, and modification of extracellular matrix.[57] Mas activation counteracts many of the pathophysiological signaling events of AT1R stimulation (Figure 1-4).[58] Therefore, the details of signaling events after AT1R and Mas activation are important because of the interplay between respective signaling cascades.

AT1R Signaling

The AT1R is a seven transmembrane G protein coupled receptor that mediates most of the known physiological and pathological actions of Ang II.[59] Many signaling pathways are activated by AT1R stimulation. Broadly, these pathways include G protein coupled pathways and non-G protein coupled pathways. The G-protein coupled signaling events are mainly mediated by phospholipase C (PLC), phospholipase A_2 (PLA_2), and phospholipase D (PLD).[60, 61] Amongst the non G-protein coupled pathways are signaling through NOX, Mitogen-Activated Protein Kinases (MAPK), Src, JAK/STAT, FAK and Pyk2, as well as Receptor Tyrosine Kinases.[62]

G protein coupled signaling pathways

AT1R activation of PLC converts (Phosphatidylinositol 4,5-bisphosphate) PIP_2 into inositol-1,4,5-triphosphate (IP3) and diacylglyerol (DAG). IP_3 can bind to a receptor on the sarcoplasmic reticulum to increase intracellular Ca2+ which binds to calmodulin and activates myosin light chain kinase (MLCK). MLCK phosphorylates the myosin light chain to cause smooth muscle cell contraction.[63] DAG activates Protein Kinase C (PKC) which stimulates the Ras/Raf/MEK/ERK pathway leading to vasoconstriction and growth promoting effects.[64] Activation of PLD can cause hydrolysis of phosphatidylcholine (PC) to choline and phosphatidic acid (PA).[57] PA is converted to DAG that contributes to PKC activation. Finally, activation of PLA2 cleaves arachidonic acid (AA) from PC. Lipoxygenase (LO) and cyclooxygenase (COX) convert AA to leukotrienes (LT) and prostaglandins (PG) respectively.[62]

Non-G protein coupled signaling pathways

The above cascade of G protein mediated events is complex. In fact, as the pathways of AT1R signaling expand, it is becoming clear that the G-protein dependent

and independent mechanisms of singling are closely integrated. For example, Ang II stimulation of AT1R activates NOX *via* phosphorylation of Src/EGFR/PI3K/Rac-1 and PLD/PKC/p47phox. Generation of superoxide by NOX leads to the production of hydrogen peroxide and other reactive oxygen species. This activity is required for phosphorylation of p38MAPK, Akt/PKB, Src, EGFR and many other components of the AT1R signaling cascade.[65] In addition, superoxide generated by AT1R activation can inactivate NO.[66] These signaling events are known to be involved in a cell proliferation, endothelial dysfunction, inflammation, and atherosclerosis. MAPK signaling pathways are also involved in AT1R signaling. In particular ERK1/2, JNK, and p38MAPK contribute to cell differentiation, proliferation, migration, and fibrosis in vessel walls. In addition, nonreceptor tyrosine kinases are activated by AT1R stimulation. For example, c-Src is a tyrosine kinase that activates many downstream components such as Ras, JAK/STAT, PLC, and AP-1. JAK and STAT proteins dimerize after AT1R stimulation and translocate to the nucleus where they have transcriptional effects. Finally, Ang II is able to transactivate several receptor tyrosine kinases, EGFR and PDGFR, as well as inhibit insulin receptor signaling *via* AT1R activation.[62] Clearly the pathways involved in AT1R signal transduction are numerous and redundant. Therefore, it should not be surprising that the RAS signaling can regulate some of these pathways *via* alternate angiotensin receptors such as AT2R and Mas.

Mas Signaling

Like the AT1R and AT2R, the Ang-(1-7) receptor, Mas, is a 7 transmembrane domain G protein coupled receptor. Mas may antagonize the AT1R through direct dimerization and inhibition.[67, 68] Ang-(1-7) stimulation of Mas can inhibit AT1R mediated phosphorylation of p38MAPK, ERK1/2, and JNK.[69, 70] SHP-2 is activated by Ang-(1-7)

signaling and this molecule is involved in the disruption of c-Src, ERK1/2, and NOX activation by Ang II.[58] In addition, Mas activation causes endothelial nitric oxide synthase (eNOS) stimulation *via* the phosphatidylinositol 3-kinase (PI3K) /Akt pathway.[71] Mas stimulation also causes AA release and PGI_2 production, as well as potentiation of bradykinin signaling.[72-75]

Targeting the Renin Angiotensin System Axes for Stroke Therapy

Among the well-documented and modifiable risk factors for stroke, hypertension and the RAS have long been key targets for pharmacotherapy and life-style modifications. Most investigations into the involvement of the RAS in stroke pathophysiology have focused on classical RAS components. Numerous studies have shown that blockade of either ACE with ACE inhibitors (ACEi) or AT1Rs with AT1R blockers (ARBs) appears to decrease cardiovascular risk and improve stroke prevention in human trials, as well as decrease infarct size and ensuing neurological deficits in animal models of stroke.[76-78] The cerebroprotective effect of reducing activation of the ACE/Ang II/AT1R axis has been hypothesized to occur *via* either decreased activation of the AT1R or an unopposed activation of the AT2R.[79-84]

While investigations of the role of the RAS in stroke have focused on Ang II and its AT1R and AT2R mediated actions, other components of this system may have potential beneficial effects in stroke. Recent progress in cardiovascular therapy suggests that stimulation of ACE2, production of Ang-(1-7), and activation of the Ang-(1-7) receptor, Mas, are viable targets for disease prevention and treatment.[85] Activation of the ACE2/Ang-(1-7)/Mas axis has revealed broad therapeutic potential for the treatment of hypertension, hypertension-related pathology, myocardial infarction, and heart failure.[86-92] It is apparent that Mas is present in the cerebrum and that activation of this receptor

with chronic and acute administration of Ang-(1-7) increases cerebral blood flow, bradykinin, bradykinin receptors, eNOS, and NO.[93-95] In summary, there is evidence for the involvement of the both ACE/Ang II/AT1R axis and ACE2/Ang-(1-7)/Mas axis components in the pathophysiology of stroke (Figure 1-5). Thus, both of these axes contain many viable targets for stroke prevention and acute therapy.

ACE/Ang II/AT1R Axis

While multiple factors influence stroke development and progression, the peptide Ang II appears to be a key player, especially in ischemic stroke. For example, there are reports that Ang II levels are increased bilaterally in the cortex and hypothalamus following stroke.[96] Additionally, systemic treatment of stroke-prone spontaneously hypertensive rats (spSHR) with ARBs reduces the occurrence of stroke.[79, 84]. Furthermore, in normal rats or spontaneously hypertensive rats (SHRs) made ischemic by middle cerebral artery occlusion (MCAO), ARBs provide a 40-50% reduction of neuronal damage (infarct size), reduce the neurological deficits, and improve recovery. [78, 82, 97-100]. These actions of ARBs are, at least in part, independent of their blood pressure lowering actions. [78, 82] More evidence for a role of AT1R in ischemic stroke comes from a report where a smaller lesion area was observed after MCAO in AT1R-deficient mice.[101] Interestingly, neither adrenergic receptor blockade nor calcium channel inhibition protects against ischemia, so it appears that inhibition of AT1R is essential to alleviate neural damage following stroke.[98, 102]

A few key facts have been revealed by previous studies utilizing candesartan and other ARBs, given both centrally and peripherally, for their cerebroprotective properties [78, 79, 81, 82, 84, 97-101, 103]. First, it is clear that ARB administration is cerebroprotective in intraluminal occlusion models of MCAO. In addition, it should be noted that certain

hydrophobic ARBs such as candesartan and valsartan cross the blood brain barrier readily and that systemic administration of these drugs for cerebroprotection has been utilized in non blood pressure lowering doses in several cases. Therefore, the cerebroprotective effect of ARBs is likely independent of blood pressure changes associated with administration. In fact, several theories exist for the mechanism of ARB cerebroprotection. The site of action of ARBs can be grossly identified as either cerebrovascular or parenchymal. For example, there is some evidence that ARB pre-treatment in a rat model of ischemic stroke increases capillary density [103], improves cerebrovascular reserve [104], and improves cerebral endothelial function [105]. These cerebrovascular effects may be mediated by unopposed agonism of the AT2 and Ang II type 4 receptors [82, 106] or AT1R alone [107]. In addition, AT2R stimulation has been shown to stimulate post stroke neurite outgrowth and improve neuron survival under hypoxic conditions [82]. Similarly, inhibition of AT1R may protect neurons during hypoxia by decreasing oxidative stress [108]. Therefore, ARB administration can either increase the cerebrovascular reserve allowing improved perfusion of tissues within the affected vascular territory, or act directly on neurons to improve their ability to survive an ischemic insult. In reality, the plethora of evidence indicates a role for both vascular and parenchymal targets in the cerebroprotection offered by ARBs.

These animal studies have been substantiated clinically by the Losartan Intervention For Endpoint (LIFE) clinical trial, which demonstrated that ARBs are more effective as anti-stroke agents than the traditionally used β-adrenergic receptor (βAR) blockers in patients with hypertension and left ventricular hypertrophy [77]. Other clinical trials such as SCOPE (Study on Cognition and Prognosis in the Elderly) have

substantiated the importance of AT1R blockade for stroke prevention [109]. Results from the Captopril Prevention Project (CAPPP) study demonstrated that angiotensin converting enzyme (ACE) inhibitors, which reduce the levels of circulating Ang II, are inferior to conventional therapy for stroke prevention [110]. This observation led to the suggestion that increased levels of Ang II in response to ARB treatment may have a role in cerebroprotection [82, 110].

Despite the focus on AT1R involvement in stroke, evidence suggests that Ang II type 2 receptors (AT2R) have a role in this disease. AT2Rs often mediate effects of Ang II that are exactly opposite to those mediated by AT1R [111], and in fact the tissue levels of AT2R are dramatically increased following injury, such as in the heart following myocardial infarction, in atherosclerotic blood vessels, in wounded skin, and in the peri-infarct region in the brain following ischemia [82, 96, 112-114]. Considering this, and the evidence that Ang II acts *via* AT2R in neurons to elicit differentiation, regeneration and neurotrophic actions [115-117], Unger and colleagues hypothesized that the increased expression of AT2R within the peri-infarct region can (in the presence of ARBs to block AT1R) be activated by the raised endogenous levels of Ang II and serve a neuroprotective role [82]. These investigators have supported this theory by demonstrating that the beneficial action of ARBs after MCAO-induced cerebral ischemia is prevented by specific AT2 receptor blockers [82]. Additional support is provided by the following experimental findings: i) ARBs are more cerebroprotective than ACE inhibitors in a rat model of ischemia and reperfusion [81]; ii) MCAO produces greater ischemic brain damage in AT2R knockout mice compared with wild-type controls [80]; and, iii) CNS delivery of an AT2R agonist provides cerebroprotection during ischemic stroke[83].

ACE2/Ang-(1-7)/Mas Axis

The expression of the Ang-(1-7) receptor Mas has been reported in both cardiovascular and non-cardiovascular control regions of the brain suggesting that the regulatory effects of Ang-(1-7)/Mas extend beyond the regulation of cardiovascular function. In fact, Mas was first characterized as a protooncogene that was found in high levels in the brain [118] and thought to be exclusively located in neurons [119]. Original reports of Mas localization revealed Mas mRNA transcripts were present broadly in areas such as hippocampus, cortex, olfactory bulbs and thalamus of rats or mice [118-121]. In addition, it has been shown that Mas is present in rat cerebral endothelial cells, but is absent from endothelial cells in the periphery [122]. Recently, a global presence for Mas in both cardiovascular and non-cardiovascular control areas of the brain was verified by immunofluorescence [93]. This study also indicates a largely neuronal localization for Mas in cardiovascular control regions, but makes no report on its cellular localization in non-cardiovascular control regions of the brain such as the motor cortex.

In accordance with these findings, ACE2 has also been identified in the brain. Two studies utilizing human tissue have detected low levels of ACE2 mRNA in the human brain [123] and ACE2 immunostaining in human brain vascular smooth muscle as well as endothelial cells [124]. More recently, ACE2 has been detected in glia during primary cell culture experiments [125]. However, in vivo analyses of mouse brains have localized both ACE2 mRNA and protein to predominately neurons, but in broad areas of cardiovascular and non-cardiovascular control including brainstem nuclei, motor cortex, and raphe [126]. Thus, it seems that a local ACE2/Ang-(1-7)/Mas axis is found in both cardiovascular and non-cardiovascular control areas of the brain.

It is also of note that activation of Mas with chronic or acute administration of Ang-(1-7) increases cerebral blood flow.[93-95] This increase in blood flow might possibly be explained by data which indicate that central administration of Ang-(1-7) shortly following stroke onset has been shown to increase bradykinin levels in the brain, as well as upregulate bradykinin receptors.[127] In addition, NO release and eNOS expression are increased with Ang-(1-7) treatment in this model.[128] Together, these results point toward a potential mechanism of cerebroprotection mediated by Ang-(1-7) stimulation of Mas and subsequent release of bradykinin, stimulation of bradykinin receptors, and release of NO, which could increase the cerebrovascular reserve during an ischemic insult. Further studies are indicated to determine the mechanism of activation of the ACE2/Ang-(1-7)/Mas Axis stroke.

Summary

Despite current knowledge of risk factors for stroke, as well as preventative and acute stroke therapy, this disease remains a leading cause of mortality and morbidity. Based on the current literature, ACE2, Ang-(1-7), and Mas are all present in the brain in various cerebrovascular and parenchymal structures. In addition, acute stimulation of Mas by peripherally administered Ang-(1-7), and chronic peripheral over-expression of ACE2 increases cerebral blood flow.

Furthermore, direct central administration of Ang-(1-7) causes increases in bradykinin, NOS activity, and NO production after stroke. In summary, there is ample evidence supporting a protective role for the ACE2/Ang-(1-7)/Mas axis in the cardiovascular system, as well as a physiological role for the presence of Mas and its activation by Ang-(1-7) in the cerebrovasculature. Therefore, we have developed the general hypothesis that activation of the brain ACE2/Ang-(1-7)/Mas axis prior to

endothelin-1 (ET-1)-induced middle cerebral artery occlusion (MCAO) will provide cerebroprotection during ischemic stroke. Three Specific Aims were designed using a combination of molecular and *in vivo* approaches.

Specific Aim 1: Establish and characterize an appropriate model for investigating the brain angiotensin system during ischemic stroke. Hypotheses: The ET-1-induced MCAO model will result in rapid occlusion of the middle cerebral artery and a gradual reperfusion. Systemic pre-treatment of rats with the ARB, Candesartan, will elicit a decrease in infarct size and in neurological deficits following ET-1-induced MCAO without altering ET-1-induced constriction of the MCA. Lastly, neurological exams will correlate with the amount of tissue damage resulting from ET-1-induced MCAO.

Specific Aim 2: Determine whether activation of the ACE2/Ang-(1-7)/Mas axis by direct central administration of Ang-(1-7) elicits cerebroprotection during ischemic stroke. Hypothesis: Central Ang-(1-7) pre-treatment will provide cerebroprotection during ET-1-induced MCAO. This effect will be mediated *via* the Ang-(1-7) receptor, Mas.

Specific Aim 3: Determine whether pharmacological activation of ACE2 can provide cerebroprotection during ischemic stroke *via* a Mas mediated action. Hypothesis: Activation of ACE2 *via* central pre-treatment with DIZE will provide cerebroprotection during ET-1-induced MCAO. This effect will be mediated by Ang-(1-7) activation of Mas.

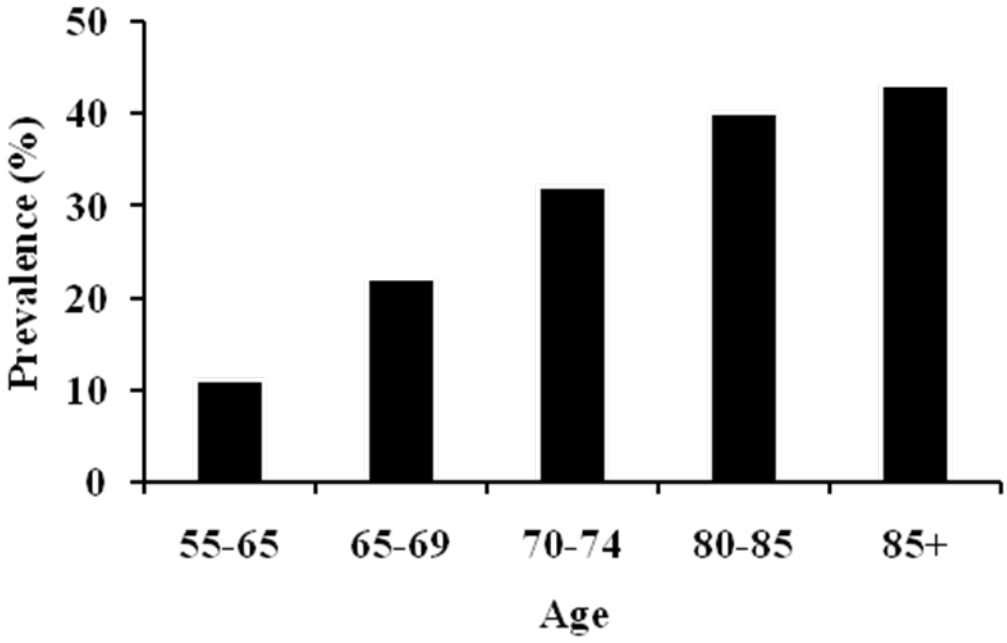

Figure 1-1. Prevalence of silent cerebral ischemia with respect to age. The prevalence of silent cerebral ischemia increases dramatically with respect to age. Data for this figure was adapted from the American Heart Association, Heart Disease and Stroke Statistics—2009 Update.[2]

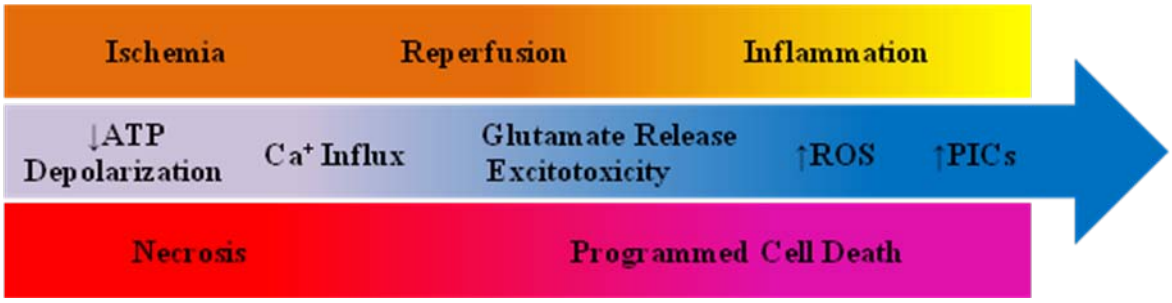

Figure 1-2. Pathophysiology of ischemic stroke. Reduction of blood flow during ischemic stroke results in tissue hypoxia and then gradual reperfusion. These events are followed by post-stroke inflammation. The molecular events involved in stroke pathophysiology include ATP depletion, cell depolarization, Ca+ influx, glutamate release, excitotoxicity, ROS production, and PIC release. The acute cell death that occurs during stroke consists primarily of necrosis, which progresses to modes of programmed cell death at later stages of pathology. PIC = proinflammatory cytokines, ROS = reactive oxygen species

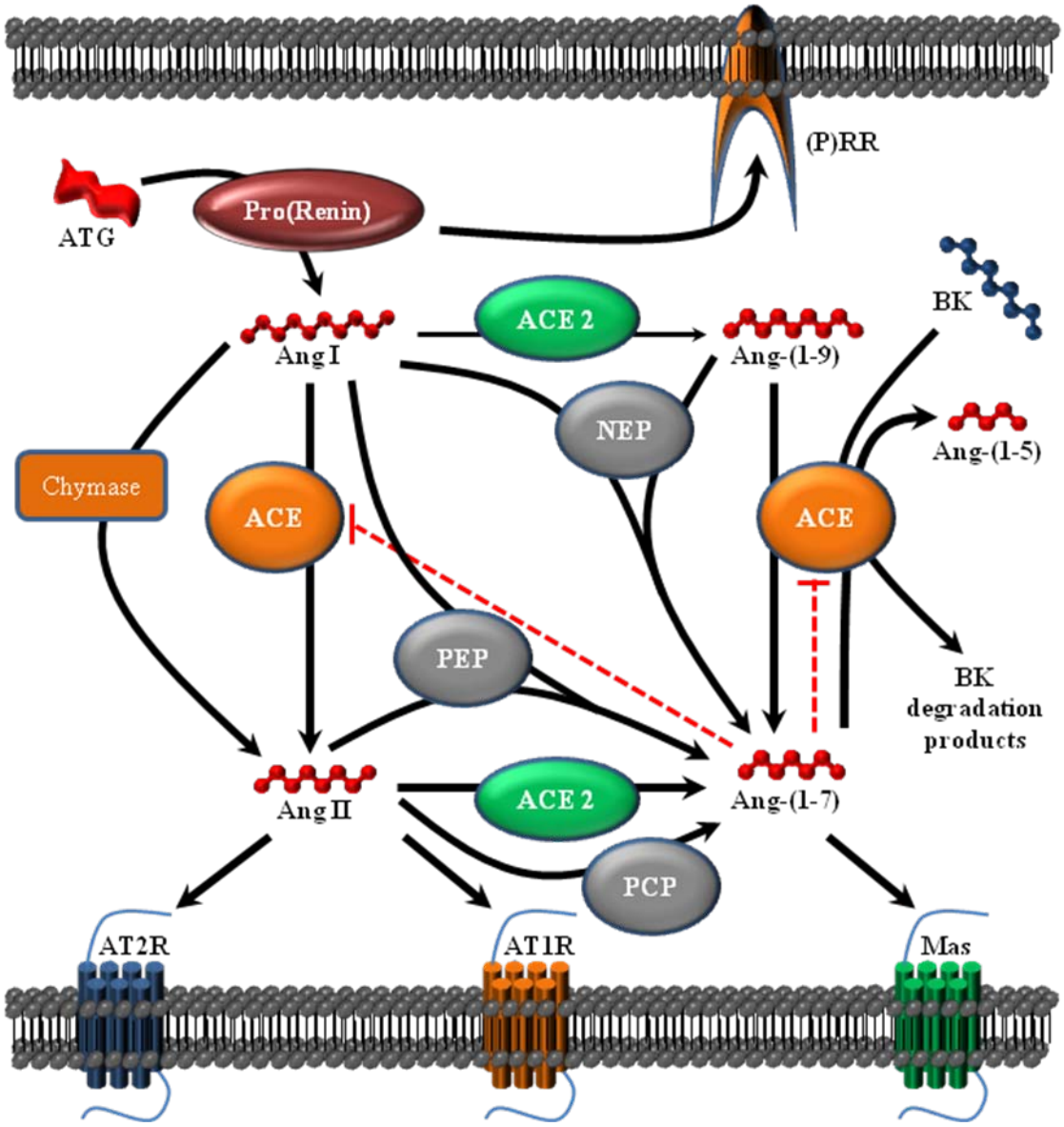

Figure 1-3. RAS components. ACE= Angiotensin Converting Enzyme, ACE2 = Angiotensin Converting Enzyme 2, ATG = Angiotensinogen, Ang = Angiotensin, AT1R = Ang II Type 1 Receptor, AT2R = Ang II Type 2 Receptor, BK = Bradykinin, NEP = Neprilysin, PEP = proyl-endopeptidase, PCP = proyl-carboxypeptidase, (P)RR = (Pro)Renin Receptor.

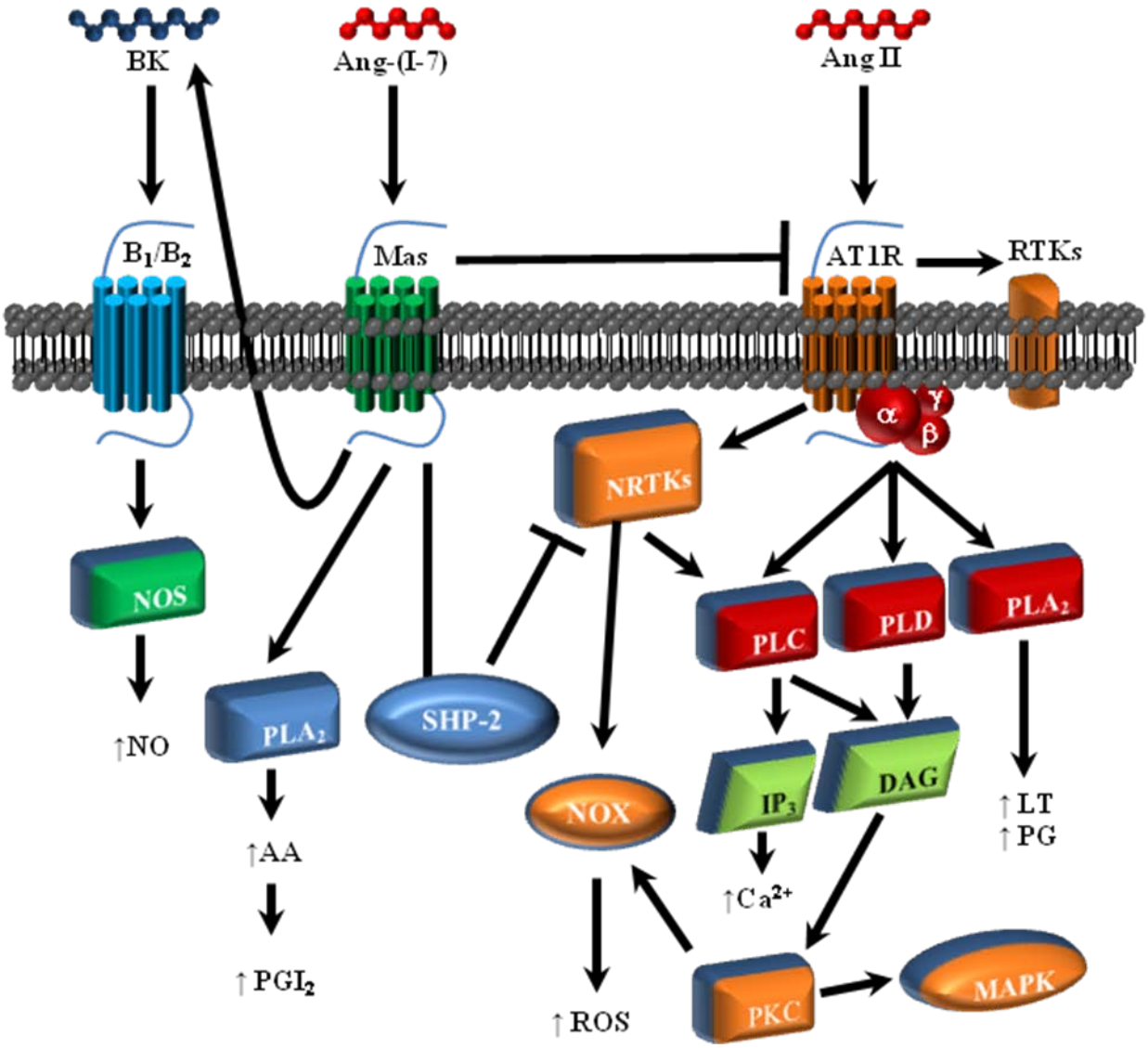

Figure 1-4. Ang-(1-7) opposes AT1R signaling. α/β/γ = G protein subunits, Ang = Angiotensin, AA = Arachidonic Acid, AT1R = Ang II Type 1 Receptor, B1/B2 = Bradykinin Receptors, BK = Bradykinin, DAG = diacylglycerol, IP_3 = inositol-1,4,5-triphosphate , LT = Leukotrienes, MAPK = Mitogen-Activated Protein Kinase, NOS = Nitric Oxide Synthase, NO = Nictric Oxide, NOX = NAD(P)H Oxidase, NRTK = Nonreceptor Tyrosine Kinase, PG, Prostaglandins, PKC = Protein Kinase C, PGI_2 = Prostacyclin, PLC = phospholipase C, PLA_2 = phospholipase A_2, PLD = phospholipase D, ROS = Reactive Oxygen Species, RTK = Receptor Tyrosine Kinase

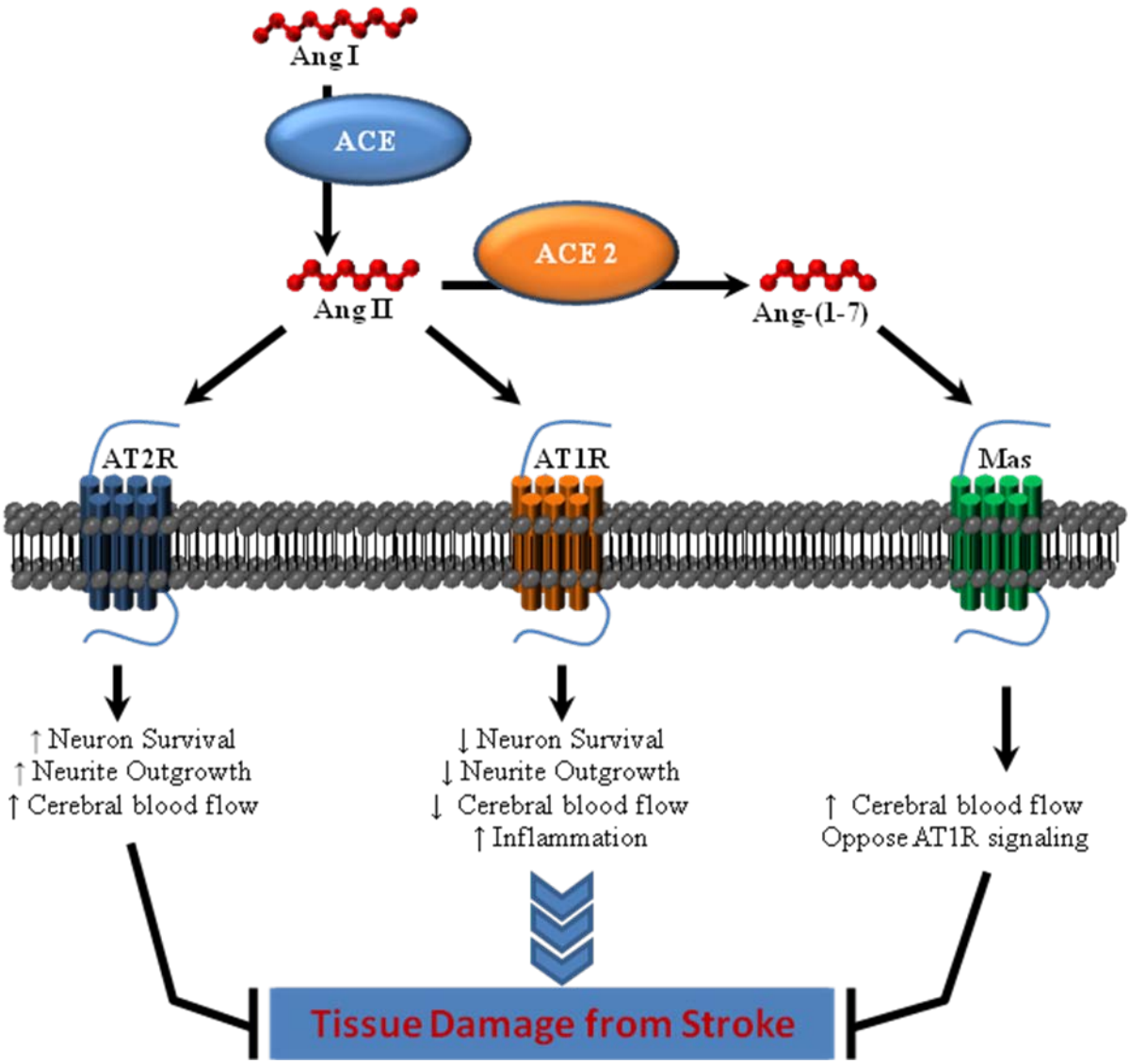

Figure 1-5. Renin Angiotensin System axes are involved in the pathophysiology of stroke. ACE= Angiotensin Converting Enzyme, ACE2 = Angiotensin Converting Enzyme 2, Ang = Angiotensin, AT1R = Ang II Type 1 Receptor, AT2R = Ang II Type 2 Receptor

CHAPTER 2
CANDESARTAN PRE-TREATMENT IS CEREBROPROTECTIVE IN A RAT MODEL OF ENDOTHELIN-1 INDUCED MIDDLE CEREBRAL ARTERY OCCLUSION

Introduction

While multiple factors influence stroke development and progression, the peptide angiotensin II (Ang II) appears to be a key player. For example, there are reports that Ang II levels are increased bilaterally in the cortex following stroke and systemic treatment of spontaneously hypertensive rats (SHR) with Ang II type 1 receptor (AT1R) blockers (ARBs) reduces the occurrence of stroke.[79, 84, 129] Additionally, ARBs provide a 40-50% reduction of infarct volume and reduce the neurological deficits in normotensive rats and SHRs.[78, 82, 97-100] These actions of ARBs are, at least in part, independent of their blood pressure lowering actions.[78, 82]

These animal studies have been substantiated clinically by the Losartan Intervention For Endpoint (LIFE) clinical trial, which demonstrated that ARBs are more effective as anti-stroke agents than the traditionally used β-adrenergic receptor (βAR) blockers in patients with hypertension and left ventricular hypertrophy.[77] Other clinical trials such as SCOPE (Study on Cognition and Prognosis in the Elderly) have substantiated the importance of AT1R blockade for stroke prevention.[109] Results from the Captopril Prevention Project (CAPPP) study demonstrated that angiotensin converting enzyme (ACE) inhibitors, which reduce the levels of circulating Ang II, are inferior to conventional therapy for stroke prevention [110]. This observation led to the suggestion that increased levels of Ang II in response to ARB treatment may have a role in cerebroprotection.[82, 110]

Despite the focus on AT1R involvement in stroke, evidence suggests that Ang II type 2 receptors (AT2R) have a role in this disease. AT2Rs often mediate effects of

Ang II that are exactly opposite to those mediated by AT1R[111], and in fact the tissue levels of AT2R are dramatically increased following injury, such as in the heart following myocardial infarction, in atherosclerotic blood vessels, in wounded skin, and in the peri-infarct region in the brain following ischemia.[82, 96, 112-114] Considering this, and the evidence that Ang II acts *via* AT2R in neurons to elicit differentiation, regenerationand neurotrophic actions[115-117], Unger and colleagues hypothesized that the increased expression of AT2R within the peri-infarct region can (in the presence of ARBs to block AT1R) be activated by the raised endogenous levels of Ang II and serve a neuroprotective role.[82] These investigators have supported this theory by demonstrating that the beneficial action of ARBs after MCAO-induced cerebral ischemia is prevented by specific AT2 receptor blockers.[82] Additional support is provided by the following experimental findings: i) ARBs are more cerebroprotective than ACE inhibitors in a rat model of ischemia and reperfusion[81]; ii) MCAO produces greater ischemic brain damage in AT2R knockout mice compared with wild-type controls[80]; and, iii)CNS delivery of an AT2R agonist provides cerebroprotection during ischemic stroke.[83]

Considering that endogenous levels of Ang II are increased in the cortex and hypothalamus following stroke and ARBs have been shown to attenuate the deleterious effects of animal stroke models utilizing intraluminal occlusion, we have developed the general hypothesis that systemic administration of an ARB prior to ET-1 induced MCAO will provide cerebroprotection during ischemic stroke. The ET-1 induced MCAO model of cerebral ischemia is thought to more closely mimic the temporal events of an embolic stroke. This model provides rapid occlusion of the middle cerebral artery and a gradual reperfusion that lasts for 16-22 h.[130] Specifically, we determined whether systemic pre-

treatment of rats with the CNS permeant ARB candesartan for 7 days would elicit a decrease in infarct size and in neurological deficits following ET-1 induced MCAO. This study aims to both solidify the cerebroprotective properties of ARBs during ischemic stroke and to validate the ET-1 induced MCAO model for examination of the role of the brain renin angiotensin system in ischemic stroke.

Methods

Animals

For the experiments described here, we used a total of 329adult male Sprague Dawley rats purchased from Charles River Farms (Wilmington, MA). Two rats from the candesartan pre-treatment group and 2 rats from the 0.9% saline pre-treatment group died following the ET-1 induced MCAO. This is consistent with the approximately 80% mortality rate we have observed with this procedure. There was no significant difference in mortality rate between groups. All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

Chemicals

Candesartan was a gift from AstraZeneca (Alderley Park, Cheshire, UK). Endothelin-1 was purchased from American Peptide Company, Inc (Sunnyvale, CA, USA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Endothelin – 1 Induced Middle Cerebral Artery Occlusion

The ET-1 induced MCAO procedure used here has been modified slightly from that published previously.[130, 131] Eight-week-old male Sprague Dawley rats were anesthetized with a mixture of O_2 (1 L/min) and 4% isoflurane, placed in a Kopf stereotaxic frame, and anesthesia was maintained for the duration of the surgery using an O_2/isoflurane (2%) mixture delivered through a nose cone attached to the frame.

The skull was exposed and a small hole was drilled in the cranium dorsal to the right hemisphere using the following stereotaxic coordinates (1.6 mm anterior and 5.2 mm lateral to the bregma). A 26 gauge needle attached to a 5 μL Hamilton microsyringe was lowered 8.7 mm ventral to bregma, after which 3 μL of 80 μM ET-1 was infused adjacent to the MCA at a rate of 1 μL/min. The needle was withdrawn 3 min after the injection was complete. Following this, the wound was closed and the rat was administered an analgesic agent (buprenorphine; 0.05 mg/kg sc) before waking.

Visualization of MCA Branches *via* a Cranial Window

Rats were anesthetized as described above, after which a temporal craniectomy was performed to visualize the primary and secondary branches of the MCA. An approximately 3-4 mm square piece of bone was removed from the left squamous portion of the temporal bone just caudal to the orbit. The dura was left in place and debris was cleared away using sterile 0.9% saline. Next, ET-1 induced MCAO was performed as described above except that the needle was left in place until all images were captured so that the focal plane would not be disturbed. The cerebral cortex and associated vessels visible through this cranial window were imaged using a Moticam 1000 digital camera (Motic; Richmond, BC, Canada) coupled to a Revelation surgical microscope (Seiler Instrument and Manufacturing; St. Louis, MO, USA). Vessel diameter was determined for 2 or 3 arterial branches per cranial window using ImageJ software (NIH). A baseline image was captured prior to ET-1 injection and subsequent images were captured each 1 min interval for 40 min. Vessel diameter at each time point was normalized to the baseline vessel diameter so that comparisons could be made using multiple MCA branches of several rats.

Implantation of Telemetry Transducers

Rats were anesthetized as described above, after which telemetry transducers (DSI, St. Paul, MN, USA) were implanted into the abdominal aorta, as detailed by us previously.[132] Rats were administered the analgesic (buprenorphine, 0.05 mg/kg s.c.) following surgery.

Neurological Deficits and Infarct Size

Neurological evaluations were performed using two separate scoring scales reported by Bederson *et al*.[133] and Garcia *et al*.[134], which cumulatively evaluate spontaneous activity, symmetry in limb movement, forepaw outstretching, climbing, body proprioception, response to vibrissae touch, resistance to lateral push, and circling behavior. Additionally, animals were evaluated for neurological deficits using a sunflower seed eating test.[135] Animals with a subarachnoid hemorrhage at postmortem examination were excluded from analysis. Infarct volume was assessed by staining brain sections with 0.05% 2,3,5-triphenyltetrazolium chloride (TTC) for 30 minutes at 37°C. Tissue ipsilateral to the occlusion, which was not stained, was assumed to be infarcted. After fixation with 10% formalin, brain sections were scanned on a flatbed scanner (Canon) and analyzed using ImageJ software (NIH). To compensate for the effect of brain edema, the corrected infarct volume was calculated using an indirect method.[129]

Implantation of Osmotic Mini-pumps

Rats were implanted subcutaneously between the shoulder blades with an osmotic pump (model 2001, ALZET, Cupertino, CA) as described previously[86], which infused candesartan (0.2 mg/kg/day) subcutaneously for 1 week.

Cardiovascular Measurements

Measurements of mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), and heart rate (HR) were made *via* DSI telemetry transducers.[132] Measurements were made prior to candesartan or 0.9% saline pre-treatment while awake, after 7 days of candesartan or 0.9% saline pre-treatment while anesthetized, during the ET-1 induced MCAO procedure while anesthetized, and 8 hours after ET-1 induced MCAO when awake.

Data Analysis

Data are expressed as means ± SEM. Statistical significance was evaluated, as specified in the figure legends, with the use of a one-way ANOVA, Mann-Whitney Test, Wilcoxon signed-rank test, or an Unpaired t test, as well as Tukey-Kramer Multiple Comparisons Test for posthoc analysis when appropriate. A Spearman nonparametric correlation was used to compare the relationship between infarct size and neurological testing data. Differences were considered significant at $p < 0.05$. Individual p values are noted in the results and figure legends.

Results

Intracranial injection of 3 μL of ET-1 (80 μM) into the brain parenchyma adjacent to the MCA resulted in abrupt constriction of the proximal MCA branches to 0% baseline vessel diameter within minutes followed by recanalizaton of the vessel (Figure 2-1, D-F and J). Rats undergoing a sham MCAO were injected with 3 μL of 0.9% saline in place of ET-1. Vessel diameter remained relatively stable at baseline values following this saline injection (Figure 2-1, A-C and J). Additionally, rats were pre-treated for 7 days with candesartan (0.2 mg/kg/day, s.c.) prior to ET-1 injection. As with the group receiving no pre-treatment, vessel diameter decreased to 0% baseline diameter in the

candesartan pre-treated group (Figure 2-1, G-I and J). There was no significant difference in vessel diameter at any time point between candesartan pre-treated and non-pretreated rats that received an injection of ET-1 adjacent to the MCA. Both groups receiving an ET-1 brain injection displayed a decrease in vessel diameter 3 min after the start of injection that was significantly greater than the group undergoing sham MCAO ($p < 0.01$). These results indicate that ET-1 injection adjacent to the MCA causes constriction of the vessel followed by recanalization. In addition, pre-treatment with candesartan does not alter the amplitude or time course of the ET-1 induced MCAO.

The effect of candesartan pre-treatment on ET-1-induced cerebral damage was assessed by TTC staining, whereby non-infarcted gray matter is stained red after incubation in TTC, delineating the infarct in white (Figure 2-2A). Pre-treatment of rats for 7 days with candesartan (0.2 mg/kg/day, s.c.) significantly reduced the infarct size to 14.35 ± 5.38% of the gray matter compared to 28.98 ± 4.38% in the 0.9% saline pre-treated control group ($p < 0.05$, Figure 2-2B). Neurological testing performed 48 h subsequent to the ET-1 induced MCAO indicated improved performance in rats pre-treated with candesartan compared to those with 0.9% saline pre-treatment (Figure 2-3, Table 2-1). Prior to ET-1 induced MCAO all rats scored the maximum of 18 on the Garcia neurological exam indicating that no deficit existed. After the ET-1 induced MCAO, the Garcia neurological exam score decreased significantly from pre-stroke values to 12.1 ± 1.1 in the 0.9% saline pre-treated group ($p < 0.001$) and 16.7 ± 0.7 in the candesartan pre-treatment group ($p < 0.05$). Two days following ET-1 induced MCAO, the Garcia exam score was significantly higher in the candesartan pre-treated

group compared to the 0.9% saline pre-treated group ($p < 0.05$). A similar pattern was observed with the Bederson neurological exam on which all rats received the minimum score of 0 prior to ET-1 induced MCAO, indicating an absence of neurological deficit. After the ET-1 induced MCAO, the Bederson neurological exam score increased from pre-stroke values to 2.1 ± 0.2 in the 0.9% saline pre-treated group ($p < 0.001$) and 0.7 ± 0.4 in the candesartan pre-treatment group. The post-stroke Bederson exam score was significantly higher in the candesartan pre-treated group compared to 0.9% saline pre-treated group ($p < 0.01$). Rats also participated in a sunflower seed-eating test where they were timed while opening 5 sunflower seeds. Prior to ET-1 induced MCAO rats performed this task in 103.7 ± 14.4 s and broke the shell into 13.6 ± 0.7 pieces. Two days following ET-1 induced MCAO, the time to eat 5 sunflower seeds increased from pre-stroke values to 215.6 ± 28.3 s in the 0.9% saline pre-treated group ($p < 0.01$) and 114.7 ± 16.5 s in the candesartan pre-treatment group (not significant, $p = 0.06$). In addition, the number of shell pieces increased from pre-stroke values to 23.8 ± 2.2 pieces in the 0.9% saline pre-treatment group ($p < 0.01$) and 14.8 ± 0.8 pieces in the candesartan pre-treatment group (not significant, $p = 0.18$). The post-stroke number of shell pieces was significantly lower for the candesartan pre-treated group compared to the 0.9% saline pre-treated group ($p < 0.05$) and a similar although non-significant pattern was observed for the time to eat 5 sunflower seeds ($p = 0.08$). Taken together, these results indicate that candesartan pre-treatment prior to ET-1 induced MCAO can reduce the size of the cerebral infarct and the extent of neurological deficits produced by this model of focal cerebral ischemia.

In order to evaluate how closely our neurological exam scores predicted the extent of brain tissue damage we grouped rats receiving both candesartan and 0.9% pre-treatment together and compared pre-stroke and post-stroke neurological test scores, as well as post-stroke scores with % infarcted gray matter. Prior to ET-1 induced MCAO both candesartan pre-treated and 0.9% saline pre-treated rats showed no neurological deficits as indicated by a minimum score of 0 on the Bederson exam, and a maximum score of 18 on the Garcia exam. As expected, the rats displayed neurological deficits 48 h subsequent to ET-1 induced MCAO with a Bederson exam score that increased to 1.6 ± 0.2 ($p < 0.0001$) and a Garcia exam score that decreased to 13.6 ± 0.9 ($p < 0.0001$) for both groups combined. Prior to ET-1 induced MCAO rats from both treatment groups combined required an average of 103.7 ± 14.4 s to eat 5 seeds and broke the shells into an average of 13.6 ± 0.7 pieces. Rats displayed significant deficit in performing this task 48 h subsequent to ET-1 induced MCAO. The time to eat 5 seeds increased to 181.9 ± 22.5 s ($p < 0.01$) and the number of shell pieces increased to 20.8 ± 1.8 ($p < 0.01$) for both groups combined compared to pre-stroke outcomes. It is clear from these data that our model of ET-1 induced MCAO causes significant neurological deficits that can be measured 48 h after stroke induction (Table 2-1 and Figure 2-3). For correlation between neurological exam results and infarct size, data showing the Garcia exam score, Bederson exam score, time to eat 5 sunflower seeds, and number of shell pieces were plotted against the associated % infarcted gray matter for each rat (Figure 2-4, A-D). A strong and significant negative correlation was seen for % infarcted gray matter vs. Garcia exam score (Spearman $r = -0.9151$, $p < 0.0001$). Additionally, strong and significant positive correlations were seen for % infarcted gray

matter vs. Bederson exam score (Spearman r = 0.8632, $p < 0.0001$), % infarcted gray matter vs. time to eat 5 sunflower seeds (Spearman r = 0.6729, $p < 0.005$), and % infarcted gray matter vs. number of shell pieces (Spearman r = 0.7241, $p < 0.005$).

Lastly, a subgroup of 5 rats was implanted with telemetry blood pressure transducers in the abdominal aorta in order to record arterial blood pressure and heart rate (Table 2-2). Cardiovascular variables consisting of mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP), and heart rate (HR) were measured. No differences in cardiovascular parameters were seen between treatment groups prior to candesartan or 0.9% saline pre-treatment or after pre-treatment for 7 days. A slight increase in all cardiovascular variables was observed within 5 min of ET-1 induced MCAO, but no difference was observed between groups. Similarly, there were no differences in cardiovascular variables between groups recorded at 8 h subsequent to ET-1 induced MCAO.

Discussion

The significant findings of this study are that pre-treatment with the CNS permeant ARB, candesartan, attenuates the neurological deficits and CNS tissue damage produced in an ET-1-induced MCAO model of ischemic stroke without altering blood pressure. In addition, we show that this model of stroke provides a rapid constriction, sustained occlusion, and then gradual reperfusion of the proximal MCA. This constriction is not altered by pre-treatment with candesartan which is a concern when using the ET-1 induced MCAO as a disease model for investigating manipulations of the brain angiotensin system during focal cerebral ischemia. Lastly, we have shown that results of the neurological testing methods used here are strongly correlated with the amount of tissue damage produced by ET-1 induced MCAO. Taken together, our

results confirm the deleterious action of Ang II in the presence of uninhibited AT1Rs in the pathophysiology of ischemic stroke and validate the ET-1 induced MCAO model for examination of the brain renin angiotensin system's role in ischemic stroke.

The ET-1 induced MCAO model has been used and characterized extensively in rodent models as a method for producing transient focal cerebral ischemia.[130, 131, 136] It has often been presented as a model that closely mimics the initial vessel occlusion in other stroke models, such as the intraluminal suture method, in that rapid and complete occlusion is initially achieved. A unique aspect of the ET-1 induced MCAO model is the reversibility of the chemically induced occlusion over time. Perfusion MRI studies in the ET-1 induced MCAO model have shown that CBF in the ipsilaterial hemisphere decreases to 30 to 50% of normal at 1 h and gradually returns to normal between 16 h and 22 h. This is followed by a period of hyperperfusion that seems to peak between 24 h and 48 h at which point the study was ended.[130] This time course for CBF has been verified by doppler flow studies of the cerebral cortex in the MCA territory.[137] However, neither vasoconstriction nor regional blood flow of the proximal MCA have been examined in this stroke model. Our data indicate that ET-1 injection adjacent to the MCA does indeed provide an initial rapid occlusion of the proximal MCA. However, vessel diameter seems to return to baseline after approximately 30 min. This profile does not match that of CBF measured previously even though infarct sizes and neurological deficits are comparable. It is possible that a cerebrovascular event distal to the proximal MCA mediates ischemia after the initial occlusion, but this fact is yet to be determined.

We have used the ET-1 induced MCAO model to verify the cerebroprotective actions of candesartan during ischemic stroke. A few key facts have been revealed by previous studies utilizing candesartan and other ARBs, given both centrally and peripherally, for their cerebroprotective properties.[78, 79, 81, 82, 84, 97-101, 103] First, it is clear that ARB administration is cerebroprotective in intraluminal occlusion models of MCAO. In addition, it should be noted that certain hydrophobic ARBs such as candesartan and valsartan cross the blood brain barrier readily and that systemic administration of these drugs for cerebroprotection has been utilized in non blood pressure lowering doses in several cases. Therefore, the cerebroprotective effect of ARBs is likely independent of blood pressure changes associated with administration. In fact, several theories exist for the mechanism of ARB cerebroprotection. The site of action of ARBs can be grossly identified as either cerebrovascular or parenchymal. For example, there is some evidence that ARB pre-treatment in a rat model of ischemic stroke increases capillary density[103], improves cerebrovascular reserve[104], and improves cerebral endothelial function.[105] These cerebrovascular effects may be mediated by unopposed agonism of the AT2 and Ang II type 4 receptors[82, 106] or AT1R alone.[107] In addition, AT2R stimulation has been shown to stimulate post stroke neurite outgrowth and improve neuron survival under hypoxic conditions.[82] Similarly, inhibition of AT1R may protect neurons during hypoxia by decreasing oxidative stress.[108] Therefore, ARB administration can either increase the cerebrovascular reserve allowing improved perfusion of tissues within the affected vascular territory, or act directly on neurons to improve their ability to survive an ischemic insult. In reality, the plethora of evidence indicates a role for both vascular and parenchymal targets in the cerebroprotection

offered by ARBs. Our observations provide support for a role of angiotensin peptides in the pathophysiology of ischemic stroke, and verify the need for further study of the RAS to identify potential targets for stroke therapy.

Table 2-1. Neurological exam results before and after ET-1 induced MCAO

	Bederson Exam Score		Garcia Exam Score		Sunflower Seed Test - Time to Eat 5 Seeds		Sunflower Seed Test - Number of Shell Pieces	
Treatment	24 h Pre-Stroke	48 h Post-Stroke	24 h Pre-Stroke	48 h Post-Stroke	24 h Pre-Stroke	48 h Post-Stroke	24 h Pre-Stroke	48 h Post-Stroke
0.9 % Saline (n = 12)	0 ± 0	2.1 ± 0.2 ***	18 ± 18	12.1 ± 1.1 ***	120.8 ± 19.5	215.6 ± 28.3 **	14.1 ± .9	23.8 ± 2.2 **
Candesartan (n = 6)	0 ± 0	0.7 ± 0.4 ‡	18 ± 18	16.7 ± 0.7 †*	69.5 ± 10.5	114.7 ± 16.5	12.7 ± 1.1	14.8 ± 0.8 †
Combined Groups (n = 18)	0 ± 0	1.6 ± 0.2 ***	18 ± 18	13.6 ± 0.9 ***	103.7 ± 14.4	181.9 ± 22.5 **	13.6 ± 0.7	20.8 ± 1.8 **

All values are reported as mean ± SEM. † $p < 0.05$ or ‡ $p < 0.05$ vs. Candesartan pre-treatment (Mann-Whitney test); * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ vs. pre-stroke value (Wilcoxon signed-rank test)

Table 2-2. Cardiovascular variables before, during, and after ET-1 induced MCAO

Treatment	MAP	SBP	DBP	PP	HR
Prior to Candesartan Pre-treatment for 7 days; Awake					
Candesartan	87.19 ± 3.74	103.00 ± 4.91	74.82 ± 3.55	28.13 ± 3.54	333.66 ± 10.01
0.9% Saline	92.36 ± 2.36	107.54 ± 1.82	80.35 ± 4.08	27.17 ± 4.22	328.08 ± 14.28
After Candesartan Pre-treatment for 7 days; Anesthetized prior to ET-1 induced MCAO					
Candesartan	80.19 ± 3.25	94.08 ± 2.70	69.35 ± 3.89	24.65 ± 1.80	336.23 ± 9.18
0.9% Saline	83.98 ± 10.01	101.08 ± 8.78	66.51 ± 15.67	35.15 ± 13.65	311.43 ± 10.75
Change from baseline during 5 min period after ET-1 induced MCAO; Anesthetized					
Candesartan	7.89 ± 3.69	8.27 ± 3.69	8.15 ± 3.79	0.47 ± 0.27	19.56 ± 6.99
0.9% Saline	10.57 ± 3.28	9.32 ± 2.42	19.51 ± 11.83	13.17 ± 10.75	27.09 ± 15.66
8 hours post ET-1 induced MCAO; Awake					
Candesartan	90.36 ± 6.45	107.06 ± 6.69	77.76 ± 6.60	29.28 ± 2.35	407.21 ± 19.20
0.9% Saline	105.80 ± 21.41	117.77 ± 21.77	96.94 ± 21.20	20.82 ± 3.23	358.96 ± 16.17

No significant differences between groups (unpaired t-test)

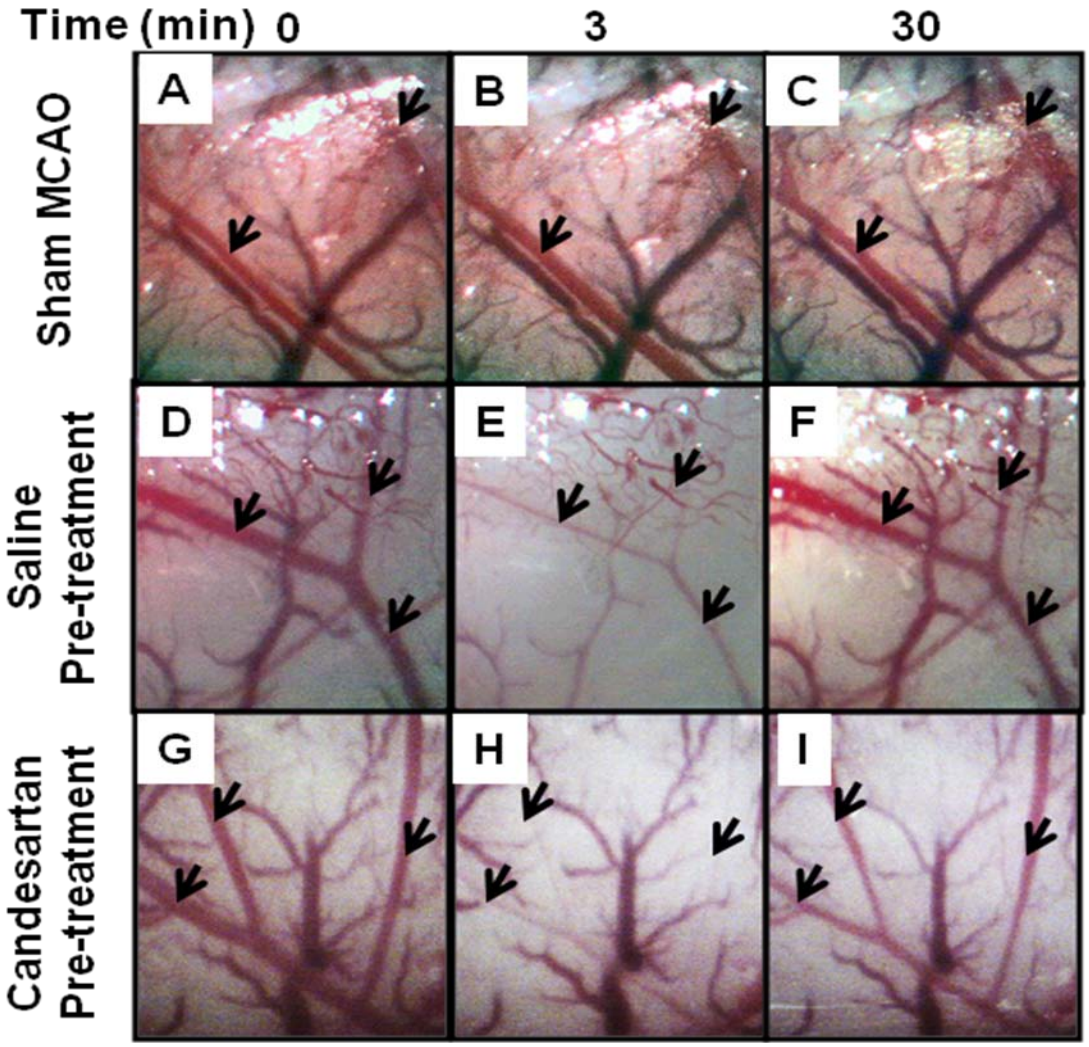

Figure 2-1. Visualization of MCA branches during ET-1 induced vasoconstriction. A-I) Visualization of MCA branches (arrows) during ET-1 induced vasoconstriction. Primary and secondary branches of the MCA were visualized with a surgical microscope after temporal craniotomy to create a cranial window. Images were captured at a rate of 1 per min starting immediately prior to ET-1 injection (0 min), throughout the ET-1 injection (3 min), and for at least 40 min after completion of ET-1 injection. Shown here are representative images captured from rats that underwent a sham (0.9% saline) injection (n = 2, A - C), an MCAO induced by 80 mM ET-1 injection (n = 3, C-E), and an ET-1 induced MCAO plus 7 days pre-treatment with candesartan (0.2 mg/kg/day, s.c.) (n = 2, G-I). J) Vasoconstriction of primary and secondary MCA branches was quantified as the % of baseline vessel diameter. Data are means ± SEM. Baseline vessel diameter was determined prior to ET-1 or 0.9% saline injection (Time = 0 min). * $p < 0.01$ for both candesartan pre-treated and non pre-treated groups compared to sham ET-1 injection (one-way ANOVA followed by Tukey-Kramer Multiple Comparisons Test).

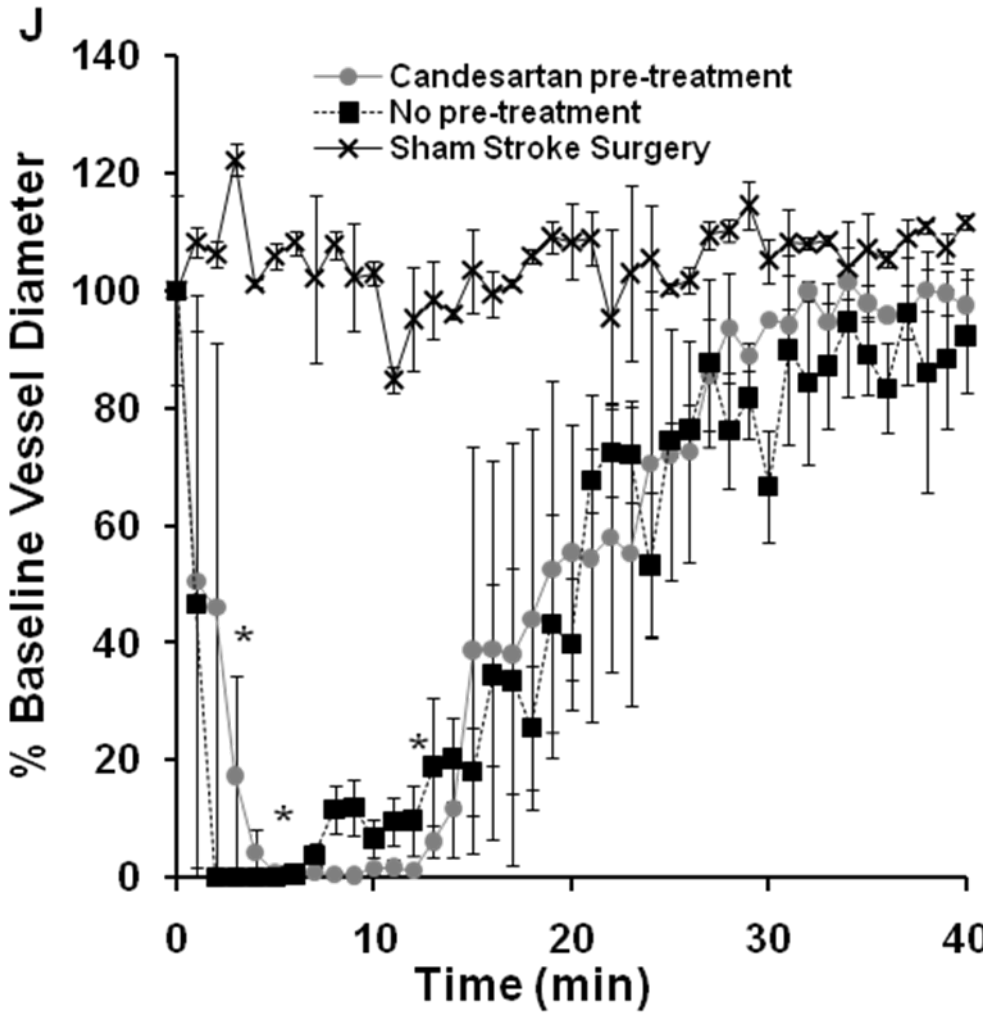

Figure 2-1. Continued

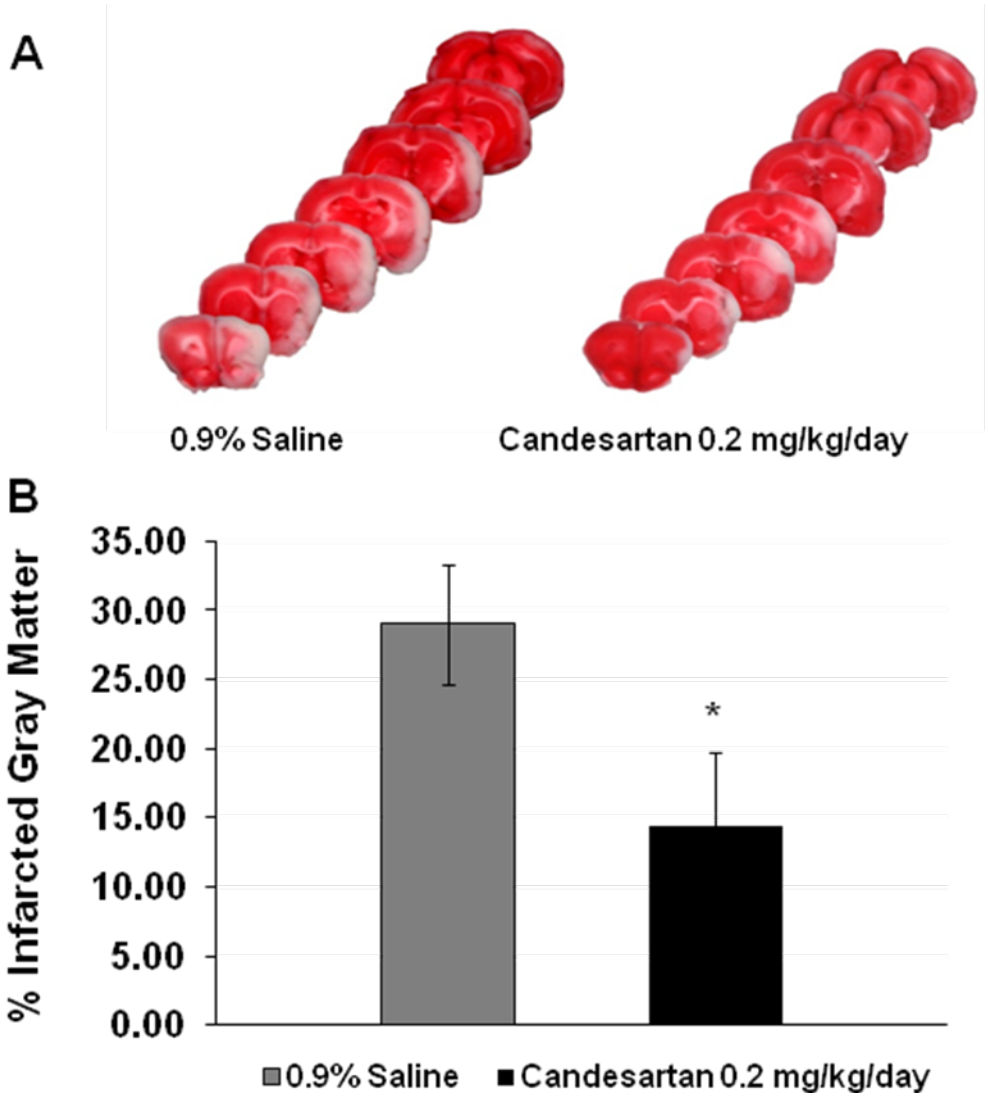

Figure 2-2. Candesartan pre-treatment decreases cerebral infarct size after ET-1 induced MCAO. Rats were pre-treated with candesartan or 0.9% saline for 7 days prior to ET-1 induced MCAO. Brains were removed and sectioned 48 h later. A) Representative brain sections from both a control (0.9% saline) pre-treated and candesartan pre-treated rat showing infarcted (white) and non-infarcted (red) gray matter. B) Bar graphs show the % infarcted gray matter in each treatment group. Data are means ± SEM from 6 (candesartan) and 11 (0.9% saline) pre-treated rats. * $p < 0.05$ compared to 0.9% saline pre-treated group (Mann-Whitney Test)

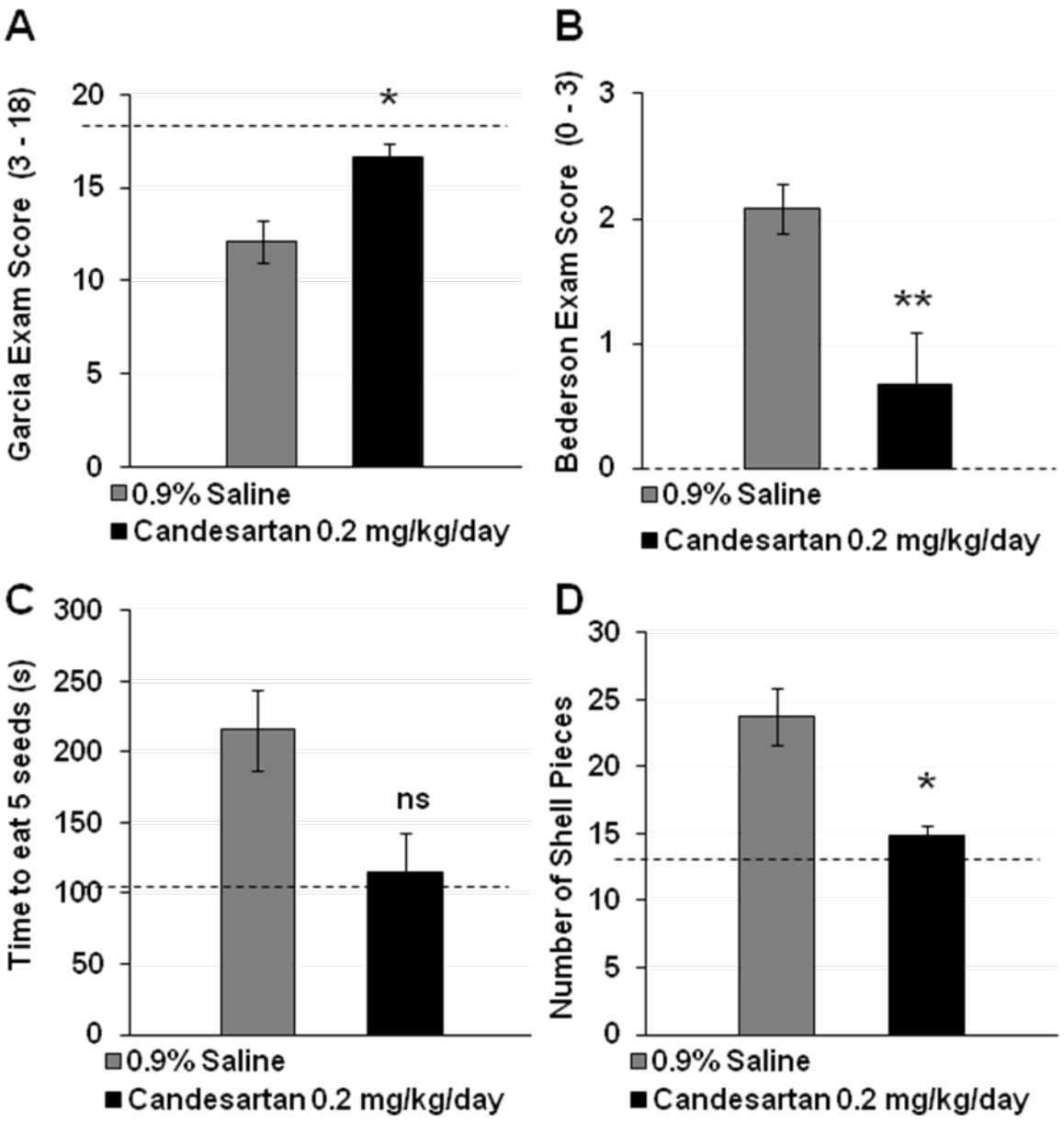

Figure 2-3. Pre-treatment with Candesartan leads to improvement of functional outcomes after ET-1 induced MCAO. Rats were pre-treated with candesartan or 0.9% saline for 7 days prior to ET-1 induced MCAO. Forty eight hours later, neurological deficits were assessed *via* the Garcia Neurological Exam (*Panel* A), the Benderson Neurological Exam (*Panel* B), and the Sunflower Seed Eating Test (*Panels* C and D). Bar graphs are means ± SEM from 6 (candesartan) and 12 (0.9% saline) pre-treated rats The dotted horizontal lines on each panel indicate the scores obtained on each test prior to ET-1 induced MCAO. * $p < 0.05$, ** $p < 0.01$, ns – not significant (Mann-Whitney Test) compared to 0.9% saline pre-treatment.

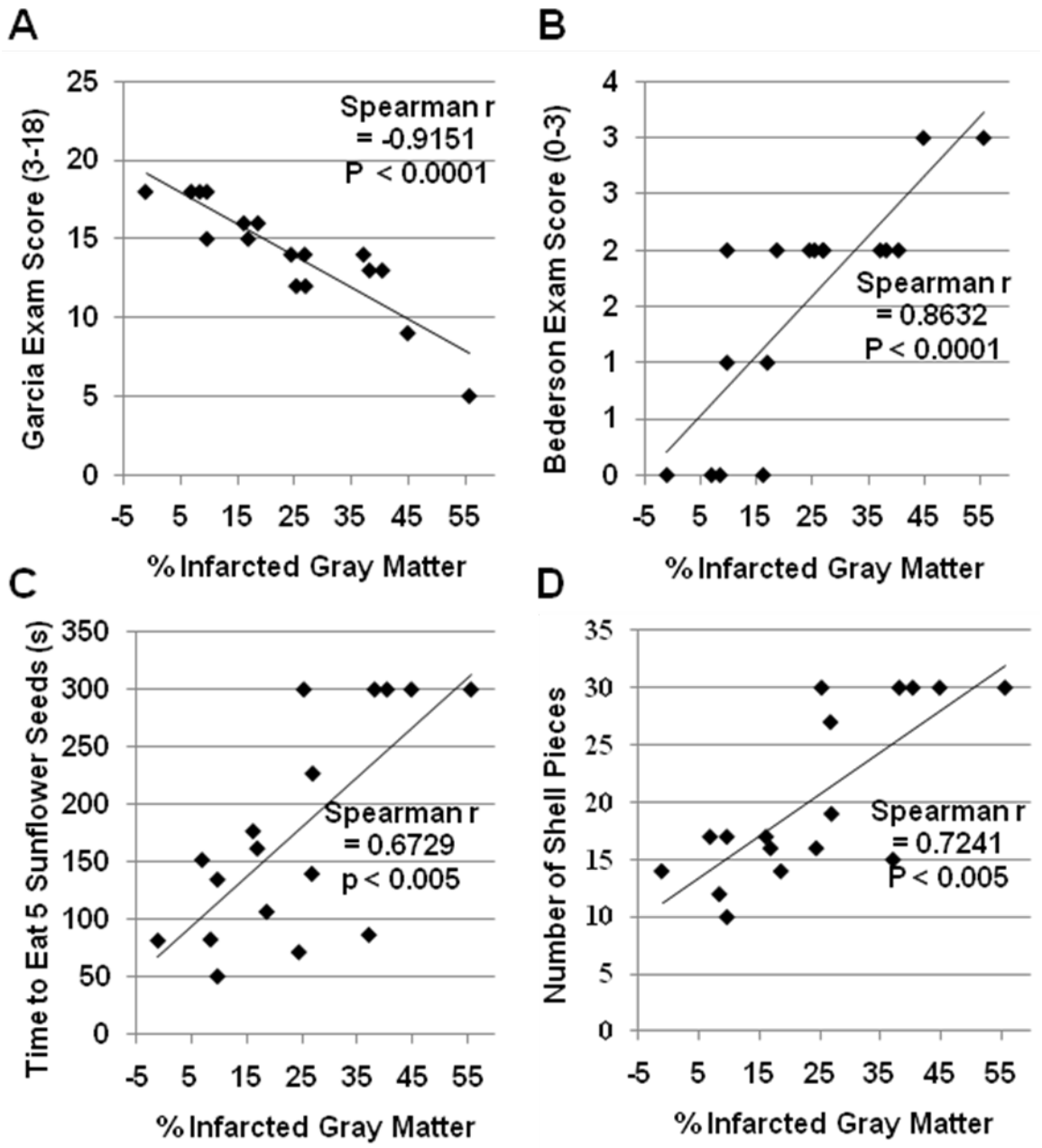

Figure 2-4. Neurological exam scores correlate with % infarcted gray matter. Neurological exams were performed 48 h after ET-1 induced MCAO. Brains were removed, sectioned and TTC stained. Correlation of Garcia Exam Scores (*Panel* A), Benderson Exam Scores (*Panel* B), and the Sunflower Seed Eating Test Scores (*Panels* C and D) with % infarcted gray matter revealed strong correlations.

CHAPTER 3
ANGIOTENSIN-(1-7) IS CEREBROPROTECTIVE IN A RAT MODEL OF ISCHEMIC STROKE

Introduction

Angiotensin-(1-7) (Ang-(1-7) is the latest member of the renin-angiotensin system (RAS) that has been shown to exert major biological activity. This heptapeptide is generated predominately from its precursor peptide, angiotensin II (Ang II), by Angiotensin Converting Enzyme 2 (ACE2) and to exert physiological effects mediated by its receptor, Mas. Together, these components make up the ACE2/Ang-(1-7)/Mas axis, which has been shown to counteract many of the physiological effects of Ang II at the angiotensin type 1 receptor (AT1R), including vasoconstrictor and proliferative actions.[85] In addition, activation of the ACE2/Ang-(1-7)/Mas axis also attenuates many of the pathophysiological states that involve increased production of Ang II by Angiotensin Converting Enzyme (ACE), and subsequent activation of the AT1R (ACE/Ang II/AT1R axis). For example, many studies targeting the ACE2/Ang-(1-7)/Mas axis have revealed its broad therapeutic potential for the treatment of hypertension, hypertension-related pathology, myocardial infarction, and heart failure.[86-88, 91, 92]

Similar to other cardiovascular diseases, it is well known that activation of the ACE/Ang II/AT1R axis contributes to the pathophysiology of stroke. Numerous studies have shown that blockade of either Angiotensin Converting Enzyme (ACE) with ACE inhibitors (ACEi) or AT1Rs with AT1R blockers (ARBs) appears to decrease cardiovascular risk and improve stroke prevention in human trials, as well as decrease infarct size and ensuing neurological deficits in animal models of stroke.[76-78] The cerebroprotective effect of reducing activation of the ACE/Ang II/AT1R axis has been

hypothesized to occur *via* either decreased activation of the AT1R or an unopposed activation of the angiotensin Type 2 Receptor (AT2R).[80, 82, 83]

Despite efforts to reduce both the incidence of stroke by targeting modifiable risk factors, as well as the morbidity and mortality associated with stroke through the use of techniques to recannulate occluded vessels, stroke remains the third leading cause of death in the United States and a leading cause of serious, long-term disability.[1] Among the well-documented and modifiable risk factors for stroke, hypertension and the RAS have long been key targets for pharmacotherapy and life-style modifications. While most investigations into the involvement of the RAS in stroke pathophysiology have focused on Ang II and its AT1R and AT2R mediated actions, it is evident that components of the ACE2/Ang-(1-7)/Mas axis may have potential beneficial effects during stroke. It is apparent that Mas is present in the cerebrum and that activation of this receptor with chronic and acute administration of Ang-(1-7) increases cerebral blood flow (CBF).[93-95] Further, central administration of Ang-(1-7) shortly following ischemic stroke onset has been shown to increase levels of the vasodilator bradykinin, upregulate bradykinin receptors, augment nitric oxide release, and increase endothelial nitric oxide synthase expression.[127, 128] The cardiovascular protective effects offered by ARB and ACEi therapy may also be due in part to ACE2/Ang-(1-7)/Mas axis activation. In fact, multiple human and animal studies have shown that ARB and ACEi treatment can increase ACE2 expression and production of Ang-(1-7).[47]

Based on the current literature supporting a protective role for the ACE2/Ang-(1-7)/Mas axis in the cardiovascular system, as well as a physiological role for the presence of Mas and its activation by Ang-(1-7) in the cerebrovasculature, we have

developed the general hypothesis that Ang-(1-7) has the ability to exert a Mas-mediated cerebroprotective action during ischemic stroke. In the current study we have demonstrated that central administration of Ang-(1-7) prior to ischemic stroke by endothelin-1 (ET-1) induced middle cerebral artery occlusion (MCAO) elicits a decrease in infarct size and neurological deficits. This is the first demonstration of a cerebroprotective action of Ang-(1-7) during ischemic stroke.

Methods

Animals

Adult male Sprague Dawley rats were purchased from Charles River Farms (Wilmington, MA). All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

Chemicals

Ang-(1-7) and A-779 (D-Ala^7)-angiotensin (1-7) were purchased from Bachem Bioscience (Torrance, CA). ET-1 was purchased from American Peptide Company, Inc (Sunnyvale, CA, USA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ang-(1-7) and A-779 were dissolved in artificial cerebral spinal fluid (aCSF). ET-1 was dissolved in 0.9% saline.

Placement of Intracerebroventricular and Guide Cannulae

Eight-week-old male Sprague Dawley rats were anesthetized with a mixture of O_2 (1 L/min) and 4% isoflurane, placed in a Kopf stereotaxic frame, and anesthesia was maintained for the duration of the surgery using an O_2/isoflurane (2%) mixture delivered through a nose cone attached to the frame. The skull was exposed and a small hole was drilled for placement of an MCAO guide cannula in the cranium dorsal to the right hemisphere using the following stereotaxic coordinates (1.6 mm anterior and 5.2 mm

lateral to the bregma). A 21 gauge stainless steel guide cannula cut to 4 mm below the pedestal was lowered into the hole and affixed to the skull with 3 mounting screws and dental cement. During the same surgery, a second hole was then drilled in the cranium dorsal to the left hemisphere for placement of an intracerebroventricular cannula (kit 1, ALZET, Cupertino, CA) coupled to a 2 week osmotic pump (model 2002, ALZET, Cupertino, CA) *via* vinyl tubing. The following stereotactic coordinates were used (1.3 mm posterior and 1.5 mm lateral to bregma, 4.5 mm below the surface of the cranium). The osmotic pump was implanted subcutaneously between the shoulder blades as described previously.[86] Osmotic pumps were used to infuse Ang-(1-7) (1 μg/h), Ang-(1-7) (1 μg/h) plus A-779 (5 μg/h), A779 (5 μg/h) alone, or aCSF into the left lateral cerebral ventricle starting at the time of cannula placement and lasting until the animals were euthanized. These doses were determined based on concentrations of Ang-(1-7) shown to increase bradykinin and NO levels in the brain following stroke.[127, 128] Following this surgery, the wound was closed and the rat was administered an analgesic agent (buprenorphine; 0.05 mg/kg sc) before waking.

Endothelin–1 Induced Middle Cerebral Artery Occlusion

Seven days after the placement of ICV and guide cannulae, the ET-1 induced MCAO procedure was performed as we have previously reported with a minor modification.[138] Eight-week-old male Sprague Dawley rats were anesthetized as described above, and anesthesia was maintained for the duration of the injection using an O2/isoflurane (2%) mixture delivered through a nose cone attached to the frame. The cannula dummy was removed after which a 26 gauge needle attached to a 5 μL Hamilton microsyringe was lowered 8.7 mm ventral to bregma. Once the needle was in

place, 3 μL of 80 μM ET-1 was infused adjacent to the MCA at a rate of 1 μL/min using a Stoelting Quintessential Injector (Stoelting Co., Wood Dale, IL, USA). The needle was left in place for 3 min after the injection was complete and then removed slowly. The cannula dummy was then replaced and the rat was administered an analgesic agent (buprenorphine; 0.05 mg/kg sc) before waking. We have characterized this model previously by showing that injection of ET-1 can cause rapid constriction of the MCA followed by gradual reperfusion. In addition, a strong and significant correlation exists between the size of infarct measured in this model and several test scores used to assess neurological deficits in the work described here.[138] In addition, we have used laser doppler flowmetry to investigate the CBF reduction that results in cortical areas both adjacent (ischemic core) and distal (ischemic penumbra) to the site of ET-1 injection (Figure 3-1). It is clear from these data that CBF decreases dramatically in tissue adjacent to the proximal MCA and that CBF is reduced to a lesser degree in tissue of more distal MCA territories.

Visualization of MCA Branches *via* a Cranial Window

An additional group of rats was anesthetized as described above, after which a temporal craniectomy was performed to visualize the primary and secondary branches of the MCA as described previously.[138] An approximately 3-4 mm square piece of bone was removed from the left squamous portion of the temporal bone just caudal to the orbit. The dura was left in place and debris was cleared away using sterile 0.9% saline. Next, ET-1 induced MCAO was performed as described above except that the needle was left in place until all images were captured so that the focal plane would not be disturbed. The cerebral cortex and associated vessels visible through this cranial

window were imaged using a Sony Handycam HDR-SR12 (Sony; Minato, Tokyo, Japan) coupled to a Revelation surgical microscope (Seiler Instrument and Manufacturing; St. Louis, MO, USA). Vessel diameter was determined by averaging one primary and two secondary MCA branches per cranial window using ImageJ software (NIH) by an individual who had been blinded to the treatment group. A baseline image was captured prior to ET-1 injection and subsequent images were captured each 1 min interval for 60 min. Vessel diameter at each time point was normalized to the baseline vessel diameter so that comparisons could be made using multiple MCA branches of several rats.

Cerebral Blood Flow Monitoring

An additional group of rats was anesthetized as described above, after which laser doppler flowmetery was used to measure CBF for a period of time starting 1 min prior to ET-1 injection and lasting for 1 h after ET-1 injection. CBF measurements were performed using a Standard Pencil Probe and Blood FlowMeter coupled to a Powerlab 4/30 with LabChart 7 (ADInstruments, Inc, Colorado Springs, CO, USA). The probe was placed either just posterior to the MCAO guide cannula at the lateral skull ridge, or 1.6 mm anterior to Bregma and on the lateral skull surface. Data was recorded in arbitrary blood perfusion units at 1000 Hz. Baseline CBF was calculated by averaging a 1 min interval just prior to ET-1 injection. Changes in CBF were calculated as a percentage of baseline by averaging a 10 s interval every 1 min (Figure 3-6), or a 1 s interval every 1 s (Figure 3-1). This procedure allowed us to further validate ET-1-induced MCAO as a method for inducing ischemic stroke, as Figure 3-1 shows that blood flow is decreased by >90% within the area adjacent to the ET-1 injection (ischemic core).

Indirect Blood Pressure Monitoring

After undergoing surgery to implant an intracerebroventricular cannula (kit 1, ALZET, Cupertino, CA) coupled to a 6 week osmotic pump (model 2006, ALZET, Cupertino, CA) *via* vinyl tubing as described above, animals were allowed to recover for 1 week. Indirect blood pressure was recorded by tail-cuff once a week for 2 weeks as previously described.[86] Animals were warmed by a 200 W heating lamp for 5 min before restraint in a heated Plexiglas cage to which the animals were previously conditioned. A pneumatic pulse sensor was attached to the tail distal to an occluding cuff controlled by a Programmed Electro-sphygmomanometer (Narco Bio-Systems, Austin TX). Voltage outputs from the cuff and pulse sensor were recorded and analyzed by a Powerlab signal transduction unit and associated Chart software (ADInstruments, Colorado Springs, CO).

Neurological Deficits and Infarct Size

Neurological deficits and infarct size were evaluated as reported previously.[138] Neurological evaluations were performed using two separate scoring scales originally described by Bederson *et al*.[133] and Garcia *et al.,*[134] which cumulatively evaluate spontaneous activity, symmetry in limb movement, forepaw outstretching, climbing, body proprioception, response to vibrissae touch, resistance to lateral push, and circling behavior. Additionally, animals were evaluated for neurological deficits using a sunflower seed eating test.[135] Infarct volume was assessed by staining brain sections with 0.05% 2,3,5-triphenyltetrazolium chloride (TTC) for 30 minutes at 37°C. Tissue ipsilateral to the occlusion, which was not stained, was assumed to be infarcted. After fixation with 10% formalin, brain sections were scanned on a flatbed scanner (Canon)

and analyzed using ImageJ software (NIH). To compensate for the effect of brain edema, the corrected infarct volume was calculated using an indirect method.[129]

Immunohistochemistry

Seventy-two hours following ET-1 induced MCAO, rats were anesthetized with isoflurane and decapitated. Brains were removed and rinsed in ice cold PBS. Brains were then sectioned coronally using a brain blocker (David Kopf Instruments, Tujunga, CA, USA). A 4 mm section starting from the frontal pole was frozen fresh in OCT Compound (Sakura Finetek; Torrance, CA, USA). Twenty µm sections were cut using a cryostat, mounted onto glass slides, and dried overnight at room temperature. Sections were then fixed for 30 min with 10 % formalin. NeuN immunostaining procedure was performed as described previously.[139] The primary antibody used was mouse monoclonal anti-NeuN antibody (1:100) (Chemicon International; Temecula, CA, USA). The secondary antibody was Alexa Fluor 594 goat anti-mouse IgG (1:500) (Molecular Probes; Eugene, OR, USA). Sections were mounted and counter stained with Vectashield Mounting Medium with DAPI (Vector Laboratories; Burlingame, CA, USA).

Real – time Reverse Transcriptase – PCR (qRT-PCR)

For analyses of endogenous Mas and ACE2 mRNA, a 2 mm thick coronal section caudal to that used for TTC staining was isolated and dissected so that left and right cortical and subcortical tissues were separated into individual samples. Total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA, USA). Isolated RNA underwent DNAse I treatment to remove genomic DNA. Mas and ACE2 mRNA were reverse transcribed with a high-capacity cDNA reverse transcription kit (Bio-Rad Laboratories, CA, USA) and then analyzed *via* qRT-PCR in a PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) as detailed by us previously.[139]

Oligonucleotide primers and TaqMan probes specific for rat Mas and ACE2 were obtained from Applied Biosystems. Data were normalized to 18S rRNA.

Data Analysis

Data are expressed as means ± SEM. Statistical significance was evaluated, as specified in the figure legends, with the use of a Kruskal-Wallis test, Two-way Row matched ANOVA, One-way ANOVA, or unpaired t-test, as well as with Dunn's Multiple Comparison Test, the Bonferroni Test, or Turkey's Multiple Comparison Test for *posthoc* analyses when appropriate. Differences were considered significant at $p<0.05$. Individual p values are noted in the results and figure legends.

Results

Cerebral Blood Flow in the Ischemic Core and Penumbra during ET-1 Induced MCAO

Laser doppler flowmetry was used to investigate the CBF reduction that results in cortical areas both adjacent (ischemic core) and distal (ischemic penumbra) to the site of ET-1 injection (Figure 3-1). Representative tracings show that CBF decreases dramatically in the ischemic core and that CBF is reduced to a lesser degree in the ischemic penumbra. CBF increases gradually toward baseline during the 1 h in which data was captured.

Localization of Mas and ACE2 in the Cerebrum

Prior to determining if Ang-(1-7) exerts beneficial actions during ischemic stroke we investigated whether transcripts of ACE2, an enzyme that is of primary importance in synthesizing Ang-(1-7), as well as Mas, an Ang-(1-7) receptor are located in the brain regions that are greatly impacted by ET-1 induced MCAO. mRNA levels of both Mas and ACE2 were measured in both frontal cortex and subcortex (Figure 3-2). Mas and

ACE2 mRNA were detected in both of these regions with a significantly higher level of Mas in the cortex ($p < 0.01$) and a significantly lower level of ACE2 in the cortex ($p < 0.05$) compared to subcortical tissue samples.

Mas mRNA Levels are increased in the Lesioned Cortex 24 h after Stroke

Mas mRNA levels were measured in the frontal cortex (Figure 3-3) in rats 24 h after either ET-1 induced MCAO or sham stroke surgery (0.9% saline injejcction). Mas mRNA was significantly higher in the lesioned (right) cortex of rats that underwent ET-1 induced MCAO compared to the right cortex of sham stroke rats ($p < 0.05$). There was no difference in Mas mRNA levels in the unlesioned (left) cortex between the two groups.

Cerebroprotective Action of Ang-(1-7)

The effect of Ang-(1-7) pre-treatment on ET-1-induced cerebral damage was assessed by TTC staining, whereby non-infarcted gray matter is stained red after incubation in TTC, delineating the infarct region in white. Seventy two hours following ET-1-induced MCAO there was a cerebral infarct can be seen in rats that had been pre-treated with aCSF (ICV infusion for 7 days) (Figure 3-4). No such infarcts were observed in rats that underwent sham MCAO (0.9% saline instead of ET-1 injection) (Figure 3-4). Central pre-treatment of rats for 7 days with Ang-(1-7) (1 μg/h, ICV) prior to ET-1-induced MCAO significantly reduced the infarct size compared to an aCSF pre-treated control group ($p<0.05$). The length of treatment was designed to efficiently determine the effectiveness of Ang-(1-7) either before, during, or after stroke. The Ang (1-7)-induced cerebroprotection was reversed significantly when Ang-(1-7) was co-infused for 7 days with its receptor antagonist, A-779 (5 μg/h, ICV) ($p<0.05$). Pre-

treatment with an ICV infusion of A-779 alone did not significantly modify the ET-1 induced cerebral damage (Figure 3-3).

The data in Figure 3-3 were reinforced by qualitative data from immunostaining which revealed that central pre-treatment with Ang-(1-7) reduced the neuronal damage 72h after ET-1 induced MCAO. Tissue adjacent to that used for TTC staining was sectioned, and stained with antibodies raised against the neuron specific protein, NeuN. The representative fluorescence micrograph shown in Figure 3-5 (*upper left panel*) is a low power view of NeuN immunoreactivity (red) in the cerebrum of a rat that had been subjected to a sham MCAO. The higher power view in Figure 3-5 (*upper right panel*) taken from the inset in the top left panel shows co-localization (pink cells) of NeuN immunoreactivity (red) with DAPI nuclear stain (blue) in a rat that received a sham MCAO. In rats that underwent ET-1 induced MCAO and ICV aCSF treatment, NeuN immunoreactivity was fragmented and there was much less co-localization with the nuclei (representative micrograph, Figure 3-5, *lower left panel*). Brain tissue from rats treated ICV with Ang-(1-7) prior to ET-1 induced MCAO displayed more co-localization of NeuN and DAPI (pink cells, Figure 3-5, *lower left panel*) compared with the rats treated ICV with aCSF.

In addition to the gross and histological evidence for cerebroprotection, central pre-treatment with Ang-(1-7) attenuated the neurological deficits attributable to ET-1 induced MCAO. For example, 72 hr following ET-1 induced MCAO there were significant behavioral deficits in rats that had been pre-treated with aCSF (ICV infusion for 7 days), according to the Bederson Exam (score>0) and the Garcia Exam (score<18) (Figures 5A and 5B). No such deficits were observed in rats that underwent

sham MCAO (0.9% saline instead of ET-1 injection). Central pre-treatment of rats for 7 days with Ang-(1-7) (1 μg/h, ICV) prior to ET-1-induced MCAO showed a trend toward a reduced Bederson Exam Score compared to the aCSF pre-treated control group (Figure 3-6A). This cerebroprotective trend was reversed when Ang-(1-7) was co-infused for 7 days with its antagonist, A-779 (5 μg/h, ICV). The trend toward cerebroprotection was also seen with an improved Garcia Exam Score compared to the aCSF pre-treated control group (Figure 3-6B). Again, this cerebroprotective trend was diminished when Ang-(1-7) was co-infused for 7 days with its antagonist, A-779 (5 μg/h, ICV). Pre-treatment with an ICV infusion of A-779 alone resulted in both Bederson and Garcia Exam Scores which were similar to the score for aCSF pre-treatment.

Utilization of a sunflower seed eating task allowed a more sensitive evaluation of neurological function that provided strong evidence for the cerebroprotective properties of Ang-(1-7) during focal cerebral ischemia. Rats were given 5 unshelled sunflower seeds and then timed while manipulating and opening the shells to eat the seeds. Rats with significant neurological deficits display longer latency to remove the shell. In addition, deficits at this task result in rats that are inefficient at removing the shell and therefore break it into many small pieces. In summary, both increasing latency to open the shell and increasing number of shell pieces are indications of more severe neurological deficit. Central pre-treatment of rats for 7 days with Ang-(1-7) (1 μg/h, ICV) prior to ET-1-induced MCAO showed a significant reduction in the time required to eat 5 sunflower seeds compared to an aCSF pre-treated control group ($p<0.01$, Figure 3-6C). This cerebroprotection was reversed when Ang-(1-7) was co-infused for 7 days with its antagonist, A-779 (5 μg/h, ICV). Pre-treatment with an ICV infusion of A-779 alone

resulted in a time to eat 5 sunflower seeds which was not significantly different than the aCSF pre-treatment group. Neurological evaluation by counting the number of shell pieces produced during this task also supported the conclusion that central pre-treatment with Ang-(1-7) is cerebroprotective during focal cerebral ischemia. Rats receiving central Ang-(1-7) prior to ET-1 induced MCAO produced significantly fewer shell pieces than the aCSF pre-treated control group ($p<0.001$, Figure 3-6D). This cerebroprotection was reversed when Ang-(1-7) was co-infused for 7 days with its antagonist, A-779. Pre-treatment with an ICV infusion of A-779 alone resulted in a number of shell pieces, which was not significantly different than the aCSF pre-treatment group. Two rats that underwent sham strokes *via* injection of 0.9% saline in place of ET-1 demonstrated a rapid ability to eat sunflower seeds while producing few shell pieces, indicating a lack of behavioral deficits (Figures 5C and 5D).

Ang-(1-7) does not Alter ET-1 Induced MCA Constriction

In order to assess alterations in ET-1 induced MCA constriction that may have occurred due to chronic central Ang-(1-7) infusion, primary and secondary MCA branches (black arrows) were viewed through a cranial window (Figure 3-7). One group of rats was pre-treated with central aCSF infusion for 7 days prior to receiving an intracranial injection with 3 μL of ET-1 (80 μM) into the brain parenchyma adjacent to the MCA. This ET-1 injection resulted in abrupt maximal constriction of the proximal MCA branches within 5 minutes followed by recanalizaton of the vessel (Figure 3-7, D-F and J). Another group of rats undergoing a sham MCAO was pre-treated with central aCSF infusion for 7 days prior to receiving an intracranial injection with 3 μL of 0.9% saline in place of ET-1. Vessel diameter remained relatively stable at baseline values

following this saline injection (Figure 3-7, A-C and J). Finally, a third group of rats was pre-treated with central Ang-(1-7) infusion for 7 days prior to receiving an intracranial injection with ET-1. Compared with the group receiving control aCSF pre-treatment prior to ET-1 injection, there was a similar, non-significantly different, and abrupt maximal vessel diameter decrease within 9 min (Figure 3-7, G-I and J). Additionally, it is of note that there was no significant difference in vessel diameter at any time point between Ang-(1-7) pre-treated and aCSF pre-treated rats that received an injection of ET-1 adjacent to the MCA. Lastly, both the aCSF and Ang-(1-7) pre-treatment groups receiving an ET-1 brain injection displayed a decrease in vessel diameter 3 min after the start of injection that was significantly greater than the group undergoing sham MCAO ($p<0.05$) and remained significantly different for 31min and 23 min respectively. These results indicate that ET-1 injection adjacent to the MCA causes constriction of that vessel followed by recanalization. In addition, pre-treatment with Ang-(1-7) does not significantly alter the amplitude or time course of the ET-1 induced MCA constriction.

Ang-(1-7) does not Alter ET-1 Induced Cerebral Blood Flow in the Cortex Distant from the Primary Branch of the MCA

To assess the effects of chronic central Ang-(1-7) infusion on blood flow in microvascular beds during ET-1 induced MCA constriction, CBF was monitored transcortically for 1 h *via* laser doppler flowmetry during stroke induction. The data presented in Figure 3-1 demonstrate the reduction in cerebral blood flow at regions of the brain corresponding to the ischemic core and the ischemic penumbra following injection of 3 μL of ET-1 (80 μM) into the brain parenchyma adjacent to the MCA. These data confirm that the ET-1 injection produces a significant ischemic action. In

rats that received an ICV infusion of Ang-(1-7) for 7 days, ET-1 injection as above resulted in abrupt reduction of CBF in the ischemic penumbra region followed by a gradual return to baseline over the period of monitoring. There were no significant differences in CBF at any time between rats infused ICV with Ang (1-7) or aCSF (Figure 3-8). Therefore, Ang-(1-7) had no effect on the reduction in CBF in the cortical areas distant from the primary branch of the MCA.

ICV Infusion of Ang-(1-7) does not Alter Systemic Blood Pressure

In order to assess SBP changes that might be caused by chronic central Ang-(1-7) infusion, two groups of 6 rats each were treated with either Ang-(1-7) (1 μg/h, ICV) or aCSF starting at eight weeks of age and SBP was recorded by the tail-cuff method after 1 and 2 weeks of treatment. The data shown in Figure 3-9 indicate that ICV infusion of Ang-(1-7) under the same conditions that attenuated the ET-1 induced cerebral infarcts and behavioral deficits produced no changes in systemic blood pressure.

Discussion

The most significant findings of this study are that central pre-treatment with Ang-(1-7) and activation of the Ang-(1-7) receptor, Mas, attenuate the neurological deficits and brain tissue damage produced in an ET-1 induced MCAO model of ischemic stroke independent of any effects on cerebral blood flow or systemic blood pressure. Previously, we have shown that ET-1 induced MCAO is a minimally invasive model of ischemic stroke that provides a rapid constriction, sustained occlusion, and then gradual reperfusion of the proximal MCA. [138] The current study provides evidence that this constriction is not altered by pre-treatment with Ang-(1-7), which was a concern when using the ET-1 induced MCAO as a disease model for investigating manipulations of the brain RAS. Our data also suggests that the cerebroprotective effect of central Ang-(1-

7) pre-treatment is not due to attenuation of the decrease in CBF in the vascular territory of the MCA. In addition, our results confirm the presence of Mas and ACE2 transcripts in relevant brain regions affected by the ET-1 induced MCAO model, as well as increased Mas mRNA levels in the lesioned cortex 24 h after stroke. This is the first report of cerebroprotection in a model of ischemic stroke elicited *via* Ang-(1-7) stimulation of its receptor, Mas.

The presence of both Mas and ACE2 in multiple cell types of the cerebral cortex and subcortical structures has been confirmed previously. Mas is expressed broadly in areas such as hippocampus, cortex, olfactory bulbs and thalamus of rats or mice.[118-121] In addition to Mas, ACE2 has been identified as a key component in the vasoprotective axis of the RAS and is known to be present in the brain. Several investigations into the CNS localization of ACE2 have detected low levels of ACE2 mRNA in the human brain[123] and ACE2 immunostaining in human brain vascular smooth muscle as well as endothelial cells.[124] More recently, ACE2 has been detected in glia during primary cell culture experiments.[125] However, *in vivo* analyses of mouse brains have localized both ACE2 mRNA and protein to predominately neurons, but in broad areas of cardiovascular and non-cardiovascular control.[126] Our data support the literature, which indicates the presence of key components of the ACE2/Ang-(1-7)/Mas axis in brain tissue affected by the ET-1 induced MCAO model of focal cerebral ischemia. Furthermore, we have demonstrated for the first time that Mas mRNA levels increase in the lesioned cortex after stroke. Increased expression of Mas implicates the Ang-(1-7) receptor as a key player with a potential protective role during stroke pathophysiology.

Based on the diverse localizations of both Mas and ACE2 described in the literature, we cannot preclude the possibility that Ang-(1-7) cerebroprotection is mediated by its actions in either the parenchymal cells of the CNS or the cells of the cerebrovasculature. Activation of Mas with chronic or acute peripheral administration of Ang-(1-7) increases cerebral blood flow.[93-95] This increase in blood flow can possibly be explained by data which indicate that central administration of Ang-(1-7) shortly following stroke onset has been shown to increase bradykinin levels in the brain, as well as upregulate bradykinin receptors.[127] In addition, nitric oxide release and endothelial nitric oxide synthase expression are increased with Ang-(1-7) treatment in this model.[128] These results point toward a potential mechanism of cerebroprotection mediated by Ang-(1-7) stimulation of Mas and subsequent release of bradykinin, stimulation of bradykinin receptors, and release of NO. Our results indicate that central Ang-(1-7) at a dose equal to that which leads to release of NO did not inhibit the decrease in CBF within the MCA territory during ET-1 induced MCAO. Therefore, we have provided evidence against alterations in cerebrovascular dynamics as a mechanism of Ang-(1-7) cerebroprotection.

Furthermore, we have begun an investigation to determine the role of CNS parenchymal cells (neurons and glia) in Ang-(1-7) cerebroprotection. Preliminary data with PCR arrays and qRT-PCR show that levels of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα and also of inducible nitric oxide synthase are increased in the ipsilateral cortex at 24 h after ET-1 induced MCAO. Our data also indicate that these effects are attenuated by central Ang-(1-7) pre-treatment, suggesting an interruption of pro-inflammatory signaling as a mechanism of cerebroprotection. We are currently

pursuing further investigations into this mechanism as well as studies to discover the cellular localization of Mas in the cerebral cortex. It is also of note that there are reports of both systemic Ang-(1-7) administration leading to a decrease in local cardiac Ang II levels,[140] as well as Ang-(1-7) inhibiting the conversion of Ang I to Ang II by ACE in kinetic studies.[141] Further experiments investigating Ang II levels in the ET-1 model of ischemic stroke could determine if a similar event is occurring in the brain due to Ang-(1-7) treatment.

In summary, our findings support a protective role for Ang-(1-7) and stimulation of Mas during cerebral ischemia. The high prevalence of stroke and its resulting morbidity and mortality indicate the importance of investigations into novel therapeutic strategies for stroke prevention and treatment. This study demonstrates the therapeutic benefit of Mas activation with Ang-(1-7) during stroke. Additional, unpublished data from our laboratory indicate that pharmacological activation of ACE2 provides a similar benefit through the activation of Mas. Finally, as the first report of Ang-(1-7) elicited cerebroprotection in a model of focal cerebral ischemia our results highlight the ACE2/Ang-(1-7)/Mas axis as a promising target for future stroke therapies.

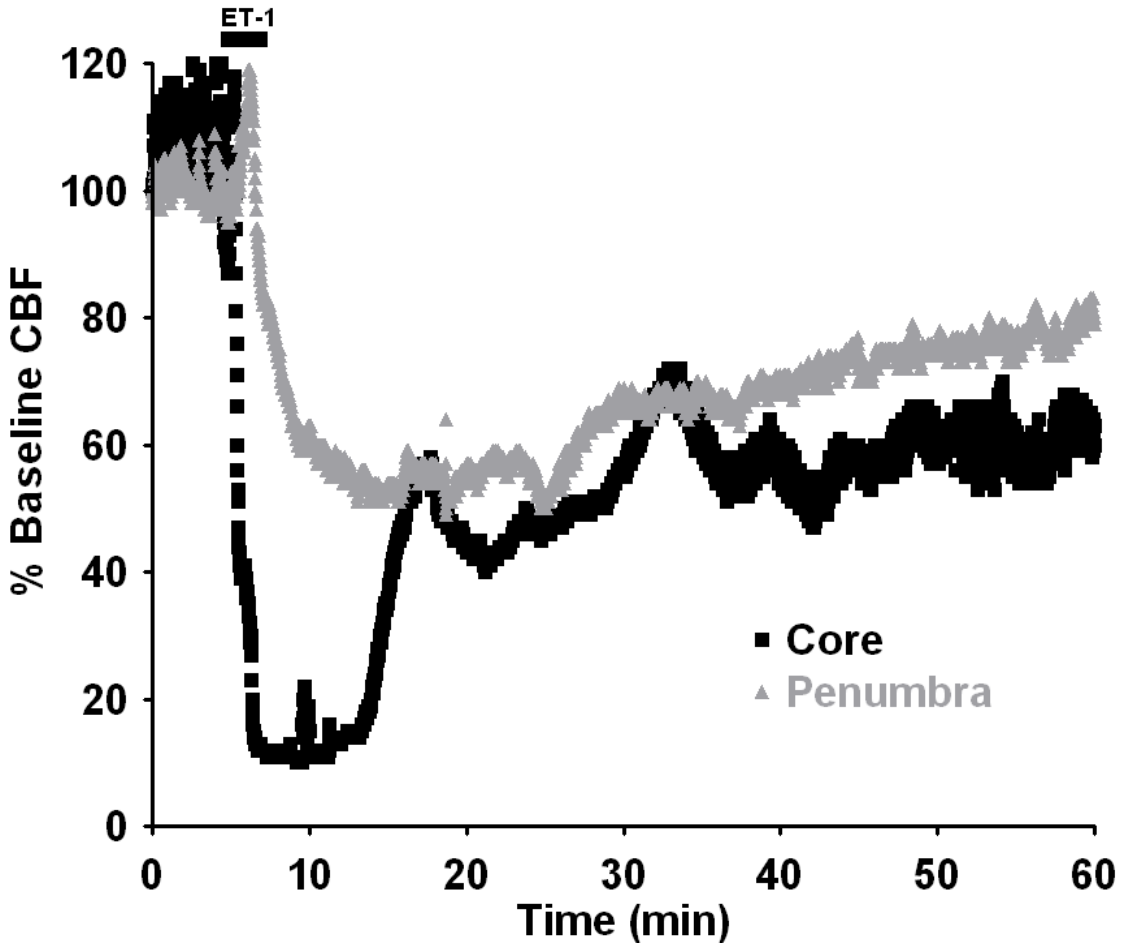

Figure 3-1. Cerebral blood flow in the ischemic core and penumbra during ET-1 induced MCAO. Laser doppler flowmetry was used to investigate the CBF reduction that results in cortical areas both adjacent (ischemic core) and distal (ischemic penumbra) to the site of ET-1 (80 mM) injection. Representative tracings from one rat are shown for each probe position. The data is presented as a percent of the baseline signal.

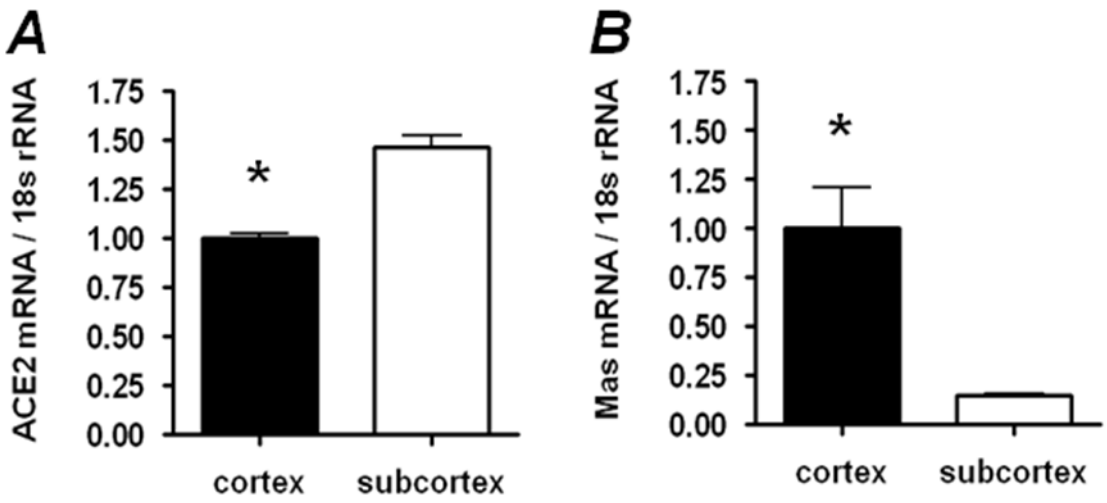

Figure 3-2. Localization of Mas and ACE2 in the cerebrum. mRNA levels of both Mas (A) and ACE2 (B) were measured by qRT-PCR in both frontal cortex (n = 4) and subcortex (n = 2) (Panels G and H). Data are presented as means ± SEM of the levels of ACE2 or Mas mRNA, normalized against 18s rRNA. * $p < 0.05$, ** $p < 0.01$ (unpaired t-test)

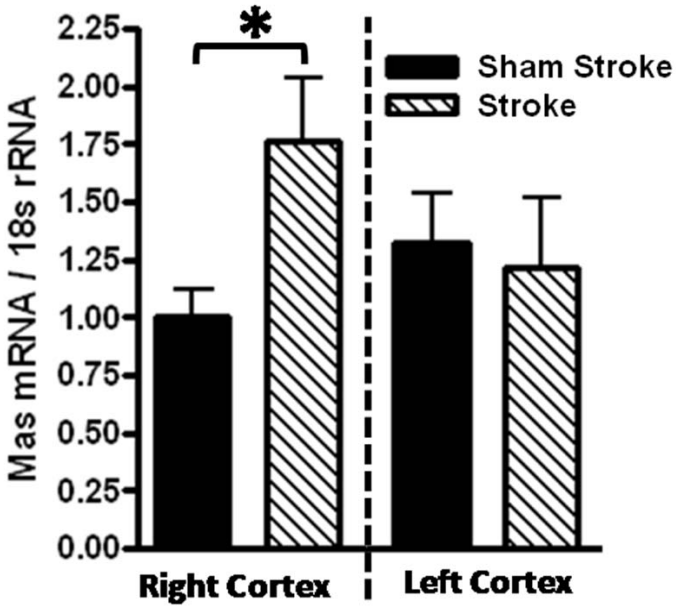

Figure 3-3. Mas mRNA levels are increased in the lesioned cortex 24 h after stroke. mRNA levels of Mas were measured by qRT-PCR in the frontal cortex of rats that underwent ET-1 induced MCAO surgery (stroke, n = 5) or 0.9% saline injection (sham stroke, n = 5). Data are represented as means ± SEM of the levels of Mas mRNA normalized against 18s rRNA. * $p < 0.05$ (One-way ANOVA with *post-hoc* Tukey's Multiple Comparison Test)

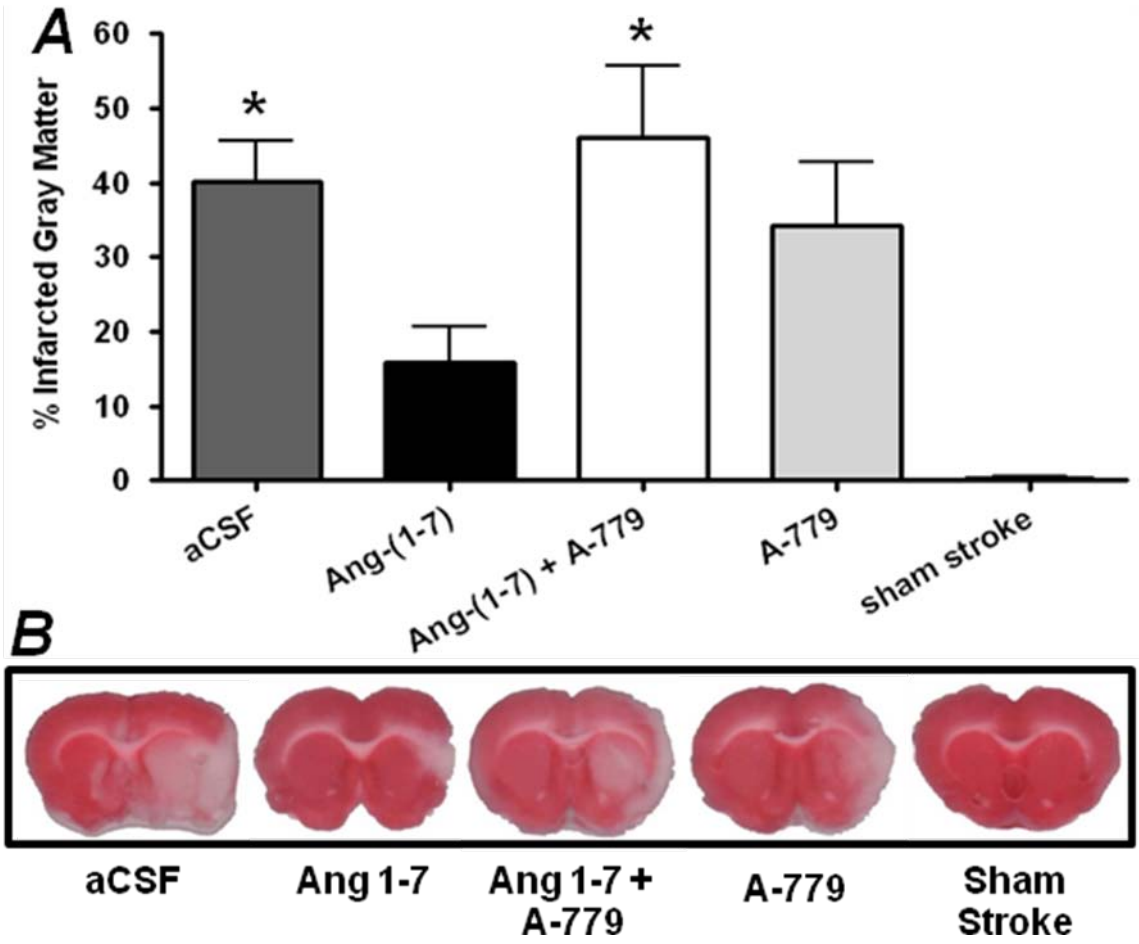

Figure 3-4. Intracerebral pre-treatment with Ang-(1-7) reduces CNS infarct size 72 h after ET-1 induced MCAO. Rats were pre-treated *via* the ICV route with either Ang-(1-7) (1 μg/h; n = 9), aCSF (n = 23), Ang-(1-7) + A-779 (5 μg/h; n = 9), or A-779 (n = 7) alone for 7 days prior to MCAO induced by intracranial injection of ET-1 (80 mM). In addition, two rats received a sham MCAO with 0.9% saline injection in place of ET-1. Brains were removed for TTC staining 72 h after stroke. (A) Bar graphs show the % infarcted gray matter in each treatment group. Data are presented as means ± SEM. Kruskal-Wallis Test ($p = 0.025$), * $p < 0.05$ vs. Ang-(1-7) (Dunn's Multiple Comparison Test) (B) Representative brain sections show infarcted (white) and non-infarcted (red) gray matter under the treatment conditions indicated.

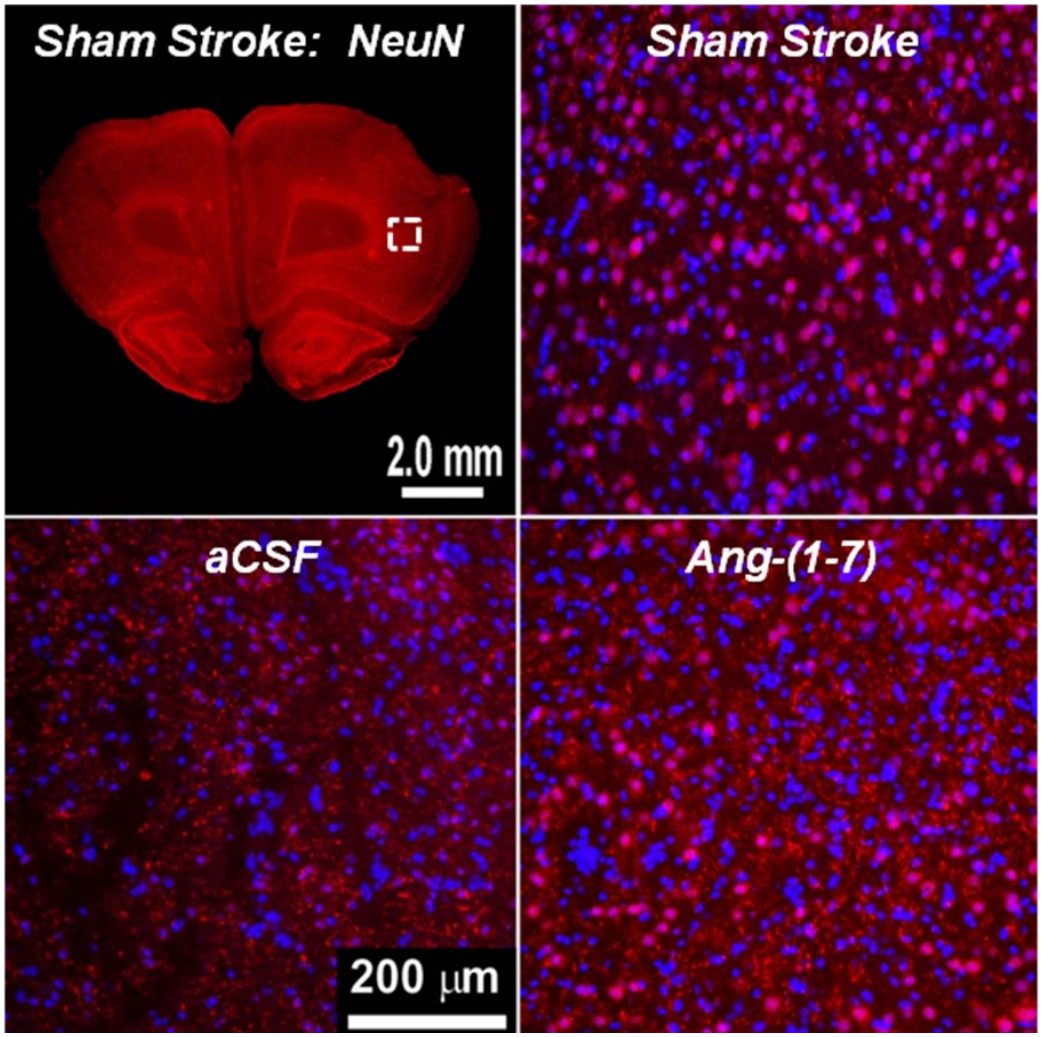

Figure 3-5. Intracerebral pre-treatment with Ang-(1-7) reduces neuronal damage 72 h after ET-1 induced MCAO. Rats were pre-treated via the ICV route with either Ang-(1-7) (1 μg/h) or aCSF for 7 days prior to ET-1 induced MCAO. In addition, a group was pre-treated with aCSF and underwent a sham stroke surgery with injection of 0.9% saline in place of ET-1. Representative fluorescence micrographs (two upper panels) are from rats that underwent sham stroke surgery. The upper left panel shows strong NeuN immunoreactivity (red) in healthy brain tissue. The remaining panels show NeuN immunoreactivity co-localized with DAPI nuclear stain (pink color cells) in areas of the frontal cortex within the MCA territory corresponding to the white dotted outline in the upper left panel. In rats that underwent MCAO (aCSF panel), NeuN immunoreactivity was fragmented and there was little co-localization (pink color) with the nuclear marker (DAPI). Tissue from rats pre-treated with Ang-(1-7) displayed neuron morphology and co-localization that was similar to tissue from the sham stroke brain tissue (Sham Stroke panel). NeuN (red), DAPI (blue), co-localization (pink). Magnifications: 20X (upper left panel), 100X (all other panels)

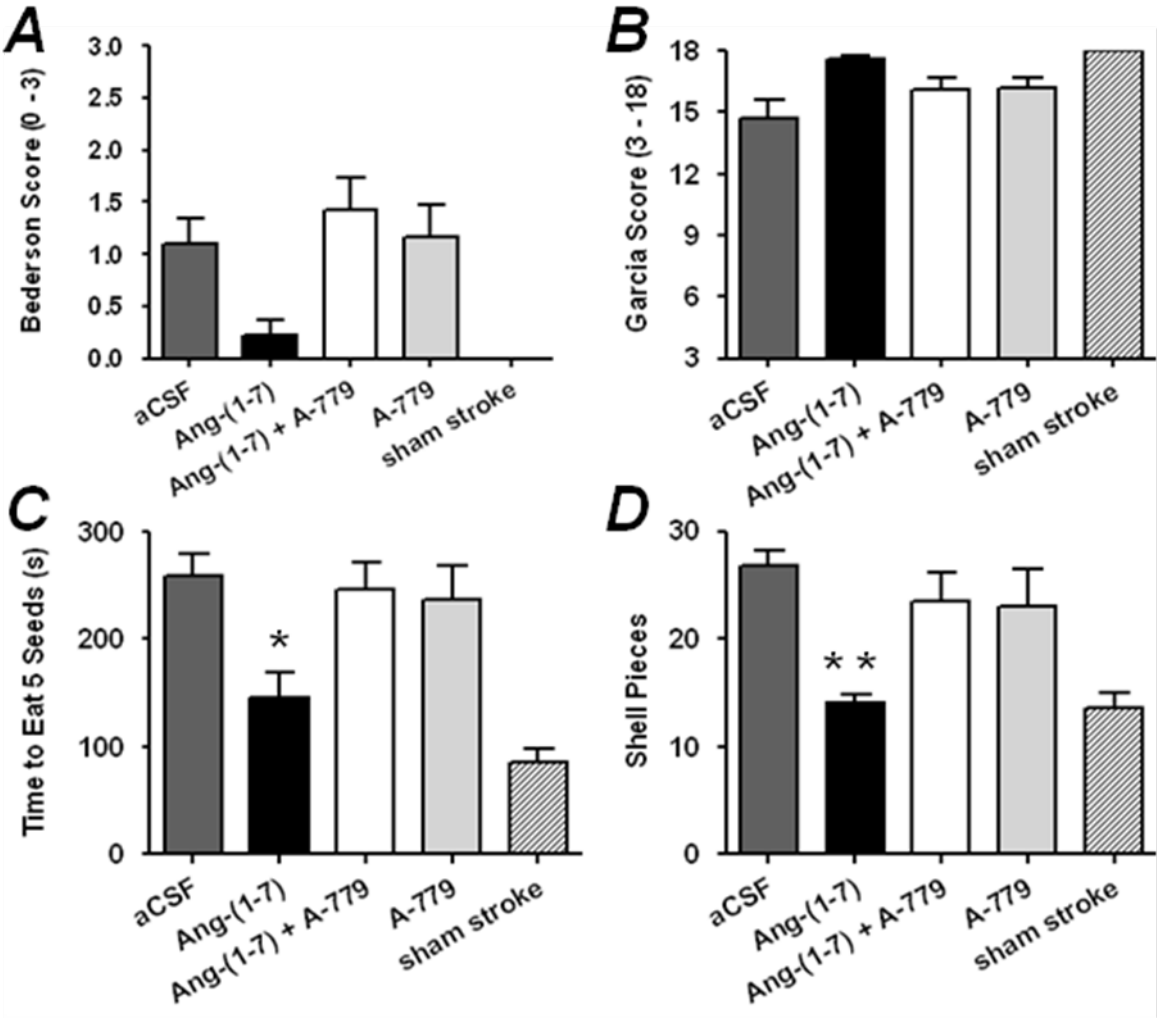

Figure 3-6. Intracerebral pre-treatment with Ang-(1-7) reduces neurological deficits 72 h after ET-1 induced MCAO. Rats were pre-treated *via* the ICV route with either Ang-(1-7) (1 μg/h; n = 9), aCSF (n = 23), Ang-(1-7) + A-779 (5 μg/h; n = 9), or A-779 (n = 7) alone for 7 days prior to ET-1 induced MCAO. Seventy-two hours later, neurological deficits were assessed *via* the Bederson Neurological Exam (*Panel A*) and the Garcia Neurological Exam (*Panel B*), as well as the Sunflower Seed Eating Test for the time to eat 5 seeds (*Panel C)* and the number of shell pieces *(Panel D).* Data are represented as means ± SEM. Bederson Exam $p < 0.05$ (Kruskal-Wallis Test) with no post-hoc significance (Dunn's Multiple Comparison Test). Garcia Exam $p = 0.08$ (Kruskal-Wallis Test). * $p < 0.01$ or ** $p < 0.001$ vs. Ang-(1-7)

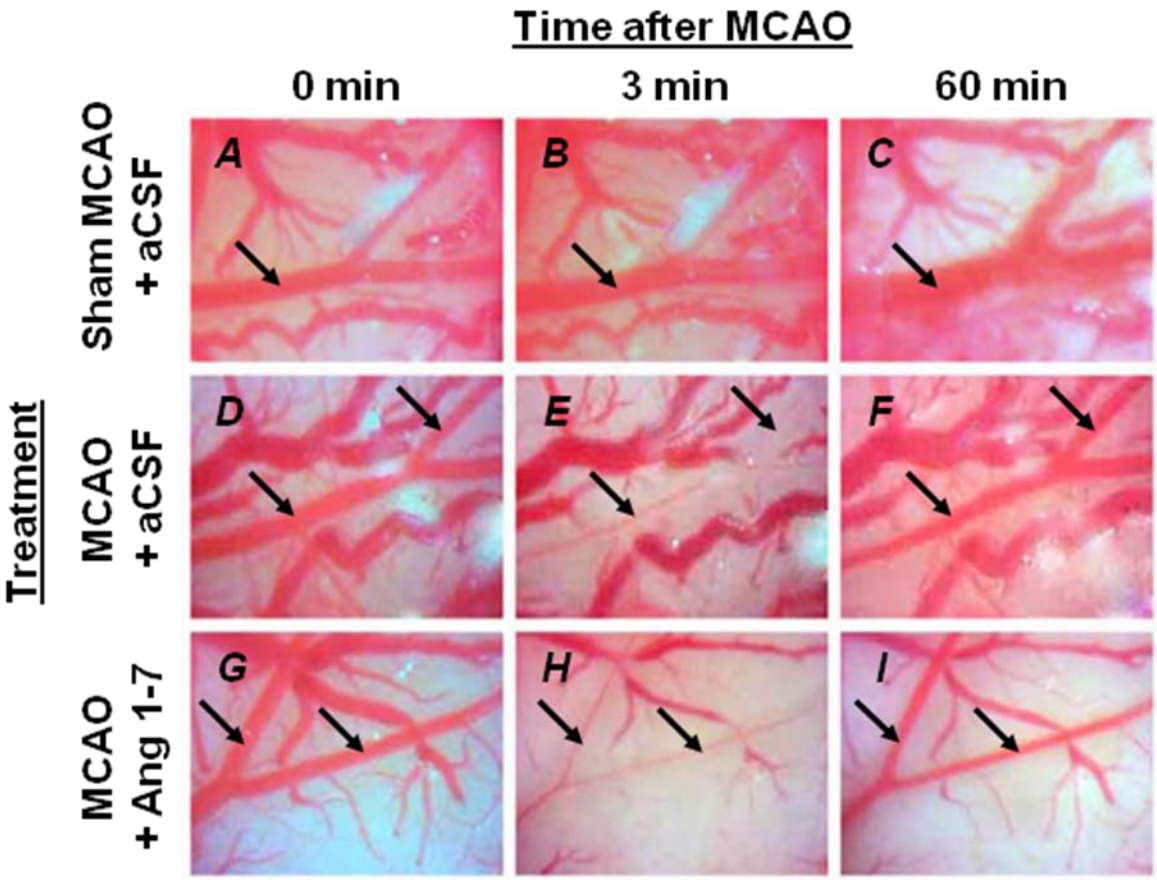

Figure 3-7. Central Ang-(1-7) does not alter ET-1 induced MCA constriction. (*A*–I) Visualization of MCA branches (arrows) during ET-1 induced vasoconstriction. Primary and secondary branches of the MCA were visualized with a surgical microscope after temporal craniotomy to create a cranial window. Images were captured at a rate of 1 min^{-1}, starting immediately prior to ET-1 injection (0 min), throughout the ET-1 injection (3 min) and for at least 60 min after initiation of the ET-1 injection. Representative images are shown for rats that underwent 7 days of ICV aCSF pre-treatment prior to a sham MCAO (3 mL of 0.9% saline injection, n = 4, A–C), 7 days of ICV aCSF pre-treatment prior to an ET-1 induced MCAO (3 mL of 80 mM ET-1 injection, n=4, D-F), and 7 days of ICV Ang-(1-7) (1 μg/h) pre-treatment prior to an ET-1 induced MCAO (n=4, G–I). (*J*) Vasoconstriction of primary or secondary MCA branches was quantified as the percentage of baseline vessel diameter. Data are means ± SEM. Baseline vessel diameter was determined prior to ET-1 or 0.9% saline injection (time = 0 min). *$p < 0.05$ for aCSF vs Sham MCAO and $^{\dagger}p < 0.05$ for Ang-(1-7) vs Sham MCAO [two-way RM ANOVA ($p < 0.001$) followed by Bonferroni post test].

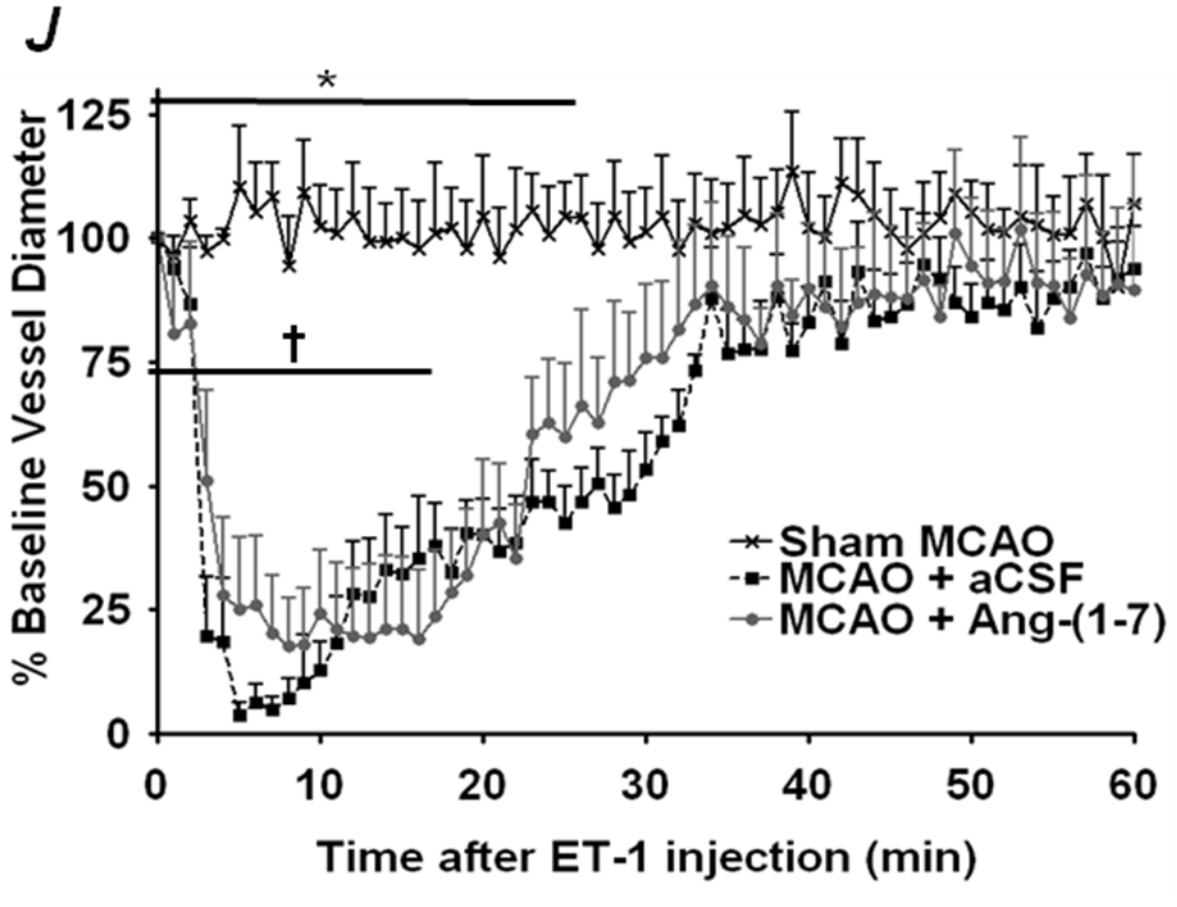

Figure 3-7. Continued

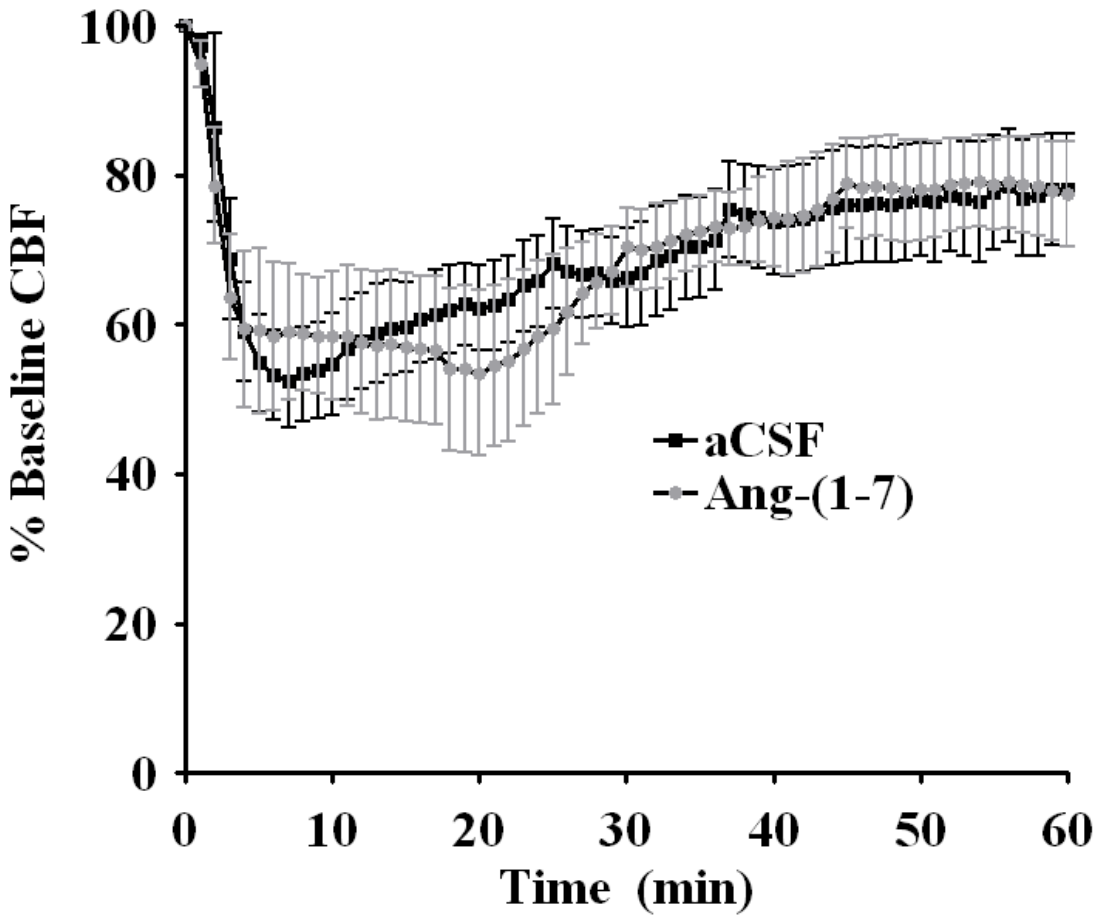

Figure 3-8. Central Ang-(1-7) does not alter ET-1 induced cerebral blood flow in the cortex distant from the primary branch of the MCA. Lasar Doppler flowmetry was used to monitor CBF in the vascular territory of the MCA distal to the site of ET-1 injection. Data are presented as means ± SEM of the percent change from baseline CBF. ET-1 injection takes place over a period of 3 minutes starting at 0 min on this graph. No significant differences exist between Ang-(1-7) (n = 6) and aCSF (n = 6) treatment groups at any time point [two-way RM ANOVA].

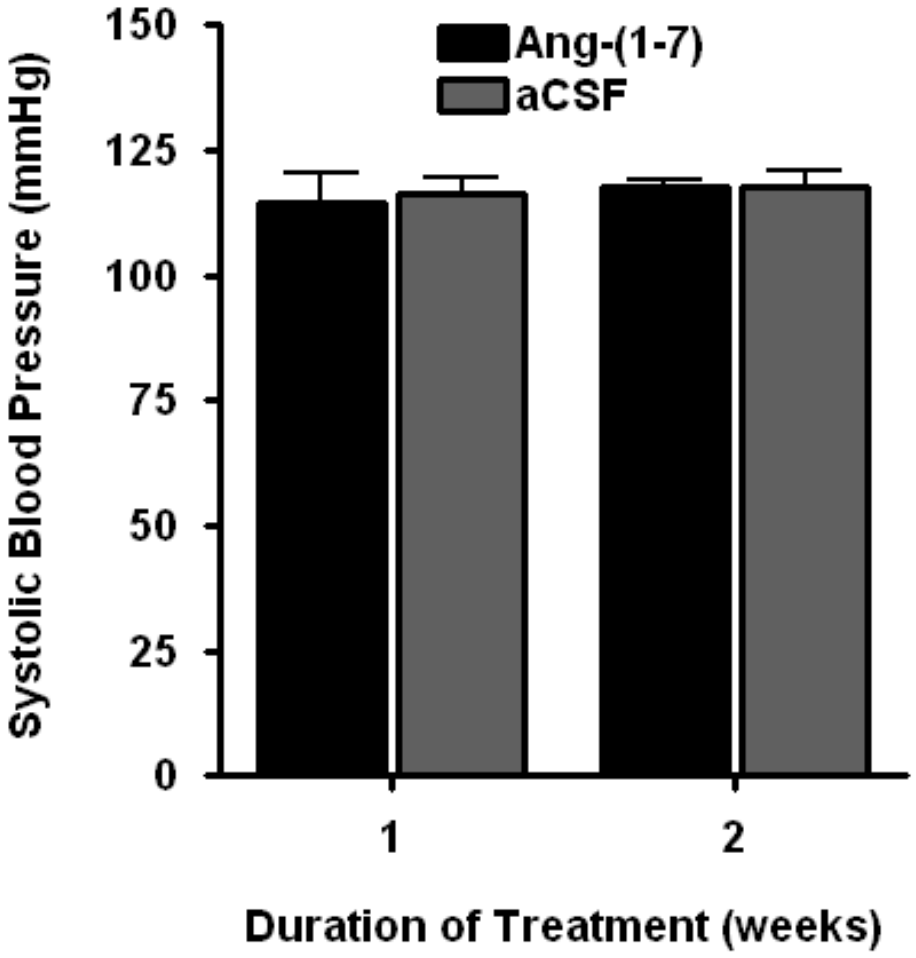

Figure 3-9. Central Ang-(1-7) pre-treatment does not alter systolic blood pressure. Rats were administered either Ang-(1-7) (1 μg/h; n = 6) or aCSF (n = 6) via the ICV route over a period of 2 weeks. SBP was measured using the tail-cuff method at weekly intervals. Data are represented as means ± SEM of SBP.

CHAPTER 4
ACE2 ACTIVATION AS A TARGET FOR CEREBROPROTECTION DURING ISCHEMIC STROKE

Introduction

We have recently shown that central pre-treatment with Angiotensin-(1-7) [Ang-(1-7)] can greatly reduce tissue damage and neurological deficits that result during focal cerebral ischemic injury.[142] These studies were performed using a rat model of endothelin-1 (ET-1)-induced Middle Cerebral Artery Occlusion (MCAO) and were the first to reveal the cerebroprotective properties of central Ang-(1-7) administration. In addition, co-administration of the Ang-(1-7) receptor antagonist, D-Alanine-[Ang-(1-7)] (A779) reduced the Ang-(1-7) cerebroprotective activity. Thus, Ang-(1-7) cerebroprotection was mediated *via* activation of its endogenous receptor, Mas. This was the first description of Ang-(1-7) activation of Mas mediated cerebroprotection during stroke. Therefore, the ACE2/Ang-(1-7)/Mas Axis is a promising target for cerebprotective therepy.

Thus far, we have focused on central administration of exogenous Ang-(1-7) to combat the pathophysiology of stroke. However, endogenous sources of Ang-(1-7), as well as several pathways for Ang-(1-7) production exist. The pathways leading to Ang-(1-7) can be either Angiotensin Converting Enzyme (ACE) dependent or ACE independent. ACE dependent pathways include conversion of Angiotensin I (Ang I) to Angiotensin II (Ang II) by ACE and subsequent conversion of Ang II to Ang-(1-7) by Angiotensin Converting Enzyme 2 (ACE2).[45] In addition, ACE2 is capable of converting Ang I to Ang-(1-9) which can then be converted to Ang-(1-7) by ACE.[46] This second ACE dependent route involving Ang-(1-9) production as an intermediate step is likely

less relevant because ACE2 is 400 times more efficient at catalyzing the conversion of Ang II to Ang-(1-7) than Ang I to Ang-(1-9).[49] In addition to the ACE dependent pathways for Ang-(1-7) production, various enzymes contribute to the production of Ang-(1-7) *via* ACE independent mechanisms. These enzymes include Neprilysin, proyl-endopeptidase, proyl-carboxypeptidase, Chymase, and Cathepsin A.[50-53] The contributions of ACE independent pathways likely become much more relevant in the presence of ACE inhibitors (ACEi). However, these enzymes lack specificity for angiotensin peptide metabolism and they are unlikely to be major contributors to Ang-(1-7) synthesis under physiological conditions. Although, multiple pathways leading to Ang-(1-7) production exist, it is clear that ACE2 is a key regulator of this angiotensin peptide.

Thus, ACE2 has recently been a target for cardiovascular disease therapy.[47] In fact, several small molecule ACE2 activators have been identified that selectively increase ACE2 activity without having an effect on ACE activity.[143, 144] One of these molecules, Diminazene aceturate (DIZE) has been shown to decrease blood pressure dramatically and dose dependently when administered acutely. In addition, a modest decrease in blood pressure and associated reductions in end organ damage are observed with chronic administration of DIZE to spontaneously hypertensive rats. Another of these activators, XNT (1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one), had similar antihypertensive effects and also prevented the pathophysiology right heart failure and pulmonary fibrosis in a rat model of pulmonary hypertension induced by monocrotaline.[90]

Therefore, these small molecule ACE2 activators are promising compounds for ACE2/Ang-(1-7)/Mas axis activation and treatment of cardiovascular disease.

Based on our previous studies supporting a protective role for ACE2/Ang-(1-7)/Mas axis during stroke, as well as a protective effect of ACE2 activation by DIZE in various models of cardiovascular disease, we have developed the general hypothesis that DIZE can activate ACE2 in the brain, leading to increased production of Ang-(1-7), and a subsequent Mas-mediated cerebroprotective action during ischemic stroke. In the current study we have demonstrated that central administration of DIZE prior to ischemic stroke by endothelin-1 (ET-1) induced middle cerebral artery occlusion (MCAO) elicits a decrease in infarct size and neurological deficits. This is the first demonstration of a cerebroprotective action of the ACE2 activator, DIZE.

Methods

Animals

Adult male Sprague Dawley rats were purchased from Charles River Farms (Wilmington, MA). All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

Chemicals

Diminazene aceturate (DIZE) was purchased from Sigma-Aldrich (St. Lous, MO, USA). A-779 (D-Ala^7)-angiotensin (1-7) was purchased from Bachem Bioscience (Torrance, CA). ET-1 was purchased from American Peptide Company, Inc (Sunnyvale, CA, USA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA). DIZE and A-779 were dissolved in H_2O. ET-1 was dissolved in 0.9% saline.

Placement of Intracerebroventricular and Guide Cannulae

Eight-week-old male Sprague Dawley rats were anesthetized with a mixture of O_2 (1 L/min) and 4% isoflurane, placed in a Kopf stereotaxic frame, and anesthesia was maintained for the duration of the surgery using an O_2/isoflurane (2%) mixture delivered through a nose cone attached to the frame. The skull was exposed and a small hole was drilled for placement of an MCAO guide cannula in the cranium dorsal to the right hemisphere using the following stereotaxic coordinates (1.6 mm anterior and 5.2 mm lateral to the bregma). A 21 gauge stainless steel guide cannula cut to 4mm below the pedestal was lowered into the hole and affixed to the skull with 3 mounting screws and dental cement. During the same surgery, a second hole was then drilled in the cranium dorsal to the left hemisphere for placement of an intracerebroventricular cannula (kit 1, ALZET, Cupertino, CA) coupled to a 2 week osmotic pump (model 2002, ALZET, Cupertino, CA) *via* vinyl tubing. The following stereotactic coordinates were used (1.3 mm posterior and 1.5 mm lateral to bregma, 4.5 mm below the surface of the cranium). The osmotic pump was implanted subcutaneously between the shoulder blades as described previously.[86] Osmotic pumps were used to infuse DIZE (5 μg/h), DIZE (5 μg/h) plus A-779 (1 μg/h), A779 (1 μg/h) alone, or H_2O into the left lateral cerebral ventricle starting at the time of cannula placement and lasting until the animals were euthanized. Following this surgery, the wound was closed and the rat was administered an analgesic agent (buprenorphine; 0.05 mg/kg sc) before waking.

Endothelin–1 Induced Middle Cerebral Artery Occlusion

Seven days after the placement of ICV and guide cannulae, the ET-1 induced MCAO procedure was performed as we have previously reported with a minor

modification.[138] Eight-week-old male Sprague Dawley rats were anesthetized as described above, and anesthesia was maintained for the duration of the injection using an O_2/isoflurane (2%) mixture delivered through a nose cone attached to the frame. The cannula dummy was removed after which a 26 gauge needle attached to a 5 μL Hamilton microsyringe was lowered 8.7 mm ventral to bregma. Once the needle was in place, 3 μL of 80 μM ET-1 was infused adjacent to the MCA at a rate of 1 μL/min using a Stoelting Quintessential Injector (Stoelting Co., Wood Dale, IL, USA). The needle was left in place for 3 min after the injection was complete and then removed slowly. The cannula dummy was then replaced and the rat was administered an analgesic agent (buprenorphine; 0.05 mg/kg sc) before waking. We have characterized this model previously by showing that injection of ET-1 can cause rapid constriction of the MCA followed by gradual reperfusion. In addition, a strong and significant correlation exists between the size of infarct measured in this model and several test scores used to assess neurological deficits in the work described here.[138] In addition, we have used laser doppler flowmetry to investigate the CBF reduction that results in cortical areas both adjacent (ischemic core) and distal (ischemic penumbra) to the site of ET-1 injection (unpublished). It is clear from these data that CBF decreases dramatically in tissue adjacent to the proximal MCA and that CBF is reduced to a lesser degree in tissue of more distal MCA territories.

Cerebral Blood Flow Monitoring

An additional group of rats was anesthetized as described above, after which laser doppler flowmetery was used to measure CBF prior to ET-1 injection and lasting for 1 h after ET-1 injection. CBF measurements were performed using a Standard Pencil

Probe and Blood FlowMeter coupled to a Powerlab 4/30 with LabChart 7 (ADInstruments, Inc, Colorado Springs, CO, USA). The probe was placed either just posterior to the MCAO guide cannula at the lateral skull ridge. Data were recorded in arbitrary blood perfusion units at 1000 Hz. Baseline CBF was calculated by averaging a 1 min interval just prior to ET-1 injection. Changes in CBF were calculated as a percentage of baseline by averaging a 10 s interval every 1 min.

Indirect Blood Pressure Monitoring

After undergoing surgery to implant an intracerebroventricular cannula (kit 1, ALZET, Cupertino, CA) coupled to a 2 week osmotic pump (model 2006, ALZET, Cupertino, CA) *via* vinyl tubing as described above, animals were allowed to recover for 1 week. Indirect blood pressure was recorded by tail-cuff once a week for 2 weeks as previously described.[86] Animals were warmed by a 200 W heating lamp for 5 min before restraint in a heated Plexiglas cage to which the animals were previously conditioned. A pneumatic pulse sensor was attached to the tail distal to an occluding cuff controlled by a Programmed Electro-sphygmomanometer (Narco Bio-Systems, Austin TX). Voltage outputs from the cuff and pulse sensor were recorded and analyzed by a Powerlab signal transduction unit and associated Chart software (ADInstruments, Colorado Springs, CO).

Neurological Deficits and Infarct Size

Neurological deficits and infarct size were evaluated as reported previously.[138] Neurological evaluations were performed using two separate scoring scales originally described by Bederson *et al*.[133] and Garcia *et al.,*[134] which cumulatively evaluate spontaneous activity, symmetry in limb movement, forepaw outstretching, climbing, body proprioception, response to vibrissae touch, resistance to lateral push, and circling

behavior. Additionally, animals were evaluated for neurological deficits using a sunflower seed eating test.[135] Infarct volume was assessed by staining brain sections with 0.05% 2,3,5-triphenyltetrazolium chloride (TTC) for 30 minutes at 37°C. Tissue ipsilateral to the occlusion, which was not stained, was assumed to be infarcted. After fixation with 10% formalin, brain sections were scanned on a flatbed scanner (Canon) and analyzed using ImageJ software (NIH). To compensate for the effect of brain edema, the corrected infarct volume was calculated using an indirect method.[129]

Data Analysis

Data are expressed as means ± SEM. Statistical significance was evaluated, as specified in the figure legends, with the use of a Kruskal-Wallis test, Two-way Row matched ANOVA, One-way ANOVA, or unpaired t-test, as well as with Dunn's Multiple Comparison Test, the Bonferroni Test, or Tukey's Multiple Comparison Test for *posthoc* analyses when appropriate. Differences were considered significant at $p<0.05$. Individual p values are noted in the results and figure legends.

Results

Cerebroprotective Action of DIZE

The effect of DIZE pre-treatment on ET-1-induced cerebral damage was assessed by TTC staining, whereby non-infarcted gray matter is stained red after incubation in TTC, delineating the infarct region in white. Seventy two hours following ET-1-induced MCAO a cerebral infarct can be seen in rats that had been pre-treated with H_2O (ICV infusion for 7 days) (Figure 4-1). Central pre-treatment of rats for 7 days with DIZE (5 μg/h, ICV) prior to ET-1-induced MCAO significantly reduced the infarct size compared to an H_2O pre-treated control group ($p<0.001$). The length of treatment was designed to efficiently determine the effectiveness of DIZE either before, during, or after stroke.

The DIZE-induced cerebroprotection was reversed by co-infusion of the Ang-(1-7) receptor antagonist, A-779 (1 μg/h, ICV). Pre-treatment with an ICV infusion of A-779 alone did not significantly modify the ET-1 induced cerebral damage compared to H_2O pre-treatment (Figure 4-1).

In addition to the gross histological evidence for cerebroprotection, central pre-treatment with DIZE attenuated the neurological deficits attributed to ET-1 induced MCAO. For example, 72 hr following ET-1 induced MCAO there were significant behavioral deficits in rats that had been pre-treated with H_2O (ICV infusion for 7 days), according to the Bederson Exam (score > 0) and the Garcia Exam (score < 18) (Figures 2A and 2B). Central pre-treatment of rats for 7 days with DIZE (5 μg/h, ICV) prior to ET-1-induced MCAO significantly reduced the Bederson Exam Score compared to the H_2O pre-treated control group ($p<0.05$, Figure 4-2A). This cerebroprotection was reversed when DIZE was co-infused for 7 days with the Ang-(1-7) receptor antagonist, A-779 (1 μg/h, ICV). A trend toward cerebroprotection was also seen with an improved Garcia Exam Score compared to the H_2O pre-treated control group (Figure 4-2B). Again, this cerebroprotective trend was diminished when DIZE was co-infused for 7 days with its antagonist, A-779 (1 μg/h, ICV). Pre-treatment with an ICV infusion of A-779 alone resulted in both Bederson and Garcia Exam Scores which were similar to the score for H_2O pre-treatment.

In addition, performance on the sunflower seed eating task was also used to evaluate neurological function. This task provided further evidence of the cerebroprotective properties of DIZE during focal cerebral ischemia. Rats were given 5 unshelled sunflower seeds and then timed while manipulating and opening the shells to

eat the seeds. Rats with significant neurological deficits display longer latency to remove the shell. In addition, deficits at this task result in rats that are inefficient at removing the shell and therefore break it into many small pieces. In summary, both increasing latency to open the shell and increasing number of shell pieces are indicators of more severe neurological deficits. Central pre-treatment of rats for 7 days with DIZE (5 μg/h, ICV) prior to ET-1-induced MCAO showed a non-significant trend toward reduction in the time required to eat 5 sunflower seeds compared to an H_2O pre-treated control group (Figure 4-2C). This cerebroprotective trend was reversed when DIZE was co-infused for 7 days with the Ang-(1-7) receptor antagonist, A-779 (1 μg/h, ICV). Pre-treatment with an ICV infusion of A-779 alone resulted in a time to eat 5 sunflower seeds which was similar to the H_2O pre-treatment group. Neurological evaluation by counting the number of shell pieces produced during this task also produced a pattern suggesting that central pre-treatment with DIZE is cerebroprotective during focal cerebral ischemia. Rats receiving central DIZE prior to ET-1 induced MCAO resulted in a non-significant trend toward reduction in the number of shell pieces compared to the H_2O pre-treated control group (Figure 4-2D). This cerebroprotective trend was reversed when DIZE was co-infused for 7 days with A-779. Pre-treatment with an ICV infusion of A-779 alone resulted in a number of shell pieces, which was similar to the H_2O pre-treatment group.

DIZE does not Alter ET-1 Induced Cerebral Blood Flow in the Cortex Distant from the Primary Branch of the MCA

To assess the effects of chronic central DIZE infusion on blood flow in microvascular beds during ET-1 induced MCA constriction, CBF was monitored transcortically for 1 h *via* laser doppler flowmetry during stroke induction. We have

previously demonstrated the reduction in cerebral blood flow at regions of the brain corresponding to the ischemic core and the ischemic penumbra following injection of 3 μL of ET-1 (80 μM) into the brain parenchyma adjacent to the MCA.[138] These data confirm that the ET-1 injection produces a significant ischemic action. In rats that received an ICV infusion of DIZE for 7 days, ET-1 injection as above resulted in abrupt reduction of CBF in the ischemic penumbra region followed by a gradual return to baseline over the period of monitoring. There were no significant differences in CBF at any time between rats infused ICV with DIZE or H_2O (Figure 4-3). Therefore, DIZE had no effect on the reduction in CBF in the cortical areas distant from the primary branch of the MCA.

ICV infusion of DIZE Decreases Blood Pressure

ICV infusion of DIZE (5 μg/h, ICV) produces a significant reduction in SBP after 7 days of treatment (Figure 4-4). These pre-treatment conditions are identical to those that attenuated the ET-1 induced cerebral infarcts and behavioral deficits.

Discussion

The most significant findings of this study are that central pre-treatment with DIZE attenuates the neurological deficits and brain tissue damage produced in an ET-1 induced MCAO model of ischemic stroke. Previously, we have shown a similar protective effect after central pre-treatment with Ang-(1-7).[142] The ET-1 induced MCAO is a minimally invasive model of ischemic stroke that provides a rapid constriction, sustained occlusion, and then gradual reperfusion of the proximal MCA which is not altered by pre-treatment with central Ang-(1-7).[142] Our current data also suggests that the cerebroprotective effect of central DIZE pre-treatment is not due to attenuation of

the decrease in CBF in the vascular territory of the MCA, which is consistent with previous studies in involving Ang-(1-7) pre-treatment. In addition, both the current DIZE cerebroprotection and previously described Ang-(1-7) cerebroprotection were attenuated in the presence of the Ang-(1-7) receptor, Mas. This is the first report of cerebroprotection in a model of ischemic stroke elicited *via* ACE2 activation and subsequent stimulation of the Ang-(1-7) receptor, Mas.

It is noteworthy that 7 days of ICV treatment with DIZE decreased BP in rats to a modest degree. This decrease was approximately 17 mmHg. It is unclear whether this change in BP contributes to the DIZE medicated cerebroprotection. However, a decrease in blood pressure would be expected to decrease the cerebrovascular reserve since this decrease is certainly well within the autoregulatory range of the cerebrovasculature. A blood pressure decrease is likely mediated by a central mechanism involving either a decrease in Ang II or an increase in Ang-(1-7). Such a decrease could potentially have an even greater antihypertensive effect in an animal strain with a well known neurogenic hypertension component. For example, central administration of DIZE to spontaneously hypertensive rats (SHRs) would likely lead to a robust decrease in blood pressure that could potentially decrease the cerebrovascular atherosclerosis and dysfunction in these animals. In addition, DIZE would likely have a protective effect separate from these BP related changes. Therefore, we predict a more robust cerebroprotective effect in this model of chronic cerebrovascular disease. Additionally, the use of a chronic cerebrovascular disease model, as opposed to the young health rats used here, would more closely mimic human stroke because of the

cardiovascular pathology present in many patients due to increased age and comorbidities.

The magnitude of cerebroprotection elicited by DIZE was similar to that elicited by Ang-(1-7) previously. However, attenuation of the DIZE-induced cerebroprotection by the Mas antagonist, A-779 seems to be less complete. Thus, either A-779 antagonism of Mas is incomplete, or Ang-(1-7) stimulation of Mas may only be a partial mechanism for DIZE-induced cerebroprotection. Since ACE2 can breakdown Ang II, one possibility is that ACE2 mediated decreases in Ang II could decrease Ang II type 1 receptor (AT1R) stimulation. AT1R antagonism is known to provide cerebroprotection during ET-1 induced MCAO. Therefore, decreased levels of Ang II could contribute to a similar mechanism.[138] In addition, ACE2 activation could actually participate in a feed forward loop of Ang-(1-7) production. Ang-(1-7) is broken down to Ang-(1-5) by ACE.[145] However, Ang-(1-7) is also an inhibitor of ACE at its c-terminal domain.[146] Therefore, activation of ACE2 can lead to increased production of Ang-(1-7) which directly inhibits the enzyme that causes its degradation, further increasing the levels of Ang-(1-7). Finally, although DIZE significantly reduced infarct size after ET-1 induced MCAO, the reduction of neurological deficits seemed to be less dramatic than our previous studies using Ang-(1-7) pre-treatment. One possible explanation of this could be an inadequate amount of Ang II substrate that would be necessary to produce Ang-(1-7) due to ACE2 activation. Alternatively, the relationship to DIZE dose and ACE2 activation *in* vivo is unknown and higher doses of DIZE might further increase levels of Ang-(1-7) and provide greater cerebroprotection. To answer these questions, further investigation into

the tissue levels of Ang II, Ang-(1-7), and Ang-(1-5) would help to understand the mechanism of DIZE-induced cerebroprotection.

It should be noted that ACE2 is able to metabolize several other non-renin angiotensin system peptides with similar efficiency to that of Ang II to Ang-(1-7). These peptides include apelin-13, neurotensin, kinetensin, dynorphin, Des-Arg^{9}-Bradykinin, and Lys-Des-Arg^{9}-Bradykinin.[49] Although the physiological significance of these reactions has not been explored, breakdown of these peptides could be involved in protection of tissue during or after cerebral ischemia. For example, ACE2 can hydrolyse the pro-inflammatory kinin, Des-Arg^{9}-Bk. This kinin cannot be broken down by ACE, which suggests that an increased ACE2/ACE ratio could contribute to a decrease in post ischemic inflammation and ensuing tissue injury. Also, important is the fact that DIZE is currently used as an antiprotozoal agent in animal and some human cases of trypanosomiasis. DIZE likely mediates this antiprotozoal activity through its ability to inhibit replication of trypanosomal mitochondrial DNA.[147] Although this activity is reportedly specific protozoal DNA, we cannot exclude a mechanism of DIZE mediated cerebroprotection that is related to this antiprotozoal activity at this time.

In summary, our findings support a protective role for ACE2 activation during cerebral ischemia. The high prevalence of stroke and its resulting morbidity and mortality indicate the importance of investigations into novel therapeutic strategies for stroke prevention and treatment. This study demonstrates the therapeutic benefit of ACE2 activation and Mas stimulation during stroke. Finally, as the first report of pharmacological activation of ACE2 for cerebroprotection in a model of focal cerebral

ischemia our results highlight the ACE2/Ang-(1-7)/Mas axis and ACE2 activating molecules as promising targets for stroke therapy.

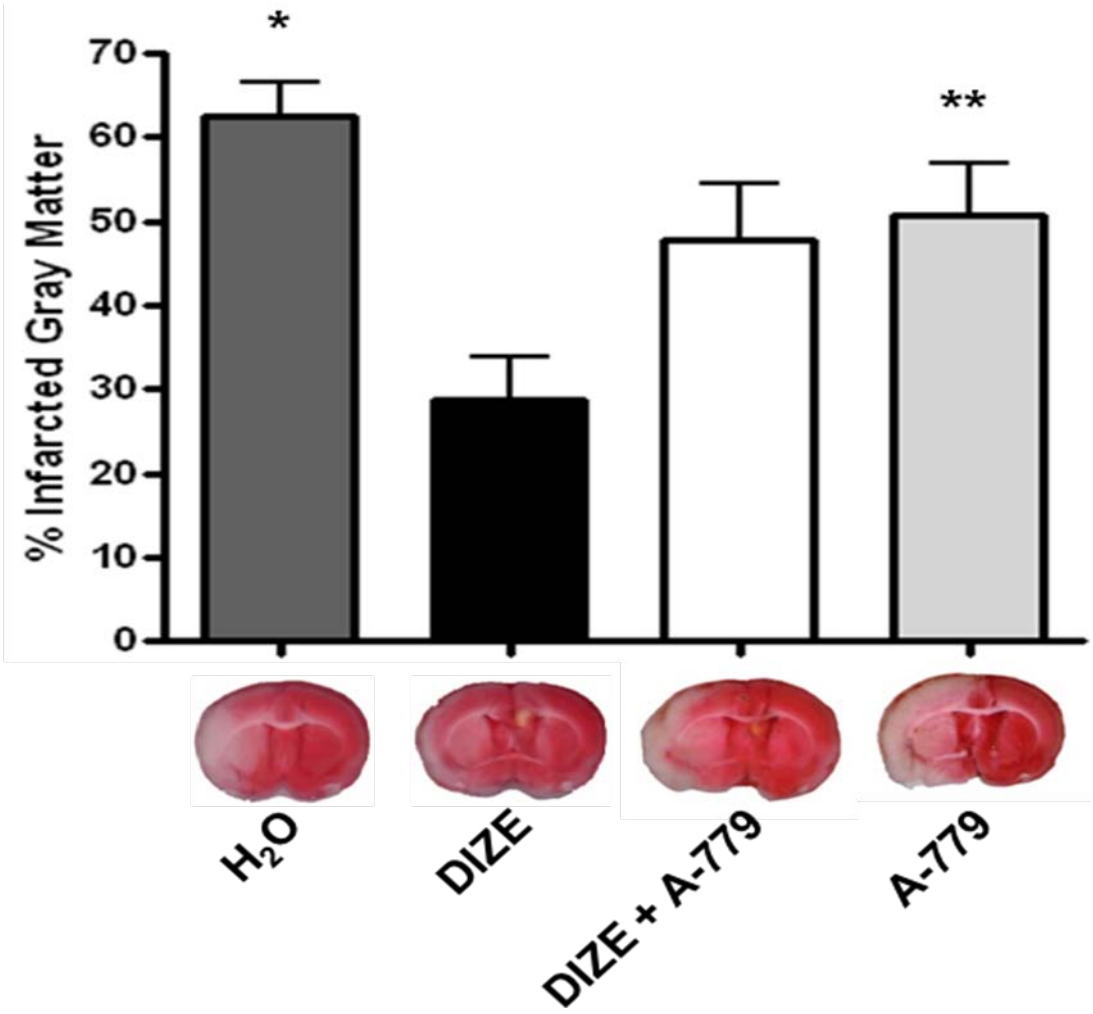

Figure 4-1. Intracerebral pre-treatment with DIZE reduces CNS infarct size 72 h after ET-1 induced MCAO. Rats were pre-treated *via* the ICV route with either H_2O (n = 15), DIZE (5 μg/h; n = 18), DIZE + A-779 (5 μg/h - 1 μg/h; n = 7), or A-779 (n = 10) alone for 7 days prior to MCAO induced by intracranial injection of ET-1 (80 mM). Brains were removed for TTC staining 72 h after stroke. Bar graphs show the % infarcted gray matter in each treatment group. Data are presented as means ± SEM. One-way ANOVA ($p < 0.0001$), * $p < 0.001$ vs. DIZE, ** $p < 0.05$ vs. DIZE (Tukey's Multiple Comparison Test). Representative brain sections show infarcted (white) and non-infarcted (red) gray matter under the treatment conditions indicated.

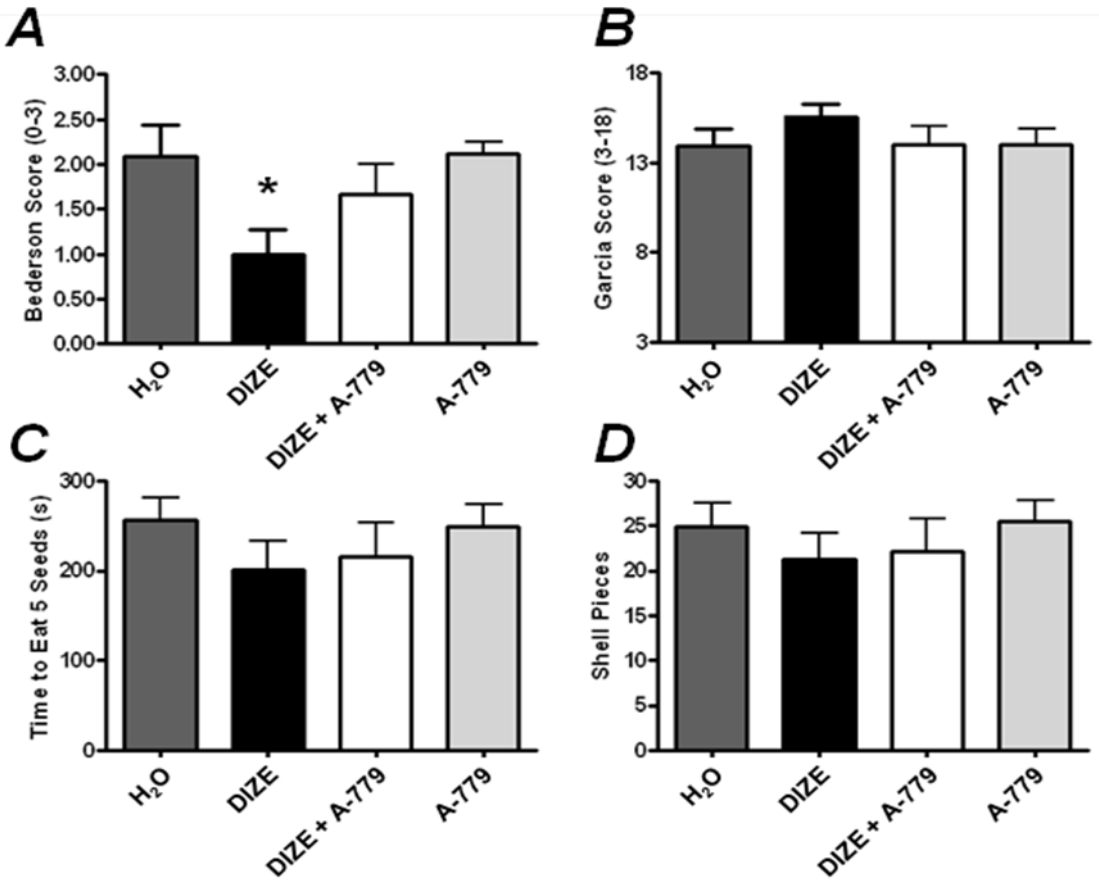

Figure 4-2. Intracerebral pre-treatment with DIZE reduces neurological deficits 72 h after ET-1 induced MCAO. Rats were pre-treated *via* the ICV route with either H_2O (n = 11), DIZE (5 μg/h; n = 16), DIZE + A-779 (5 μg/h - 1 μg/h; n = 6), or A-779 (n = 8) alone for 7 days prior to MCAO induced by intracranial injection of ET-1 (80 mM). Seventy-two hours later, neurological deficits were assessed *via* the Bederson Neurological Exam (*Panel A*) and the Garcia Neurological Exam (*Panel B*), as well as the Sunflower Seed Eating Test for the time to eat 5 seeds (*Panel C)* and the number of shell pieces *(Panel D).* Data are represented as means ± SEM. Bederson Exam $p < 0.05$ (Kruskal-Wallis Test), *$p<0.05$ vs. Ang-(1-7) (Dunn's Multiple Comparison Test)

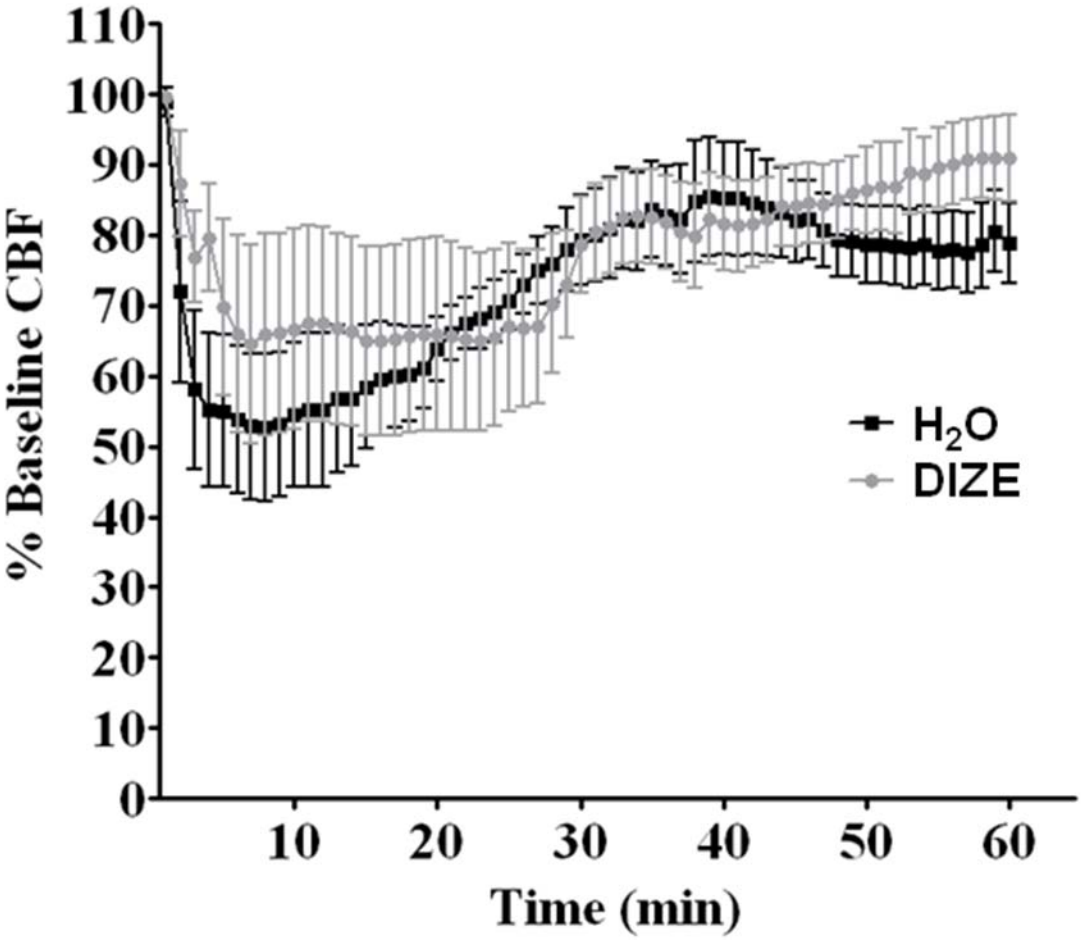

Figure 4-3. Central DIZE does not alter ET-1 induced cerebral blood flow in the cortex distant from the primary branch of the MCA. Lasar Doppler flowmetry was used to monitor CBF in the vascular territory of the MCA distal to the site of ET-1 injection. Data are presented as means ± SEM of the percent change from baseline CBF. ET-1 injection takes place over a period of 3 minutes starting at 0 min on this graph. No significant differences exist between DIZE (n = 6) and H_2O (n = 6) treatment groups at any time point (two-way RM ANOVA).

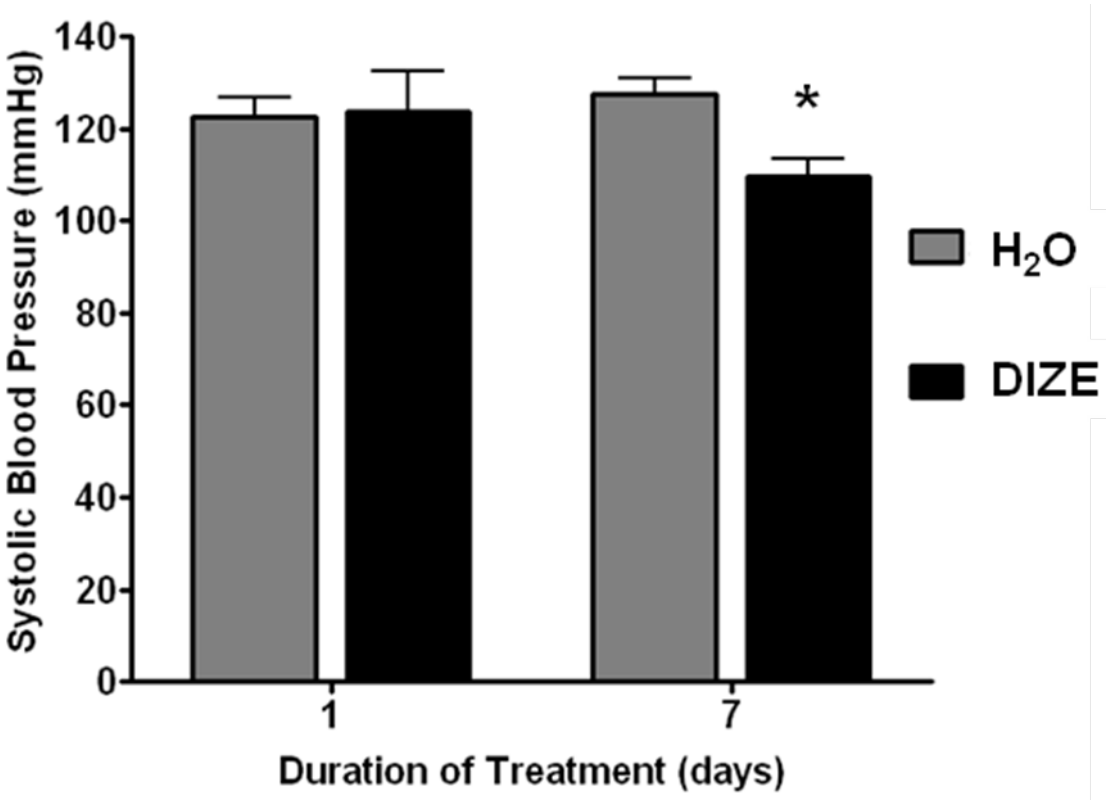

Figure 4-4. Central DIZE pre-treatment decreases systolic blood pressure. Rats were administered either H_2O (n = 6) or DIZE (5 μg/h; n = 6) *via* the ICV route over a period of 2 weeks. SBP was measured using the tail-cuff method at weekly intervals. Data are represented as means ± SEM of SBP. * $p < 0.01$ vs. H_2O (un-paired t test)

CHAPTER5
SUMMARY AND CONCLUSIONS

Summary

Specific Aim 1

Endogenous levels of Angiotensin II (Ang II) are increased bilaterally in the cortex and hypothalamus following stroke and systemic treatment of spontaneously hypertensive rats (SHR) with Ang II type 1 receptor (AT1R) blockers (ARBs) reduces the occurrence of stroke.[79, 84, 129] Additionally, ARBs provide a 40-50% reduction of infarct volume and reduce the neurological deficits in normotensive rats and SHRs that received a middle cerebral artery (MCA) occlusion *via* intraluminal occlusion.[78, 82, 97-100] However, the endothelin-1 (ET-1) induced middle cerebral artery occlusion (MCAO) model of cerebral ischemia is thought to more closely mimic the temporal events of an embolic stroke. This model provides rapid occlusion of the middle cerebral artery and a gradual reperfusion that lasts for 16-22 h.[130] Aim 1 was designed to evaluate whether systemic administration of an ARB prior to ET-1 induced MCAO will provide cerebroprotection during this model of ischemic stroke. Injection of 3 μL of 80 μM ET-1 adjacent to the MCA resulted in complete occlusion of the vessel that resolved over a period of 30 min to 40 min. Following ET-1 induced MCAO, rats had significant neurological impairment, as well as, an infarct that consisted of approximately 30% of the ipsilateral gray matter. Systemic pre-treatment with 0.2mg/kg/day candesartan for 7 days attenuated both the infarct size and neurological deficits caused by ET-1 induced MCAO without altering blood pressure. The effect of candesartan pre-treatment on ET-1 induced vasoconstriction of the MCA was also evaluated by visualization of the MCA through a cranial window. It was determined that candesartan pre-treatment did not

alter ET-1 induced constriction of the MCA, which validates the use of this stroke model during ARB pharmacotherapy. In addition, strong correlations were observed between infarct volumes and neurological deficits. This study solidifies the the view that ARBs can exert a cerebroprotective action during ischemic stroke and validates the ET-1 induced MCAO model for examination of the brain renin angiotensin system's (RAS) role in this disease.

Specific Aim 2

Recent progress in cardiovascular therapy suggests that stimulation of Angiotensin Converting Enzyme 2 (ACE2), production of Angiotensin-(1-7) [Ang-(1-7)], and activation of the Ang-(1-7) receptor, Mas, are viable targets for disease prevention and treatment. The ACE2/Ang-(1-7)/Mas axis has been shown to counteract many of the physiological effects of the AT1R, including vasoconstrictor and proliferative actions.[85] In addition, activation of the ACE2/Ang-(1-7)/Mas axis also attenuates many of the pathophysiological states that involve increased production of Ang II by Angiotensin Converting Enzyme (ACE), and subsequent activation of the AT1R (ACE/Ang II/AT1R axis). For example, many studies targeting the ACE2/Ang-(1-7)/Mas axis have revealed its broad therapeutic potential for the treatment of hypertension, hypertension-related pathology, myocardial infarction, and heart failure.[86-88, 91, 92] Aim 2 was designed to test whether central administration of Ang-(1-7) *via* lateral ventricular cannula would provide cerebroprotection during ET-1 induced MCAO, a rat model of ischemic stroke. Sprague Dawley rats were treated *via* the intracerebroventricular route with Ang-(1-7) (1 μg/h) or artificial cerebrospinal fluid (aCSF) prior to ET-1 induced MCAO. Ang-(1-7) treatment reduced the cerebral infarct size, neuronal damage and

neurological deficits measured 72 h after MCAO induction. Infarct size was reduced to 15.78 ± 5.54% of ipsilateral gray matter in the Ang-(1-7) treated rats compared with 40.11 ± 5.48% in aCSF treated controls. Ang-(1-7) treatment also reduced the neurological deficits produced by ET-1-induced ischemic stroke, as indicated by a battery of neurological tests including the Bederson Exam, Garcia Exam, and the sunflower seed eating task. These protective actions of Ang-(1-7) were reversed by blockade of the Ang-(1-7) receptor, Mas, with A-779 (1 μg/h). In addition, the effect of Ang-(1-7) pre-treatment on ET-1 induced vasoconstriction of the MCA was also evaluated by visualization of the MCA through a cranial window. It was determined that central Ang-(1-7) pre-treatment did not alter ET-1 induced constriction of the MCA, which validates the use of this stroke model during Ang-(1-7) pharmacotherapy. In order to investigate alterations in cerebral blood flow (CBF) as a mechanism of Ang-(1-7) induced cerebroprotection, we measured CBF in the penumbra during ET-1 induced MCAO. Ang-(1-7) did not affect the reduction of CBF in the penumbra which ruled out the possibility of a protective mechanism of Ang-(1-7) mediated through improved CBF during MCAO. This is the first demonstration of cerebroprotective properties of Ang-(1-7) during ischemic stroke.

Specific Aim 3

Investigations of the ACE2/Ang-(1-7)/Mas axis has revealed broad therapeutic potential for the treatment of hypertension, and hypertension-related pathology such as stroke, myocardial infarction, and heart failure. For example, we have shown the beneficial actions of central pre-treatment with Ang-(1-7) in a rat model of ischemic stroke. Furthermore, ACE2 can form endogenous Ang-(1-7) from Ang II and has

recently been a target for cardiovascular disease therapy.[47] In fact, several small molecule ACE2 activators have been identified that selectively increase ACE2 activity without having an effect on ACE activity.[143, 144] One of these molecules, Diminazene aceturate (DIZE) has been shown to decrease blood pressure dramatically and dose dependently when administered acutely. In addition, a modest decrease in blood pressure and associated reductions in end organ damage are observed with chronic administration of DIZE to spontaneously hypertensive rats. Another of these activators, XNT (1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one), had similar antihypertensive effects and also prevented the pathophysiology right heart failure and pulmonary fibrosis in a rat model of pulmonary hypertension induced by monocrotaline.[90] Therefore, these small molecule ACE2 activators are promising compounds for ACE2/Ang-(1-7)/Mas axis activation and treatment of cardiovascular disease. Based on our previous studies supporting a protective role for ACE2/Ang-(1-7)/Mas axis during stroke, as well as a protective effect of ACE2 activation by DIZE in various models of cardiovascular disease, we have developed the general hypothesis that DIZE can activate ACE2 in the brain, leading to increased production of Ang-(1-7), and a subsequent Mas-mediated cerebroprotective action during ischemic stroke. Aim 3 was designed to test whether central pre-treatment with DIZE will provide cerebroprotection in a rat model of ET-1 induced MCAO. Adult male Sprague Dawley Rats were pre-treated with intracerebroventricular DIZE (5 μg/h) or H_2O for 7 days prior to ET-1 induced MCAO. DIZE treatment reduced neurological deficits and infarct size measured 72 h after MCAO induction. Specifically, infarct size was reduced to 28.75 ± 5.05% of ipsilateral gray matter in DIZE-treated rats

compared with 62.40 ± 4.08% in H_2O controls. Additionally, neurological deficits were reduced in DIZE treated rats as indicated by a lower Bederson Exam Score of 0.9 ± 0.3 compared to 2.1 ± 0.3 in control rats, a higher Garcia Exam Score of 15.9 ± 0.7 compared with 13.9 ± 1.0 in control rats, and improvement in a sunflower seed eating task to 187 ± 33 s and 20 ± 3 shell pieces compared to 256 ± 5 s and 25 ± 3 pieces in H_2O control rats. Furthermore, the histological and neurological benefits of pre-treatment with DIZE were attenuated when DIZE was co-administered with the Ang-(1-7) receptor antagonist, A-779 (1 μg/h). In order to investigate alterations in CBF as a mechanism of DIZE induced cerebroprotection, we measured CBF in the penumbra during ET-1 induced MCAO. DIZE did not affect the reduction of CBF in the penumbra which ruled out the possibility of a protective mechanism of DIZE mediated through improved CBF during MCAO. This data indicates that central administration of DIZE prior to stroke is cerebroprotective and extends the known cardiovascular protective effects elicited by stimulation of the ACE2/Ang-(1-7)/Mas axis.

Discussion

Mechanism of ACE2/Ang-(1-7)/Mas Cerebroprotection

Our results demonstrate that either direct stimulation of Mas with Ang-(1-7) or indirect stimulation through ACE2 activation with DIZE improves the neurological outcomes after ET-1 induced MCAO. However, the cell types and molecular mechanisms involved in this cerebroprotection have not been determined. Previous reports have demonstrated that Ang(1-7) administered centrally after a stroke can increase bradykinin release, bradykinin receptor stimulation, endothelial nitric oxide synthase (eNOS) activity, and NO production.[127, 128] This suggests that Ang-(1-7) may be able to increase NO availability and cerebrovascular vasodilation. In addition, both

acute and chronic peripheral administration of Ang-(1-7) has been shown to increase cerebral blood flow. However, the peripheral route of administration should not deliver effective doses of Ang-(1-7) across the blood brain barrier (BBB).[148] Despite these findings, the data presented in Chapter 2 demonstrate that central infusion of Ang-(1-7) at a dose which affords cerebroprotection does not alter cerebral blood flow. Therefore, the mechanism for peripheral Ang-(1-7)-induced increases in cerebral blood flow cannot be synonymous with the mechanism of central Ang-(1-7) potentiation of the bradykinin/NO pathway activity after stroke.

Some insight into the protective mechanism of Ang-(1-7) might be gained from examining the cellular localization of Mas. There has not been an organized investigation into the cell types that express Mas in the brain. Mas was first characterized as a protooncogene that was found in high levels in the brain [118] and thought to be exclusively located in neurons [119]. In addition, it has been shown that Mas is present in rat cerebral endothelial cells, but is absent from endothelial cells in the periphery [122]. Recently, a global presence for Mas in both cardiovascular and non-cardiovascular control areas of the brain was verified by immunofluorescence [93]. This study also indicates a largely neuronal localization for Mas in cardiovascular control regions, but makes no report on its cellular localization in non-cardiovascular control regions of the brain such as the motor cortex. Taken together, these results suggest a diverse localization for Mas in the brain. However, none of these studies have reported various Mas expressing cell types simultaneously. This can be addressed with studies to co-localize various cell types with Mas using immunohistochemistry. Identification of cell types expressing Mas as endothelial cells, smooth muscle cells, neurons,

astrocytes, oligodendrocytes, microglia, or peripheral leukocytes (post ischemic stroke) will help lead us toward the mechanism of action of Ang-(1-7).

Although limited in scope, we have made several advancements in the understanding of Ang-(1-7) mediated cerebroprotection. First, we have investigated the cerebral blood flow changes of the penumbra that result from MCAO. It is clear that with either Ang-(1-7) or DIZE pre-treatment, the reductions of blood flow in the penumbra remain unaltered compared with vehicle pre-treated control rats. These experiments rule out a protective mechanism of Mas stimulation that involves improved cerebral blood flow to tissue in the vascular territory of the MCA. Although, the participation of smooth muscle and endothelial cells of the cerebrovasculature is not ruled out by these experiments, it is clear that their involvement in vasodilatory events is not a factor. In addition, our preliminary studies indicate Ang-(1-7) mediated interruption of the post stroke inflammatory cascade as a potential mechanism of cerebroprotection. Our results indicate that ET-1 induced stroke can increase levels of mRNAs for pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα within the infarct regio̲n̲. In addition, inducible nitric oxide synthase (iNOS) expression is increased in the ipsilateral cortex at 24 h after ET-1 induced MCAO, consistent with what is observed in other models of ischemic stroke.[149, 150] Ang-(1-7) attenuates these increases in proinflammatory cytokines and iNOS, providing potential loci for the cerebroprotective actions of this peptide.

These findings are supported by reports of proinflammatory cytokine release by astrocytes, microglia, smooth muscle cells, and endothelial cells during stroke.[8] These cytokines, including TNF-α, IL- 1β and IL-6, are associated with an increase in iNOS, as

well as early invasion of neutrophils and transmigration of adhesion molecules. These proinflammatory mediators are critical to the pathogenesis of tissue damage in cerebral infarction. For example, after middle cerebral artery occlusion TNF-α, IL-1ß, IL-6 and iNOS, as well as phosphorylated ERK1/2 are increased in smooth muscle cells of the middle cerebral artery and in associated intracerebral microvessels. Inhibition of ERK phosphorylation decreases the tissue damage, as well as the cytokine and iNOS production after cerebral ischemia. [32] Therefore, disruption of post stroke inflammatory pathways may be a mechanism of Ang-(1-7) cerebroprotection. Further investigations of the time course and protein expression levels of these inflammatory mediators are underway.

Common Mechanisms for AT2R and Mas Mediated Cerebroprotection

AT2Rs often mediate effects of Ang II that are exactly opposite to those mediated by AT1R [111], and in fact the tissue levels of AT2R are dramatically increased following injury, such as in the heart following myocardial infarction, in atherosclerotic blood vessels, in wounded skin, and in the peri-infarct region in the brain following ischemia [82, 96, 112-114]. Considering this, and the evidence that Ang II acts *via* AT2R in neurons to elicit differentiation, regeneration and neurotrophic actions [115-117], Unger and colleagues hypothesized that the increased expression of AT2R within the peri-infarct region can (in the presence of ARBs to block AT1R) be activated by the raised endogenous levels of Ang II and serve a neuroprotective role [82]. These investigators have supported this theory by demonstrating that the beneficial action of ARBs after MCAO-induced cerebral ischemia is prevented by specific AT2 receptor blockers [82]. Additional support is provided by the following experimental findings: i) ARBs are more cerebroprotective than ACE inhibitors in a rat model of ischemia and reperfusion [81]; ii) MCAO produces

greater ischemic brain damage in AT2R knockout mice compared with wild-type controls [80]; and, iii) CNS delivery of an AT2R agonist (CGP 42112) provides cerebroprotection during ischemic stroke[83]. Although most studies have focused on Ang II stimulation of the AT2R during stroke, there is also evidence that conversion of Ang II to Ang IV which activates the AT4R might be involved in ARB mediated cerebroprotection.[106] Similarly, it has been shown that Ang II conversion to Ang III is required for AT2R mediated naturetic effects of ARBs.[151] Overall, these studies demonstrate a variety of RAS components that counteract AT1R stimulation and lead to cerebroprotection during ischemic stroke. Our studies have added the components of the ACE2/Ang-(1-7)/Mas axis to these AT1 opposing mechanisms during stroke.

The AT2R is a 7 transmembrane domain G protein coupled receptor with 34% sequence homology to the AT1R.[152] Stimulation of the AT2R activates signaling cascades that counteract many of the AT1R mediated events.[153] For example, several phosphatases such as MAP Kinase Phosphatase-1 (MPK-1), SHP-1, and PP2A are activated following AT2R stimulation by Ang II[41, 154]. These phosphatases serve to deactivate several of the AT1R signaling members such as ERK1/2, STAT, and JAK. The increase in phosphatase activity involved in AT2R signaling can be both G protein dependent and independent. In addition to increasing phosphatase activity, AT2R stimulation increases NO, cyclic GMP formation, and bradykinin release.[155, 156] The interplay between these events is somewhat uncertain, but soluble adenylate cyclase and the bradykinin B_2 receptor are involved.

Like the AT2R, the Ang-(1-7) receptor, Mas, is a 7 transmembrane domain G protein coupled receptor that antagonizes the AT1R through direct similar mechanisms.

For example, Ang-(1-7) stimulation of Mas can inhibit AT1R mediated phosphorylation of p38MAPK, ERK1/2, and JNK.[69, 70] SHP-2 is activated by Ang-(1-7) signaling and this molecule is involved in the disruption of c-Src, ERK1/2, and NOX activation by Ang II.[58] In addition, Mas activation causes eNOSstimulation *via* the phosphatidylinositol 3-kinase (PI3K) /Akt pathway.[71] Mas stimulation also causes AA release and PGI_2 production, as well as potentiation of bradykinin signaling.[72-75] Thus, it is clear that both AT2R and Mas stimulation can contribute to phosphatase activation, bradykinin receptor activation, and NO release. Similar mechanisms of AT1R signaling disruption by these receptors may indicate common mechanisms of cerebroprotection.

CNS Pharmacotherapy

Targeting the CNS with pharmacotherapy has proven difficult because the BBB excludes many molecules and limits their activity centrally.[148] In fact, less than 5% of drugs are active in the CNS due to exclusion by the BBB.[157] The limitations of many small molecules to cross the BBB are due to the relatively narrow set of characteristics that seem to allow for BBB transport. Most of the small molecules that cross the BBB have a molecular mass less than 400 to 500 Da and are very lipophilic.[148] These concerns were the rationale for designing our studies using transcranial drug delivery directly into the cerebral ventricle. Ang-(1-7) is an 899 Da peptide and is hydrophilic. These characteristics are extremely likely to limit its transport across the BBB. In addition, DIZE is approximately 515 Da and is also hydrophilic. In fact, there is direct evidence of the limited ability of DIZE delivered peripherally to cross the BBB.[158] Therefore, we have administered Ang-(1-7) and DIZE centrally with a preventative treatment strategy in order to bypass the BBB. This route of administration is more

invasive and less sustainable than peripheral delivery of drugs. Therefore, alternative strategies to activate the central ACE2/Ang-(1-7)/Mas axis should be explored.

Modeling Middle Cerebral Artery Occlusion

There are several methods of inducing focal cerebral ischemia in rodents and each method has numerous benefits and drawbacks. The specific method used to induce experimental ischemia is important to interpreting results of cerebroprotection studies. This difficulty in reproducing the pathophysiology of human stroke has likely led to the failure of many cerebroprotective agents during clinical trials.[159, 160] Some of the most common models of focal cerebral ischemia include the intraluminal thread induced MCAO, ET-1 induced MCAO, surgically induced MCAO (clipping, electrocauterization, ligation), photothrombosis, embolization (blood clots, microspheres).

The intraluminal thread induced MCAO consists of inserting a filament into the common carotid artery and passing it through the internal carotid up to the junction of the anterior middle cerebral arteries.[161] Benefits of this method include the absence of craniotomy and the ability to control the duration of ischemia and onset of reperfusion if it is desired. Drawbacks to using the intraluminal thread model include a high rate of subarachnoid hemorrhage, external carotid artery hypoperfusion, retinal damage, and involvement of the hypothalamus.[159]

The ET-1 induced MCAO consists of a stereotactical injection of ET-1 into the brain parenchyma adjacent to the proximal portion of the middle cerebral artery.[136] Benefits of this method include the ability to control artery constriction by altering the dose of ET-1 delivered, as well as no manipulation of the extracranial vessels supplying blood to the brain.[162] In addition, ET-1 delivery allows for a gradual as opposed to

abrupt reperfusion of the middle cerebral artery.[138] Drawbacks to this method are the need for a craniotomy as well as high variability in stroke volume.[159] Our experiments show that ET-1 induced vasoconstriction is at a maximum within a few minutes and gradual recanalization occurs over 30 to 40 min. Although, this clearly causes a significant amount of tissue damage and neurological deficit, the duration of occlusion may not closely mimic that of human stroke where most patients have at least partial reperfusion over a period of hours to days following occlusion.[6, 7] It should also be noted that the rapid constriction and gradual reperfusion of the ET-1 induced MCAO might closely mimic the vasospasms seen following subarachnoid hemorrhage. Therefore, this model could also be a useful tool for studying the ischemithat occurs during vasospasm events.

Surgically induced MCAO consists of occluding the middle cerebral artery using clips, electrocauterization, or ligation. These methods require removal of part of the mandible and zygomatic arch, as well as a craniotomy. Complex and invasive surgical procedures are among the drawbacks of this model that have led many researchers to prefer alternative methods.[163]

Photothrombisis can be used to induce cortical ischemia through the use of a photosensitive dye and then irradiation of the cortex.[164] Irradiation can be performed either transcortically or after performing a craniotomy. Benefits of this method are that it is a non-invasive, efficient, and reproducible procedure. Drawbacks include doubts that photothrombosis damages tissue through an ischemic mechanism and generates an ischemic penumbra.[165-167]

Embolization can also be used to induce ischemic stroke. Methods of embolization include injection of microspheres or an ex vivo prepared thrombus into either the common carotid or middle cerebral artery.[159] The major benefit of this model is the similarity to human stroke. In fact, most human ischemic strokes are embolic in nature. In addition, use of an ex vivo prepared thrombus is a good model for testing thrombolytic therapies. The drawbacks to embolizaton models are the high variability of infarct and difficulty in placing the embolus directly into the middle cerebral artery in models of MCAO. In addition, embolization procedures have a high rate of subarachnoid hemorrhage.[168-170]

It is clear that many factors must be taken into consideration when developing animal models of ischemic stroke. For example, anesthesia is a possible source of variability when modeling ischemic stroke. Isoflurane can protect brain tissue from injury during ischemia and thus differences in dose and duration of anesthesia can affect the tissue damage that occurs during stroke.[171] The experiments of each aim were performed sequentially and the brain tissue damage increased during the ET-1 induced MCAO experiments of each aim. One possible explanation for the increase in tissue damage could be decreased times to complete surgical manipulations with experience in the required surgical techniques. Normalization of the data using a log of anesthesia duration should be performed in subsequent experiments to reduce variability. Consideration of factors such as anesthesia, as well as the benefits and drawbacks to various models of focal cerebral ischemia, must be taken into consideration when attempting to translate results from animal stroke experiments to human clinical trials.

Strategies for Targeting the Brain ACE2/Ang-(1-7)/Mas Axis in Humans

Medicinal chemistry can be used to alter a compound in one of two ways to increase BBB permeability. The drug can either be altered to be more lipophilic or altered to utilize active transport mechanisms.[148] The latter is more desirable because extremely lipid permeable drugs would cross the BBB, but also be eliminated faster leading to difficulties achieving an effective concentration in the brain. BBB disruption can be used to effectively deliver drugs to the CNS. Strategies for BBB disruption include the use of poorly diffusible osmotic compounds such as mannitol to shrink endothelial cells, and detergents to destabilize membranes. The use of either of these methods is associated with pathological changes due to the BBB disrupting compounds or neurotoxic plasma proteins.[148, 172] In experiments using hyperosmolar lithium chloride to disrupt the BBB, concentrations of DIZE in the brain were increased compared to a vehicle control.[158] Interestingly, a hyperosmolar treatment with sucrose in the same study was not effective. The discrepancy in effectiveness between sucrose and lithium to increase DIZE delivery to the brain is interesting and co-administration of lithium and DIZE should be investigated further for cerebroprotective properties. More recent strategies to delivery therapy across the BBB have included transcranial ultrasound and liposome assisted delivery of drugs.[173, 174] In addition to more conventional strategies of drug delivery to the CNS, gene therapy approaches with ACE2 or secretable Ang-(1-7) should be investigated. Viral delivery and endothelial specific expression of ACE2 or Ang-(1-7) in the brain could provide a means of by-passing the BBB.

Additional Considerations about Timing and Dose of Therapy

Many investigations into stroke therapeutics have focused on acute therapy after onset of stroke symptoms. However, a preventative approach is not unrealistic due to the well defined and strong risk factors for stroke, such as age and hypertension. Our experiments were designed so that treatment was administered prior to and throughout cerebral ischemia and subsequent pathology. This approach was used to efficiently determine the cerebroprotective potential of ACE2/Ang-(1-7)/Mas activation. Further experiments should be performed to test the effect of acute therapy after stroke using Ang-(1-7) and DIZE. If acute therapy is beneficial, than a peripheral administration strategy may be warranted due to the BBB disruption that occurs during stroke. This would circumvent the need to investigate strategies for traversing the BBB. In addition, it is unclear whether administration of DIZE at the dose used in these experiments activates ACE2 and generates a comparable dose of Ang-(1-7) when compared to that delivered directly. Proof of principle experiments should be performed to determine the ability of DIZE to alter angiotensin peptide metabolism in the brain. Measurement of Ang II, Ang-(1-7), and Ang-(1-5) *via* high performance liquid chromatography and mass spectrometry would be an appropriate way to test this hypothesis.

Conclusion

Our studies have extended the known cardiovascular protective roles of the ACE2/Ang-(1-7)/Mas axis (Figure 5-1). Specifically, we have shown that the ET-1 induced MCAO model of ischemic stroke can provide a rapid onset of ischemia caused by constriction of the MCA. This constriction is followed by a gradual period of vessel relaxation and reperfusion of the involved vascular territory. Our studies, have confirmed that the ET-1 induced MCAO model is appropriate for studies investigating

the involvement of RAS components in stroke. For example, we have shown that neither candesartan, nor Ang-(1-7) can alter the ET-1 induced vasoconstriction of the MCA. Most importantly, we have determined that central administration of Ang-(1-7) prior to ET-1 induced MCAO can reduce the brain damage and neurological deficits that occur due to ischemic injury. The effects of Ang-(1-7) were mediated *via* its receptor, Mas. A similar Mas mediated cerebroprotective action was elicited when DIZE, an ACE2 activator, was administered prior to stroke. Neither ACE2 activation, nor Ang-(1-7) administration was shown to alter CBF reductions resulting from MCAO. Thus, the mechanism of ACE2/Ang-(1-7)/Mas mediated cerebroprotection remains undetermined, but preliminary studies suggest interruption of post ischemic inflammatory responses as a possibility. In summary, stimulation of the ACE2/Ang-(1-7)/Mas components is a viable target for the prevention and treatment of ischemic stroke.

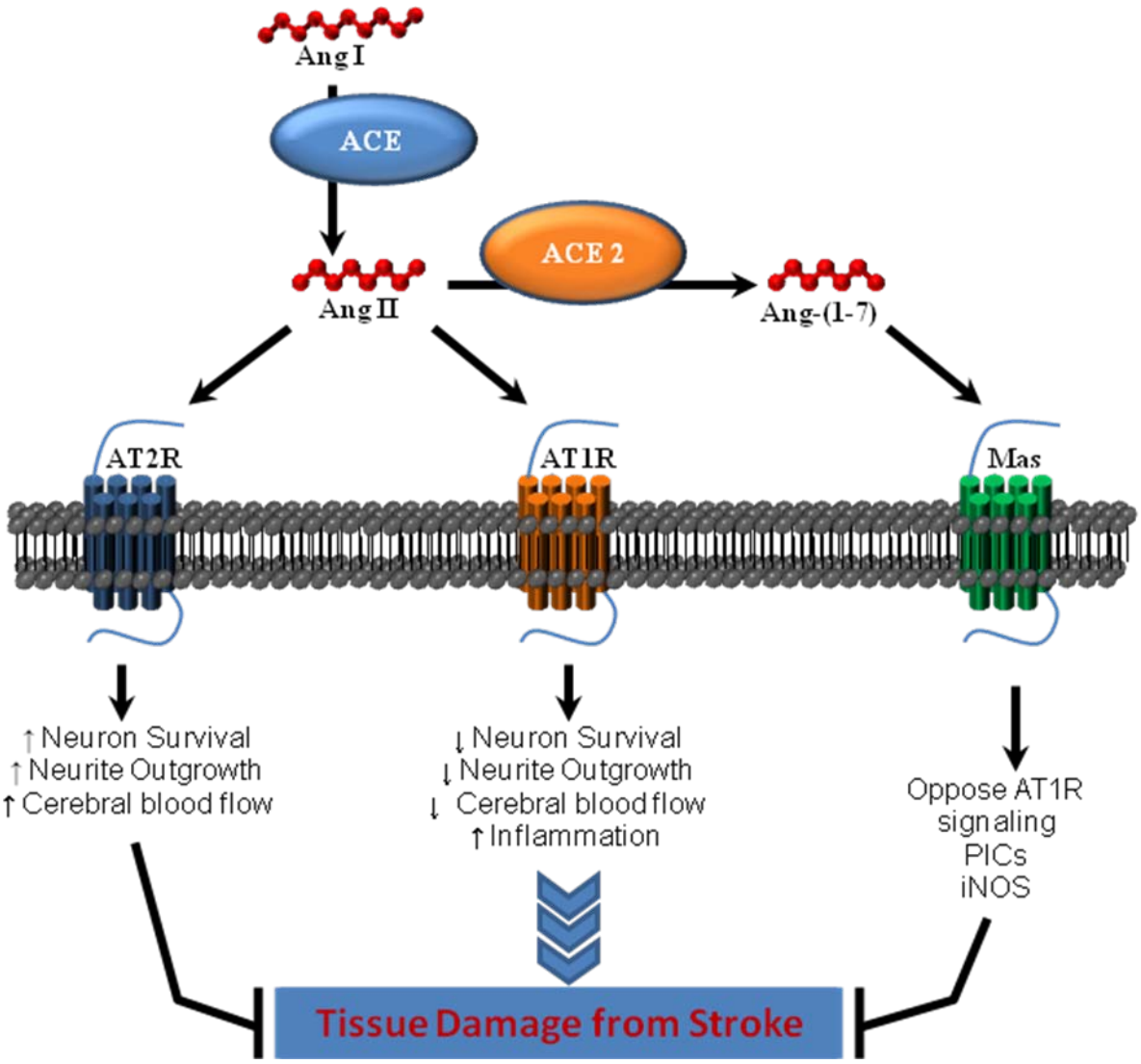

Figure 5-1. ACE2/Ang-(1-7)/Mas axis is cerebroprotective during stroke. ACE= Angiotensin Converting Enzyme, ACE2 = Angiotensin Converting Enzyme 2, Ang = Angiotensin, AT1R = Ang II Type 1 Receptor, AT2R = Ang II Type 2 Receptor, iNOS = inducible nictric oxide synthase, PIC = proinflammatory cytokines

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BIOGRAPHICAL SKETCH

Adam P. Mecca was born in West Palm Beach, Florida, USA in 1983. He graduated with highest honors from the University of Florida in 2005 with a Bachelor of Science and a double major in chemistry and microbiology. He performed his undergraduate honors thesis research with Michael Katovich, Ph.D. and studied the cardioprotective properties of Angiotensin-(1-7) in animal models of chronic hypertension. Adam began medical school at the University of Florida, College of Medicine in 2005 and started his graduate studies as an M.D.—Ph.D. student working with Colin Sumners, Ph.D., and Michael Katovich, Ph.D. His research interests include the renin angiotensin system, cardiovascular disease, and stroke. His doctoral thesis is titled "Targeting the ACE2/Ang-(1-7)/Mas axis for cerebroprotection during ischemic stroke." Adam completed his graduate research in 2010 for this work which focused on activating endogenous biological pathways in the brain to prevent or treat stroke. He is currently completing the clinical portion of his Medical Doctorate training.

In addition to his research interests, Adam is a Co-Director of the Equal Access Clinic, a student run free medical clinic in downtown Gainesville. The clinic serves the under-insured populations of Gainesville and Alachua County. Adam became interested in medically under-served populations when he began volunteering at the Equal Access Clinic as an undergraduate student in 2002. Since then, Adam has worked with faculty and student volunteers across the University of Florida health professions to establish and expand the patient services offered by the Equal Access Clinic. Adam is interested in utilizing student run free clinics to enhance the educational experience of health professional students and provide high quality care to patients in need. He is also the Co-founder and Conference Coordinator for the Society for Student Run Free Clinics,

an organization dedicated to assisting international collaboration between student run free clinics. Adam aspires to be an effective physician scientist, educator, and healthcare provider.