TARGETING THE PROTECTIVE RENIN ANGIOTENSIN SYSTEM IN ISCHEMIC STROKE: STEPS FROM BENCH TOWARD BEDSIDE

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2016
To my grandfathers
ACKNOWLEDGMENTS

The work of a successful course of Ph.D. study is accomplished not by an individual, but by a village. To the many dozens of villagers that I do not here mention by name, I offer my sincerest thanks. The first seeds of my love for research were planted and carefully cultivated by Dr. Jeffrey G. Edwards, my undergraduate research professor, continuing mentor, and close friend. Other mentors who have notably influenced me include Drs. Jocelyn Gravlee, R. Whit Curry, Jr., Thomas Rowe, Bill Allen, Patrick Duff, Alfred Lewin, Paul Gulig, Peter Sayeski, and Shawn Christensen. I am also deeply grateful to have been the recipient of truly exceptional mentoring from not one, but two MD-PhD Training Program directors, Drs. Stephen I. Hsu and W. Strat May. Each of them has imparted advice and wisdom in ways and at critical times that have given me the courage to start close in and take the steps I didn’t want to take.

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<td>ACEi</td>
<td>Angiotensin converting enzyme inhibitor</td>
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<td>ACEi_p</td>
<td>Angiotensin converting enzyme inhibitor previously prescribed</td>
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<td>ARB</td>
<td>Angiotensin receptor blocker</td>
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<td>ARB_p</td>
<td>Angiotensin receptor blocker previously prescribed</td>
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<td>AT1R</td>
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<td>CI</td>
<td>Confidence interval</td>
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<td>Contralateral</td>
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<td>DBP</td>
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<td>DIZE</td>
<td>Diminazene</td>
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<td>DUSP-1</td>
<td>Dual specificity phosphatase 1</td>
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<td>ECA</td>
<td>External carotid artery</td>
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<td>eNOS</td>
<td>Endothelial cell nitric oxide synthase</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<td>ET-1</td>
<td>Endothelin-1</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<td>GFP</td>
<td>Green fluorescence protein</td>
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<tr>
<td>HuC/D</td>
<td>Human neuronal protein</td>
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<td>Iba-1</td>
<td>Ionized calcium-binding adapter molecule 1</td>
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<td>IC</td>
<td>Inflammatory cell</td>
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<td>ICV</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>INR</td>
<td>International normalized ratio</td>
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<td>IQR</td>
<td>Interquartile range</td>
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<td>IP</td>
<td>Intraperitoneal</td>
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<td>Ipsi</td>
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<td>IS</td>
<td>Ischemic stroke</td>
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<td>IV tPA</td>
<td>Intravenous tissue plasminogen activator</td>
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<td>LCN-2</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
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<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>mRS$_d$</td>
<td>Modified Rankin scale score at discharge</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>NEP</td>
<td>Neprilysin</td>
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<tr>
<td>NeuN</td>
<td>Neuron specific protein</td>
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<tr>
<td>NF-$\kappa$B</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NIHSS$_d$</td>
<td>National Institutes of Health stroke scale score at discharge</td>
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<tr>
<td>NIHSS$_p$</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>PCP</td>
<td>Prolyl carboxy peptidase</td>
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<tr>
<td>PEP</td>
<td>Prolyl endopeptidase</td>
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<tr>
<td>PIC</td>
<td>Proinflammatory cytokine</td>
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<tr>
<td>Protime</td>
<td>Prothrombin time</td>
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<td>RAS</td>
<td>Renin angiotensin system</td>
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<td>RFU</td>
<td>Relative fluorescence unit</td>
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<tr>
<td>rhACE2</td>
<td>Recombinant human angiotensin converting enzyme 2</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RVLM</td>
<td>Rostral ventrolateral medulla</td>
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<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
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<td>SBP$_p$</td>
<td>Systolic blood pressure at presentation</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>SEM</td>
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<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
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<td>spSHR</td>
<td>Stroke prone spontaneously hypertensive rat</td>
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<td>TACE</td>
<td>Tumor necrosis factor alpha converting enzyme</td>
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<td>TIA</td>
<td>Transient ischemic attack</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
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<td>TTC</td>
<td>2,3,5-triphenyltetrazolium chloride</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion protein 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WBC</td>
<td>White blood cell count</td>
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TARGETING THE PROTECTIVE RENIN ANGIOTENSIN SYSTEM IN ISCHEMIC STROKE: STEPS FROM BENCH TOWARD BEDSIDE

By

Douglas Martin Bennion

May 2016

Chair: Colin Sumners
Major: Medical Sciences – Physiology and Pharmacology

As a leading cause of worldwide death and disability, stroke stands as a disease for which most victims have no effective treatment options. The renin angiotensin system (RAS) is highly involved in the multi-factorial pathophysiology of stroke. In contrast with the deleterious actions of over activation of angiotensin II type 1 receptors by angiotensin II, stimulation of angiotensin II type 2 receptors (AT2Rs) has beneficial effects in stroke. Likewise, activation of the angiotensin converting enzyme 2 (ACE2)–angiotensin-(1-7)[Ang-(1-7)]–Mas axis of the RAS is neuroprotective. A major hurdle in translating these results to stroke patients is devising non-invasive strategies to activate the neuroprotective RAS. To this end, we devised specific aims to test whether there are stroke-induced changes within the RAS that can be targeted with post-stroke therapies administered non-invasively to induce neuroprotection.

Specific Aim 1 evaluated stroke-induced changes in the RAS in the absence of targeted interventions in preclinical models and an observational human stroke study. ACE2 activity in the ischemic cerebral cortex was doubled in the first day after stroke and returned to control levels by three days. Serum ACE2 activity was decreased by
~25% initially, with convalescent increases back to pre-stroke or control levels at three days post-stroke in both animals and humans. In humans lower levels of serum ACE2 activity were significantly correlated with ischemic stroke. Post-stroke distribution and cellular localization of AT2Rs in the brain were not affected at one or two weeks post-stroke. Specific Aim 2 was designed to test therapies that might target these stroke-induced changes with investigational compounds. Post-stroke injections of diminazene, which activated ACE2, resulted in ~25% reductions in infarct sizes and improved neurological function. Testing of post-stroke treatments with other compounds including orally active Ang-(1-7) and bioencapsulated ACE2 also resulted in significant improvements. Post-stroke injections of Compound 21, an AT2R agonist, resulted in ~25% reduced infarct volume and improved neurological function durable to three weeks post-stroke. These findings suggest that stroke therapeutics that target the protective RAS may interact cooperatively with endogenous stroke-induced changes, lending promise to their study as neuroprotective agents to treat patients for whom current therapies are lacking.
CHAPTER 1
REIN ANGIOTENSIN SYSTEM PATHWAYS IN STROKE NEUROPROTECTION

Stroke Epidemiology

As a cause of worldwide mortality, stroke ranks second only to ischemic heart disease.\(^1\) In association with increased use of statins and blood pressure medications, stroke recently fell from the fourth to the fifth leading cause of death in the United States. Despite this, almost one million Americans still suffer a stroke each year.\(^2\) This translates to one person having a stroke every 40 seconds, with one person dying from stroke every 4 minutes, which accounts for \(~1\) of every 20 deaths.\(^2\) As the U.S. population ages along with an increasing number of stroke survivors, the prevalence of stroke is expected to increase 20.5% by the year 2030.\(^2\) By that time, the financial burden of stroke as a per capita cost, which is compounded by the likelihood of extended hospital stays and long-term nursing care for many stroke patients, is expected to reach more than $20,000 per person.\(^2\) Outside of the U.S., the burden of stroke is even heavier, with rates of disability and mortality from stroke that are at least 10 times greater in medically underserved developing nations.\(^3\)

Stroke has been singled out by the World Health Organization as the incoming epidemic of the 21\(^{st}\) century.\(^4\) Several demographic factors are known to be associated with increased risk for stroke. Advancing age is associated with an approximate

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Doubling of incident stroke rates per decade of life, and a tripling of the rate for those 85 or older.\textsuperscript{2} The lifetime risk of stroke and overall stroke mortality is higher for women than for men, although this is due mainly to the larger number of elderly women.\textsuperscript{2} Family history and genetics also plays an important role, with a 3-fold increase in stroke risk for those with a parental ischemic stroke by the age of 65 years.\textsuperscript{2} Several gene regions and alleles (e.g. specific apolipoprotein E alleles) have been associated with specific stroke subtypes.\textsuperscript{2} The effect of race on stroke risk is also well-documented. Age-adjusted incidence of first ischemic stroke among blacks and among Hispanics is approximately double that of whites,\textsuperscript{2} and while adults of all races have seen a decline in the age-adjusted stroke death rate over the past three decades, African Americans continue to experience rates that are \~50\% higher than other races.\textsuperscript{2} Although the reasons for these discrepancies are not fully understood, they are under intense study and likely include differences in the prevalence and management of modifiable stroke risk factors, including hypertension,\textsuperscript{5} diabetes,\textsuperscript{6} hyperlipidemia,\textsuperscript{7} and diet.\textsuperscript{8} In addition to these factors, residence within the southeast region of the U.S. known as the stroke belt is also associated with increased risk for incident stroke,\textsuperscript{2} an association which may be most profound during the years of adolescence.\textsuperscript{9}

While these demographic factors contribute to the risk of stroke, it is thought that the large majority of strokes could be prevented by improved control over modifiable risk factors,\textsuperscript{10} which include high blood pressure, both pre-hypertension and hypertension, current smoking/tobacco use, obesity, nutrition, physical inactivity, diabetes mellitus, alcohol intake, psychosocial stress and depression, atrial fibrillation, hyperlipidemia, chronic kidney disease, sleep apnea, and hormone supplementation.\textsuperscript{2, 10} As the leading
modifiable risk factor for stroke, high blood pressure represents an especially important target for stroke prevention strategies. A 25% reduction in stroke has been demonstrated with just a 2 mm Hg reduction of systolic blood pressure (SBP), a relationship which persists even in pre-hypertension ranges of SBP.\(^4\) Perhaps more in the case of stroke than in many others, Franklin’s adage holds true: an ounce of prevention is worth a pound (or more) of cure.

**Stroke Pathophysiology**

Stroke can be broadly classified into two types: ischemic and hemorrhagic strokes. Ischemic stroke occurs when blood supply to the brain is decreased, often due to thromboembolism or to atherosclerotic occlusion, depriving the brain of oxygen and nutrients.\(^1^1\) This process results in an ischemic core of necrotic tissue within minutes of an ischemic event. Outside this core exists tissue known as the penumbra that is somewhat buffered by collateral blood flow. In the hours and days following the ischemic event, the cells in this region offer a target for therapies that promote reperfusion and/or resolution pathways.\(^1^1,^1^2\) Hemorrhagic stroke, in contrast, occurs when a blood vessel within the brain ruptures and releases blood.\(^1^3\) Of all reported strokes, 80-90% are ischemic strokes and 10-20% are intracerebral hemorrhagic strokes and subarachnoid hemorrhages. Regardless of type, the outcome of stroke is essentially the same: neuronal death and consequent behavioral and motor symptoms result from the absence of oxygen and nutrients and/or sudden bleeding into the brain. Decades of research in the field of stroke therapy have successfully yielded only one FDA-approved treatment for ischemic stroke, the use of thrombolytic or “clot-busting” drugs to re-establish blood perfusion.\(^1^4\) Unfortunately, less than 5% of stroke patients receive this treatment due to contraindications for anticoagulation and a narrow
therapeutic time window.\textsuperscript{15, 16} In addition, approximately one in twenty patients treated with tissue plasminogen activator or other anti-coagulant strategies for clot fibrinolysis experience symptomatic bleeding.\textsuperscript{14} A large number of preclinical and human clinical trials have been conducted in an effort to discover novel stroke treatments, but the clinical unpredictability and multi-factorial nature of the disease have hampered these efforts.\textsuperscript{17} Despite their failure to identify a clinically-validated drug target, these studies have provided vastly improved insight into the pathophysiology of stroke and helped to define characteristics that may determine successful testing of future targets.\textsuperscript{18}

**Stroke Core and Penumbra**

The damage that occurs in stroke involves a progression of cell death that begins in the area of greatest ischemia or toxicity, called the core, and over time spreads to surrounding at-risk brain tissue, known as the penumbra, which is still supported by some blood supply from collateral circulation.\textsuperscript{11} In the first minutes following disrupted blood flow, the brain tissue at the core of the stroke experiences profound ischemia and cells that are now deprived of oxygen and glucose become overloaded with calcium and begin undergoing necrotic cell death. This results from rapid disruptions to their membrane ionic homeostasis, an essential element to their function and survival that requires constant energy-dependent maintenance. These dying cells, which include neurons and glial cells, release large amounts of glutamate, an excitatory neurotransmitter, which can act on ionotropic and metabotropic receptors on nearby cells to propagate recurrent membrane depolarization and calcium overload.\textsuperscript{12} As these peri-infarct depolarizations spread outward, penumbral tissue begins to collapse as calcium within cells activates cell death pathways. This process is exacerbated by oxidative damage that results from increased production of free radicals by damaged
tissues. A part of a highly metabolically active organ, brain cells contribute radicals from mitochondrial oxidative metabolism, nitric oxide synthases, and through the action of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Reperfusion of the ischemic tissue with oxygen and glucose-rich blood, whether spontaneously or following thrombolytic therapy, contributes to increasing levels of oxidative stress. Oxidative damage results to essential cellular components, including fatty acids and other membrane lipids, deoxyribonucleic acid, and proteins.

While these early mechanisms of cell death and ischemic brain damage have proven difficult to successfully counteract with neuroprotective treatments due to their rapid time course, a set of later events also occurs that may contain more promising targets. Necrotic cell death in the core within the early minutes gives way to apoptotic cell death in penumbral tissues over the ensuing hours and days, and interventions that interrupt this apoptotic process hold promise. Neuroinflammatory cascades are also initiated as part of the late events in stroke, which are driven by detrimental proinflammatory cytokine signaling by activated glial cells and by peripheral leukocytes that invade across a compromised blood brain barrier.

**Blood Brain Barrier Breakdown**

As treatment strategies are designed that may salvage at risk penumbral tissue, consideration should also be given to the characteristic pattern of BBB permeability following stroke during which these or any peripherally administered therapies may gain access to the brain. The BBB has the dual role of preventing unregulated exchange of solutes, protein, and other neuro-immune substances between the blood and the CSF while facilitating efficient exchange of essential nutrients. It accomplishes this via tight junctions between endothelial cells and via an impressive array of protein transporters.
During an ischemic event, energy failure from a lack of oxygen and glucose spurs a cascade of events involving swelling of endothelial cells and subsequent disruption of the essential astrocytic foot processes that help form the BBB. Matrix metalloproteinases and other proteases become activated and begin to degrade the basal lamina surrounding vessels, and down regulation of tight junction proteins further contribute to increases in paracellular permeability. Reperfusion facilitates additional oxidative stress as well as recruitment of peripheral leukocytes to the ischemic tissue, which secrete additional proteases and inflammatory cytokines that increase the disruption of the BBB.

As these and other mechanisms compromise the barrier’s integrity in response to ischemic injury, leakiness of the barrier develops in a characteristic pattern of immediate hyperemic reperfusion permeability followed by a biphasic period of permeability. The first phase is 3-8 hours post ischemia/reperfusion and the second phase is from 18-96 hours post injury. During the first clinically modifiable phase of BBB leakiness in response to stroke, permeability is greatest to small molecules less than ~200 kDa, such as aminoisobutyric acid, [14]C-Sucrose, albumin tagged with Evans blue dye, and lower molecular weight dextrans. At this early phase, the barrier remains relatively less permeable to red blood cells and larger molecules such as higher weight dextrans (2000 kDa). The second phase of BBB permeability after stroke is characterized by increased number and size of openings of the tight junctions to allow greater permeability to both lower and higher molecular weight molecules for a longer period of time, including RBCs and higher weight dextrans that were not readily permeable in the early phase. Overall, the second permeability phase extends
the trends of the first phase. It is longer lasting, has more tight junction openings, and allows extravasation of larger molecules – all important factors impacting therapy targeted to ischemic brain tissue. Thus the development of therapeutic strategies for stroke that involve manipulation of the ACE2–Ang-(1-7)–Mas axis must take into account the BBB and its altered integrity following cerebrovascular insult.

**Infiltration and Activation of Immune Cells**

In addition to allowing access of small and large molecules carried in the blood, compromise of the BBB facilitates entry of peripheral leukocytes into ischemic brain regions. The infiltration of brain tissue by neutrophils, monocytes/macrophages, and lymphocytes begins early after stroke onset and contributes significantly to the progression of ischemic damage and eventual resolution of inflammation. This invasion by circulating immune cells begins with neutrophils, which invade as early as 30 minutes after stroke,\(^{32}\) and secrete matrix metalloproteinases that contribute to the further breakdown of the BBB.\(^{33}\) By 24 hours after stroke, circulating monocytes have begun invading and the resulting macrophage population adopts an activated phenotype that allows them to act as antigen presenting cells to induce activation of invading T cells.\(^{34}\)

Over the next several days, increasing numbers of monocyte/macrophages infiltrate into ischemic regions to phagocytose debris and secrete cytokines. Interestingly, a recent study found that the population of resident microglia may actually decline, at least initially, in the ipsilateral hemisphere following stroke in response to the ischemia, and that those microglia that remain produce higher levels of pro-inflammatory cytokines and oxidative stress than the invading monocyte/macrophage population. Although these activated inflammatory microglia are thought to contribute to
the ischemic damage in the peri-infarct zone, another population of healing and repair-oriented microglia typical within the core of the stroke has also been identified.\textsuperscript{35} By one week after stroke, the monocytes/macrophages decline and resident microglia remain as the predominant phagocytes in the brain.\textsuperscript{36}

Recruitment and regulation of peripheral leukocytes by ischemic tissues is coordinated, at least in part, by astrocytes. The resistance to ischemic damage that these support cells exhibit, relative to neurons and endothelial cells, allows them to perform important functions within and around ischemic lesions. They are initially involved in the recruitment of leukocytes to damaged areas where they can phagocytose tissue debris and potentially promote repair processes.\textsuperscript{37} Astrocytes can also limit the spread of infiltrating inflammatory cells by becoming activated to form a glial scar through a process called astrogliosis. These functions are essential to the natural repair processes following damage to the central nervous system, as disruption of astrocytes has been shown to have association with larger infarct sizes, increased inflammation, and worse function.\textsuperscript{37}

Different populations of T cells that are recruited and persist in the stroke brain appear to both contribute to and inhibit the inflammatory and apoptotic processes that occur during stroke recovery. As the main source of interferon gamma, infiltrating effector T cells exert delayed neurotoxic effects, and depletion of CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells results in smaller infarct sizes in preclinical studies.\textsuperscript{38} Populations of T regulatory cells, however, may be protective in stroke, as their depletion results in exacerbated ischemic damage and increased pro-inflammatory signaling among brain microglia and invading effector T cells.\textsuperscript{38} Research in this area is moving forward, and a clearer
understanding of these complex immune interactions and their consequences may provide mechanisms for potential neuroprotective treatments.

The Classical Renin Angiotensin System (RAS)

High blood pressure is a powerful driver of increased risk for both ischemic and hemorrhagic stroke,\(^2\) and as one of the primary regulators of blood pressure, the renin-angiotensin system (RAS) has a profound impact in stroke (see Figure 1-1). One factor that has been consistently linked to deleterious actions in stroke is the peptide angiotensin II (Ang II), which is the best known member of the renin angiotensin system (RAS). Ang II, formed from angiotensin I primarily by the action of angiotensin converting enzyme (ACE) and secondarily by chymase, plays a critical physiological role in blood pressure regulation, fluid balance, and electrolyte homeostasis, effects that are mediated by its type 1 receptor (AT1R).\(^{39-41}\) The original view of the RAS as a circulating endocrine system involving primarily the liver, vasculature of the kidney, and lung endothelium has been revised in recent decades to include the discovery of its ubiquitous expression in many tissues, including the brain,\(^42\) where both autocrine signaling and paracrine signaling contribute to its tissue specific effects.\(^42,43\)

Derangements in the RAS that result in chronic increases in circulating levels of Ang II and sustained over activation of AT1Rs contribute to various cardiovascular diseases, including hypertension, cardiac hypertrophy, cardiac fibrosis, atherosclerosis, and myocardial infarction, many of which are risk factors for stroke.\(^44\)

Decreased Stroke Risk by RAS Blockade

Since the development of the first orally-active ACE inhibitor (ACEi), captopril, in 1975, the RAS has been a primary therapeutic target for the treatment of hypertension and related diseases. The original view of the RAS holds that conversion of angiotensin
I into Ang II by ACE leads to activation of AT1Rs in multiple tissues to induce various effects that act in concert to defend and increase blood pressure, elicit electrolyte and water retention by the kidney, and increase thirst via central mechanisms. In the case of prolonged overstimulation of AT1Rs, however, Ang II signaling increases inflammation, fibrosis, and cellular hypertrophy, which contribute to disease pathophysiology. High blood pressure and cerebrovascular health are inextricably linked, with hypertension as the leading modifiable risk factor for stroke. A meta-analysis of 10 placebo-controlled trials found that RAS blockade significantly reduced the incidence of stroke, with a larger and more significant reduction by ACEi versus AT1 receptor blocker (ARB) therapy. In a separate meta-analysis of 10 trials conducted among patients with diabetes and hypertension, neither ACEi nor ARB therapy was found to be associated with a significant reduction in stroke, although there was a reduction in overall cardiovascular mortality. A newly published study found that patients with resistant hypertension who switched to treatment olmesartan, a newer ARB, experienced improvements in blood pressure control as well as in levels of plasma biomarkers of cardiovascular health and stroke risk, including increased levels of angiotensin-(1-7). Trials to study the effects of ACEi’s and ARBs, including the PROGRESS and MOSES trials, have demonstrated the efficacy of these therapies at reducing the risk of stroke recurrence. The potential reduction in stroke risk that results from therapy with RAS blockers in humans is likely closely tied to the beneficial effects of decreases in blood pressure, although other protective actions of decreased AT1R activation are also likely to be involved.
Neuroprotection by RAS Blockade

Neuroprotection is defined here as an enhancement of the brain’s resilience to an injury, such as ischemia, in order to improve clinical outcome. This definition is not limited to actions only on neurons, but extends to include glial and other cells within the brain. Recent research has shown that in addition to having efficacy in treating hypertension and decreasing stroke risk, ACEi’s and ARBs are neuroprotective in stroke.45,50-55 The evidence for neuroprotective effects in humans is scarce but encouraging. One recent study that analyzed the preadmission use of ACEi’s or ARBs among over 100,000 stroke patients found that patients who were current users of ACEi’s/ARBs at the time of incident ischemic stroke were at 15% lower relative risk of 30 day mortality than non-users.56 One randomized trial57 has evaluated the effect of irbesartan therapy (n=23 patients), an ARB, initiated within 4 days after stroke onset versus placebo (n=20 patients) on infarct growth and markers of inflammation. This study did not demonstrate a significant effect of ARB therapy, which was initiated at an average of 53 hours post-stroke, at reducing infarct volume or improving levels of cerebral blood flow at 3 or 30 days post-stroke, but did find a significant reduction of levels of C-reactive protein, a surrogate marker of inflammation, at 30 days after stroke. While no effect of ARB therapy on infarct volume was found in this study, initiating therapy much earlier (e.g. within four hours post-stroke) is far more likely to uncover potential neuroprotective effects. Carefully conducted early phase clinical trials to test the efficacy of post-stroke treatment with ACEi’s or ARBs are needed to begin a more thorough assessment of the potential for these approved and relatively safe therapies for their neuroprotective efficacy in humans.
The preclinical evidence for stroke neuroprotection induced by RAS blockade is substantial and positive. ARBs prevent the occurrence of hemorrhagic stroke in stroke prone spontaneously hypertensive rats (spSHRs) even at doses that did not prevent hypertension. In preclinical models of ischemic stroke, ARBs have repeatedly been shown to induce neuroprotection when given before or after stroke. Additionally, many other studies have demonstrated neuroprotective efficacy as measured by decreased infarct volumes and improved neurological function when RAS blockers are administered either peripherally or centrally. In one study, there were significantly increased areas of salvageable penumbral tissue in brains from AT1R-deficient mice than from wild-type littermates, while angiotensinogen overexpression resulted in a more rapid penumbral collapse, results that demonstrate clearly the deleterious actions of Ang II at AT1Rs in stroke. The ability of RAS blockade to induce neuroprotective effects even in the absence of effects on blood pressure is intriguing and suggests a reliance on other protective mechanisms that may be initiated at the level of the brain-specific RAS. This notion is corroborated by studies employing β-adrenergic receptor blockers and calcium channel blockers which do not protect against ischemic stroke despite lowering blood pressure.

**Alternative Pathways in the Renin Angiotensin System**

In contrast to the harmful actions of the ACE/Ang II/AT1R arm of the RAS, there is a growing body of evidence that more recently discovered components of the RAS such as Ang II type 2 receptors (AT2Rs), which have Ang II as their ligand, and angiotensin-(1-7) [Ang-(1-7)] and its receptor Mas exert protective anti-AT1R effects in a number of cardiovascular diseases. The classical view of the RAS has therefore been expanded to include both an alternative receptor for Ang II, the AT2R, and an
alternative pathway for Ang II metabolism and signaling in which Ang II is hydrolyzed by angiotensin converting enzyme 2 (ACE2) to form Ang-(1-7). In cases of chronic overstimulation of the ACE – Ang II – AT1R pathway, ACE2 might be considered a safety valve that metabolizes excessive Ang II into Ang-(1-7). Alternative pathways for angiotensin peptide metabolism and Ang-(1-7) production have also been identified and are detailed in Figure 1-1. This heptapeptide binds to and activates a unique G-protein coupled receptor known as Mas to exert effects that are largely in direct opposition to those of AT1R activation. The next sections will explore evidence regarding the protective actions of these alternative RAS pathways axis in stroke.

**The ACE2–Angiotensin-(1-7)–Mas Axis and Stroke**

As a leading cause of death and disability worldwide, stroke is an exceptionally important disease for continued translational research. The ACE2–Ang-(1-7)–Mas pathway has many characteristics that lend promise to its potential as a target for treatments to successfully induce stroke neuroprotection.

First, activation of this axis has been demonstrated to have therapeutic potential for hypertension and related pathology, myocardial infarction, heart failure, several types of cancer, and other diseases.

Second, all of the components of the ACE2–Ang-(1-7)–Mas axis are present in the CNS endogenously. In rodent models, Mas mRNA has been detected throughout the brain, including but not limited to the hippocampus, cortex, thalamus, and vasculature. Mas protein was demonstrated to be present in the cortex, basal ganglia, thalamus, hypothalamus, hippocampus, amygdala, circumventricular organ, and blood vessels. Co-localization studies in rats showed that within the cerebral cortex and striatum, Mas is present on neurons, microglia, and endothelial cells.
Within the human brain, ACE2 mRNA has been detected\(^9^0\) and immunofluorescence studies have shown ACE2 immunoreactivity in vascular smooth muscle cells and endothelial cells.\(^9^1\) Although cell culture experiments have suggested ACE2 to be expressed in glia,\(^9^2\) mouse *in vivo* studies show ACE2, at the mRNA and protein level, to be most predominantly expressed by neurons within cardiovascular control regions, brainstem, raphe, motor cortex, and many other areas.\(^9^3\)

Third, this axis undergoes dynamic changes in stroke,\(^9^4, 9^5\) which may allow targeted treatments to act synergistically with endogenous mechanisms. This makes the idea of manipulating this system in a favorable way an attractive therapeutic strategy.

Fourth, its effects are robust; neuroprotection has been observed in various models of ischemic and hemorrhagic stroke from different laboratories, as detailed below.

And lastly, it is hypothesized that drug targets with multiple therapeutic effects such as those described below for the Ang-(1-7)/Mas axis, as opposed to a single mechanism of action, will prove more likely to translate.\(^9^6\) For all of these and other reasons, it has been recently suggested that the Ang-(1-7)/Mas axis in stroke represents an especially promising candidate for targeted stroke therapy.\(^9^7\)

**Stroke and the brain RAS**

Importantly for stroke, the cells of the CNS express all of the components of the ACE2–Ang-(1-7)–Mas pathway.\(^9^8\) Until recently, it was not entirely clear whether expression levels of components of this protective axis were altered following stroke. Studies had shown that levels of Ang II are increased after stroke in the ventral cortex\(^9^9\) and the rostral ventrolateral medulla (RVLM).\(^9^5\) In addition, AT1R expression was shown to be decreased in the cerebral cortex following transient middle cerebral artery
occlusion (MCAO), but increased in the RVLM. In a rat model of middle cerebral artery occlusion, these components were shown to be involved in a dynamic process during ischemic stroke. Ang-(1-7) was shown to be increased in the ischemic brain and in circulation at 6-48 hours after stroke. Furthermore, ACE2 and Mas mRNA and protein were increased in the ischemic brain following stroke. Within the RVLM, stroke resulted in decreased Ang-(1-7) levels for up to three days post-stroke and an initial decrease in Mas one day following stroke, followed by significant increases three and seven days after middle cerebral artery occlusion (MCAO) in rats. Gene array results from this study indicated an increase in ACE2 expression one day following stroke. Another study using samples from human stroke patients found that serum ACE2 activity was significantly higher among cardioembolic strokes, and concluded that changes in ACE2 activity might be useful in diagnosing stroke subtype and predicting outcome. Further research in this area is needed to substantiate the changes observed in these studies, and to clarify ways in which the Ang-(1-7)/Mas axis can be targeted to act in synergy with endogenous post-stroke alterations in the components of this system.

**Ang-(1-7) and stroke neuroprotection**

A growing number of studies have now demonstrated neuroprotective actions of Ang-(1-7) in both ischemic and hemorrhagic stroke. The first look at the role of the protective ACE2-Ang-(1-7)-Mas axis in stroke found that ICV administration of Ang-(1-7) in stroke resulted in increased nitric oxide release via increases in endothelial nitric oxide synthase expression as well as increased bradykinin receptor expression. In 2011, our group was the first to find that rats that were infused centrally via the intracerebroventricular (ICV) route with Ang-(1-7) performed better on neurological
function testing and had an ~50% reduction in infarct sizes following ischemic stroke by endothelin-1 (ET-1)-induced MCAO.\textsuperscript{106} These effects were unlikely to have been a result of changes in hemodynamics, as during the stroke procedure, Ang-(1-7) had no effect of cerebral blood vessel diameter or cerebral blood flow. Also, this neuroprotection was prevented by co-administration of the Mas antagonist A-779. Of note, these findings have subsequently been verified in models of permanent MCAO.\textsuperscript{107-109}

Alternative means of activating this axis have also proven effective. In a study using Ang II overexpressing mice subjected to permanent focal ischemic stroke, neuronal ACE2 overexpression resulted in similar cerebroprotection in vivo\textsuperscript{110} and in vitro.\textsuperscript{111} Importantly, this effect was more pronounced in 8-month old mice compared to 3-month old mice, indicating that the neuroprotective effects of Ang-(1-7) are preserved and may even be enhanced in aged animals.\textsuperscript{112} To add another level of complexity, human renin and angiotensinogen double transgenic mice with neuronal ACE2 overexpression have decreased infarct volume, increased cerebral blood flow, increased neurological function, and increased microvascular density compared to the same double transgenic mice with normal ACE2 expression.\textsuperscript{110} Additionally, lenti-viral ACE2 priming of endothelial progenitor cells enhanced the ability of these cells to reduce infarct size and improve neurological function.\textsuperscript{113} These findings are especially relevant from a translational perspective, as systemic treatment infusions were not started until two hours after stroke. One recently published study found that administration of a receptor Mas agonist, AVE0991, was not effective at reducing infarct size when
measured at 24 hours after stroke, whereas AVE0991 administered in vitro reduced neuronal cell death by ~60% in a culture of primary cortical neurons.\textsuperscript{114}

In addition to ischemic stroke, activation of this axis also appears to have therapeutic potential in hemorrhagic stroke. Ang-(1-7) had several therapeutic effects when given centrally for 6 weeks to stroke-prone Spontaneously Hypertensive Rats (spSHR) fed a high salt diet. These rats develop intracerebral hemorrhages throughout the brain spontaneously secondary to severe hypertension and chronic vascular pathology. The group that was treated with Ang-(1-7) showed increased survival, decreased number of hemorrhages in the striatum, less lethargy, and improved sensorimotor function.\textsuperscript{115} Similar Ang-(1-7)-induced cerebroprotection has been demonstrated in models of collagenase-induced intracranial hemorrhage.\textsuperscript{98, 116} In summary, there is a growing body of evidence that activation of the central Ang-(1-7)/Mas axis can exert profound protective effects in stroke. A discussion of the potential mechanisms of Ang-(1-7)-induced neuroprotection follows in Chapter 4.

**Angiotensin II Type 2 Receptors and Stroke**

Within the pathways of the protective RAS, the AT2R has emerged as a promising target for stroke research. A growing body of evidence indicates that activation of AT2Rs, which results in effects that generally oppose AT1R effects,\textsuperscript{75} induces neuroprotection in preclinical models of stroke. These AT2R-dependent protective effects may even be responsible for the cerebroprotective action of ARBs in stroke, which could be triggered by permissive unopposed activation of AT2Rs in the presence of AT1Rs antagonism.\textsuperscript{58, 59, 70, 71, 117, 118} Expression of AT2Rs is increased in many disease tissues, such as in myocardial tissue post-infarction, atherosclerotic blood vessels, wounded skin, and may even be increased in cerebral cortex following
ischemia.\textsuperscript{70, 99, 119-122} These higher levels of AT2Rs may be activated to exert neuroprotective effects by the increased endogenous levels of Ang II that can result from ARB treatment.\textsuperscript{70} In support of this idea, one study demonstrated that the neuroprotective effects of treatment with an ARB were abolished by an AT2R antagonist.\textsuperscript{70} In addition, when AT2R knockout mice exhibit larger strokes compared to wild-type controls.\textsuperscript{123} Taken together, these results point to the balance of AT1R and AT2R activity as an important element in the regulation of tissue recovery after stroke.

Recently, a number of studies have tested the efficacy compounds to directly activate AT2Rs in stroke. The first of these employed intracerebroventricular (ICV) administration of a small peptide agonist of AT2R called CGP42112 beginning five days prior to inducing stroke in conscious spontaneously hypertensive rats. This resulted in reduced infarct size and improved performance on the ledged beam test, an assessment of neurological function, at 72h post-stroke.\textsuperscript{118} These protective effects were shown to be AT2R-dependent, as they were blocked by co-administration of the AT2R antagonist PD123319. Later, this group demonstrated that initiation of treatment with CGP42112 starting 6 hours after stroke by either ICV infusion or intraperitoneal (IP) injection was likewise neuroprotective,\textsuperscript{124} results that are confirmed by a study in mice in which the agonist was given by IP injection at the time of reperfusion to neuroprotective effect.\textsuperscript{125}

Our group was the first to demonstrate AT2R-dependent neuroprotective effects in rats using a recently developed non-peptide AT2R specific agonist called Compound 21 (C21, N-Butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl)- 5-isobutythiophene-2-sulfonamide\textsuperscript{126}), which was shown to induce reduced infarct damage and improve
neurological function when given by ICV infusion before and after stroke as well as when given by IP injections starting four hours after stroke\textsuperscript{127}. These findings were confirmed in mice where a higher dose of C21 was also shown not to induce changes in blood pressure\textsuperscript{128} and in conscious hypertensive rats with treatments starting six hours post-stroke.\textsuperscript{129} The neuroprotective effects of AT2R activation by systemic administration of C21 were shown to be sustained for up to seven days as induced by a single IP injection at 90 minutes post-stroke.\textsuperscript{130} Activation of AT2Rs has also been shown to protect against stroke damage induced by intracranial hemorrhage and to improve survival in stroke-prone SHRs.\textsuperscript{131} A recent study from our group that used transgenic AT2R reporter mice localized AT2R expression within the forebrain specifically to neurons within the cortex and not the striatum.\textsuperscript{132} Activation of neuronal AT2Rs might induce a variety of neuronally-mediated mechanisms to affect the neuroprotection that we and others have observed, as discussed in more detail in Chapter 4. While these early studies of the benefits of AT2R activation in stroke are encouraging, additional translationally-oriented testing is needed to validate approaches that could be tested clinically.

**Summary**

As one of the world’s most debilitating and widespread diseases, stroke represents an incredibly important target for research and improved clinical treatment options. The demographics of stroke are made reality when it is considered that as many as 1 in every 6 Americans will suffer a stroke in their lifetime, leaving practically no family untouched. While our understanding of the risk factors and pathophysiology of stroke is extensive, the translation of this body of knowledge into operative therapies has fallen far short of success. The identification of protective pathways within the RAS
and the demonstration of clear neuroprotective benefits from activation of these pathways should give reason for hope. The foundation of preclinical evidence showing efficacy of targeted activation of the protective RAS in stroke is becoming well-established. The focus of the work contained in the following chapters is to provide important data to help translate the promising results from earlier preclinical experiments into valid bedside therapeutics. To this end, we developed the general hypothesis that during ischemic stroke, neuroprotective pathways within the RAS including AT2Rs and the ACE2–Ang-(1-7)–Mas axis, undergo changes that can be targeted with newly developed therapeutics that are administered non-invasively in the hours after stroke onset to neuroprotective effect. This was assessed by two specific aims that combined studies in experimental models of stroke as well as an observational study in humans.

**Specific Aim 1. Characterize the dynamic alterations in the expression and activity of components of both the classical and protective RAS that occur during stroke in rodents and in humans.** Hypothesis: RAS components will be regulated during ischemic stroke similarly in rodents and humans in ways and over a time-course that may uncover important points for targeted interventions with novel therapeutics.

**Specific Aim 2. Determine the neuroprotective effects of post-stroke systemic administration of treatment compounds designed to activate the protective RAS in rodents.** Hypothesis: Administration of compounds to activate ACE2, the Ang-(1-7) receptor Mas, or AT2Rs will result in decreased infarct size and improved neurological function, effects will be sustained for up to several weeks and inducible in both young and aged rats.
Figure 1-1. Components of the renin angiotensin system. ACE: angiotensin converting enzyme; ACE2: angiotensin converting enzyme 2; Ang I: angiotensin I; Ang II: angiotensin II; Ang-(1-7): angiotensin-(1-7); Ang-(1-9): angiotensin-(1-9); NEP: neprilysin; PEP: prolyl endopeptidase; PCP: prolyl carboxy peptidase.
CHAPTER 2
DYNAMIC CHANGES IN THE EXPRESSION AND ACTIVITY OF THE RENIN ANGIOTENSIN SYSTEM IN ISCHEMIC STROKE: MOVING TARGETS FOR STROKE TREATMENTS

Introduction

Despite the important role of the renin angiotensin system in regulating blood pressure and cardiovascular health, surprisingly little is known regarding its activity during acute ischemic stroke (IS). The recent discovery and characterization of a protective pathway within this system, the angiotensin converting enzyme 2/angiotensin-(1-7)/Mas [ACE2–Ang-(1-7)–Mas] axis, has increased the potential impact of an improved understanding of the regulation of this system in IS. By catalyzing the conversion of angiotensin II (Ang II) into Ang-(1-7), ACE2 counteracts the deleterious effects that result from sustained over-activation of the Ang II type 1 receptor (AT1R). Binding of Ang-(1-7) to its receptor Mas initiates anti-inflammatory, anti-oxidative, and vasodilatory effects in a variety of disease states. In the context of preclinical studies of IS, activation of the protective RAS by targeted interventions has been proven to reproducibly induce neuroprotection. We found that treatments with Ang-(1-7) or with an activator of ACE2 induce significant neuroprotection in a rat model of ischemia when administered via intracerebroventricular infusion before and after stroke. Activation of the angiotensin II type 2 receptor (AT2R) has been shown to induce similar beneficial effects in neurological injury and disease and specifically in stroke. Further, recent studies of AT2R activation in stroke from our lab have

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demonstrated profound neuroprotection when treatments are given centrally or peripherally, before or after stroke.\textsuperscript{127}

An important consideration for the continued study of the therapeutic effects of the ACE2–Ang-(1-7)–Mas axis in stroke is the characterization of changes that occur in the endogenous components of this system during stroke in the absence of targeted interventions. Studies of the role of ACE2 in human disease, especially in stroke, are limited. In rats, it has recently been reported that expression of this protective axis is altered following stroke in the rat cerebral cortex\textsuperscript{94} and rostral ventrolateral medulla,\textsuperscript{95} although it is unknown whether ACE2 activity levels are affected or whether this is accompanied by changes in the deleterious angiotensin converting enzyme/angiotensin II/angiotensin II type 1 receptor (ACE/Ang II/AT1R) axis. Several reports indicate that AT2R expression is increased in ischemic brain regions after stroke,\textsuperscript{70, 99, 120, 122} although localization of AT2Rs has been problematic due to limitations in available immunostaining and autoradiography techniques. Exploring these changes further in experimental models and in human disease will provide further support for targeting ACE2 with neuroprotective interventions. The objective here in our preclinical studies here was to test whether components of the RAS, including the ACE2–Ang-(1-7)–Mas pathway and AT2Rs, are altered in rodents following stroke. In a human observational study, we assessed the changes in endogenous ACE2 activity in human serum following IS or intracerebral hemorrhage (ICH) as compared to transient ischemic attack, conditions mimicking stroke (TIA/mimic) or to control participants. Furthermore, we explored the activity of ACE and associations between changes in enzyme activity and other relevant clinical measures.
Methods

Ethical Approval

The Institutional Animal Care and Use Committee approved all procedures adhering to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The ARRIVE Guidelines\textsuperscript{137} to improve the design, analysis, and reporting of research using animals were applied in all experiments. The collection and use of human samples and clinical data was by written informed consent in a manner approved by the Institutional Review Board of the University of Florida and according to institutional guidelines. This preliminary observational study has been registered with clinicaltrials.gov (NCT02409043).

Animals and Housing

For these experiments, we used nine week old male Sprague Dawley rats (275-300g) purchased from Charles Rivers Farms (Wilmington, MA, USA) and male AT2R reporter mice\textsuperscript{132} from the laboratory of Eric Krause (Department of Pharmacodynamics, University of Florida). This transgenic mouse model was generated to express green fluorescence protein (GFP) driven by AT2R regulatory sequences, with the result that all AT2R+ cells fluoresce green.\textsuperscript{132} All animals were drug and test naïve prior to inclusion in this study and housed in well-ventilated, specific pathogen-free, temperature-controlled facilities (24 ± 1 °C; 12–12 h light–dark cycle) with ad libitum access to water and standard rodent chow.

Anesthesia, Analgesia, and Euthanasia

Anesthesia for animal surgeries was induced using 100% O\textsubscript{2}/4% isoflurane and maintained using 100% O\textsubscript{2}/2% isoflurane. Post-operative analgesia was provided using buprenorphine (0.05 mg/kg, subcutaneously, Hospira Inc., Lake Forest, IL, USA).
Animals were euthanized by decapitation under deep anesthesia (5% isoflurane), with immediate brain removal and tissue processing and storage.

**Endothelin-1 Induced Middle Cerebral Artery Occlusion**

Adult Sprague Dawley rats underwent transient ischemia by occlusion of the right middle cerebral artery (MCAO) induced by stereotaxic microinjection of endothelin-1 (3µl of 80µmol/L solution) under isoflurane anesthesia as described previously. This model of focal ischemic stroke was selected for several reasons: 1) the gradual reperfusion of the tissue more closely resembles cerebral blood flow in human stroke than other temporary occlusion models associated with surge reperfusion and hyperemia; 2) the relatively non-invasive surgical procedure rarely results in surgical complications thus reducing experimentally-related mortality; and 3) we have effectively utilized the endothelin-1 model in this species and strain to establish the neuroprotective effects of manipulating of the ACE2–Ang-(1-7)–Mas axis pre-stroke, and we sought to build upon and extend these findings in the same model for comparison. To induce temporary occlusion of the middle cerebral artery (MCAO), a Hamilton syringe was lowered 7.5mm below the skull surface through a burr hole drilled stereotaxically at 5.2mm lateral and 1.6mm anterior to Bregma, and endothelin-1 (ET-1, 3µl of 80µmol/L solution) or sterile saline (3µl of 0.9% solution) was injected (1µl/min). For rats in Experiment 2 described below, we additionally implanted a stainless steel guide cannula 5 days before ET-1 MCAO at the same coordinates used for the burr hole through which the Hamilton syringe was lowered to induce stroke. During surgery body temperature and depth of anesthesia were controlled, and the latter was monitored using the eye blink reflex, reaction to paw pinch, and visual monitoring of depth and...
frequency of respirations. Relative cerebral blood flow was monitored by laser Doppler flowmetry as described below.

**Intraluminal Transient Middle Cerebral Artery Occlusion**

Mice underwent transient middle cerebral artery occlusion using the intraluminal filament method as described previously. This procedure was performed under isoflurane anesthesia. Through a ventral midline neck incision, the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were carefully isolated. Silk suture, size 4-0, was used to permanently ligate the common carotid artery and the ECA was temporarily blocked using a vascular clip. A second silk suture was then loosely tied around the bifurcation of the CCA, followed by a small incision in the CCA approximately 2 mm proximal to the carotid bifurcation. Through this incision, a 7-0 silicone-coated filament was inserted and gently directed 8-10 mm (normally 18-20 mm for rats) distally along the ICA until a mild resistance was encountered indicating the branching of the anterior and middle cerebral artery. This occluding filament was tied in place for 45 minutes, during which time the animal was allowed to recover from anesthesia under close monitoring in a temperature controlled environment. Several minutes prior to the end of the occlusion period, animals were re-anesthetized, and the silicone-coated filament was gently removed to allow reperfusion of the MCA territory, followed by permanent ligation just proximal to the bifurcation of the ECA and the ICA from the CCA using the previously placed silk suture. Sham surgeries consisted of isolation of the CCA and permanent suture ligation without monofilament insertion. Animals receive post-operative care including subcutaneous fluids and continued analgesic administration.
Animal Experimental Protocols

The rodent experiments in this study were designed to assess post-stroke changes in the RAS and related systems in the absence of targeted interventions, with ACE2 activity levels assessed as the primary outcome measure and other molecular markers as secondary outcomes.

Experiment 1. Rats were randomly assigned to undergo sham stroke (n = 31) or ischemic stroke (n = 35) by endothelin-1 MCAO. Tail clip blood samples (~150μl) were collected at one pre-stroke time point and again at 4h, 12h, 1d, 2d, and 3d after stroke in the same animal, where possible, followed by centrifugation (13,200rpm for 15m) and decanting of serum, which was stored at -80 °C until analysis. Groups of rats were euthanized at 4h, 12h, 1d, and 3d after stroke for brain tissue collection. Brains were immediately placed in cold 0.9% saline and cut into 2mm coronal sections. Two sections from 1 to 5mm rostral to bregma were separated into left (contralateral to the stroke) and right (ipsilateral to the stroke) cortex and striatum before tissue homogenization and storage at -80 °C for use in mRNA and enzymatic activity assays. For the 1d group, a 2mm section from 1mm caudal to 1mm rostral to bregma was immediately saved in optimal cutting temperature compound (Sakura, #4583) and frozen at -20 °C in preparation for immunohistochemical analyses.

Experiment 2. AT2R reporter mice were subjected to sham surgery or 45 minutes of intraluminal temporary middle cerebral artery followed by euthanasia and tissue fixation by cardiac perfusion with saline followed by 4% paraformaldehyde (~50mL each) at 7 days post-stroke (n=6 stroke and 3 sham) or 14 days post-stroke (n=5 stroke and 3 sham). Brains were harvested and post-fixed in 4% paraformaldehyde overnight followed by 30% sucrose until sectioning.
mRNA Analyses

Angiotensin converting enzyme (ACE), angiotensin II type 1 receptor (AT1R), angiotensin converting enzyme 2 (ACE2), Mas, angiotensin type 2 receptor (AT2R), ADAM metallopeptidase domain 17 (ADAM17 or TACE), cluster of differentiation 11b (CD11b), and lipocalin-2 (LCN2) mRNA levels were assessed using real-time reverse transcription-PCR (qRT-PCR) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as detailed previously.\textsuperscript{127} Oligonucleotide primers and Taqman probes were from Applied Biosystems. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.

Immunohistochemistry and Semi-Quantitative Analysis

Mas immunoreactivity and its co-localization with neurons was assessed as we have described previously\textsuperscript{89} using 20µm brain sections obtained from fresh frozen brains from sham and stroke rats in optimal cutting temperature compound. Sections were primary labeled with mouse anti-NeuN (Neuron specific protein; 1:100) and rabbit anti-Mas (1:100), and secondary labeled with Alexafluor donkey anti-mouse 488 (1:500) and goat anti-rabbit 594 (1:750) before mounting in DAPI vectashield followed by fluorescence imaging. In order to perform a semi-quantitative analysis of levels of NeuN and Mas immunofluorescence, we averaged the fractional area of immunopositive staining from three standardized 40x imaging fields from each of six 20 µm sections through the ischemic region, for a total of 18 fields from each brain. For each section, three standardized fields were imaged from the infarcted cerebral cortex and included images 1) from near midline, 2) a region of cortex at the dorsal edge (penumbra) or 3) fully within (core) the infarct area as typically defined by infarct volume analysis.
Fractional area was calculated using ImageJ software (NIH) to identify immunopositive versus total area.

GFP immunoreactivity (measured as a direct index of AT2R expression) and its co-localization with neurons, microglia, or astrocytes was assessed using 30µm brain sections obtained from AT2R reporter mice brains fixed by perfusion with 4% paraformaldehyde solution as described above in Experiment 2 of the Animal Experimental Protocols section. Sections were primary labeled with mouse anti-HuC/D (human neuronal protein, 1:500), rabbit anti-Iba-1 (ionized calcium binding adaptor molecule 1, 1:1000), rabbit anti-GFAP (glial fibrillary acidic protein, 1:100), or chicken anti-GFP (green fluorescence protein, 1:500), and secondary labeled with Alexafluor donkey anti-mouse 488 or donkey anti-rabbit Cy3 before mounting in polyvinyl alcohol followed by fluorescence imaging. Mosaic images were produced using Adobe Photoshop®.

**Measurement of Relative Cerebral Blood Flow**

To measure relative cerebral blood flow by laser Doppler flowmetry, a Standard Pencil Probe was placed on the surface of the skull over the right middle cerebral artery territory, and using a Blood Flow Meter coupled to a Powerlab 4/30, we measured several minutes of baseline flow before stroke induction followed by continuous flow monitoring during vessel occlusion, as described previously.\(^{51,106}\) We routinely observed 40-60% reductions from baseline in the levels of relative cerebral blood flow following endothelin-1 induced MCAO in rats using this method (see Figure 3-2A) and 75-95% reductions from baseline in intraluminal temporary MCAO in mice.
Intracerebral Infarct Volume Determination

We measured cerebral infarct size by staining with 2,3,5-triphenyltetrazolium chloride (TTC; 0.05%) for 30 minutes at 37 °C as our group has detailed previously. Brain sections were scanned on a flatbed scanner (Canon) and the volume of healthy (stained) tissue from the contralateral hemisphere was compared to the volume of infarcted (unstained) tissue from the hemisphere ipsilateral to the ET-1 MCAO using ImageJ software (NIH). We assessed the infarct volume using two 2mm coronal sections from 1 to 5mm rostral to bregma which contain the stroke core and penumbra. Infarct volume analyses were performed by a blinded investigator.

Inclusion and Exclusion Criteria

Of the rats that underwent MCAO, five died shortly after stroke induction. Mouse surgeries were without mortality or exclusion.

Randomization and Allocation Concealment

Animals were identified by an assigned number and randomized using the randomize function in Microsoft Excel. Neurological assessments and analyses of all samples were performed by investigators blinded to group allocation.

Human Subjects and Sample Collection

Samples of peripheral blood, collected in serum tubes, were obtained between July 2014 and October 2015 and participant characteristics are described in Table 2-1. Samples were from n=38 IS and n=8 ICH patients at UF Health & Shands hospital within six hours of symptom onset (average of 3.6 hours), from n=20 control participants consisting primarily of patient spouses and adult children (control group), and from n=24 patients presenting with transient ischemic attack or symptoms mimicking stroke that were later determined to be of non-stroke etiology (TIA/mimic group). Patients with
subarachnoid hemorrhage or subdural hematoma were not included. A second phlebotomy was performed in n=15 IS and n=3 ICH patients who remained hospitalized at 3 days post-stroke, and volumetric analysis was calculated from magnetic resonance diffusion weighted images from 17 IS patients at ~24h after stroke. Samples were immediately placed on ice, followed by centrifugation for 10 minutes at 3000 rpm. Serum aliquots were frozen and stored at -80 °C until analysis. Plasma ACE2 activity was assessed in samples from an additional cohort of healthy young adults obtained as described previously. Clinical and demographic variables were obtained from patient interviews and medical records. Modified Rankin score at follow-up one to six months after stroke was available in 28 IS patients.

ACE2, ACE, and TACE Activity Assays

Activities of ACE2, ACE, and TACE in tissue and serum samples from rats were assessed by enzyme activity assay involving continuous fluorometric cleavage of specific substrates. Substrate concentrations were selected following determination of the optimal amount of sample (See Figure 2-1) and fluorogenic substrate (See Figure 2-2) using control samples and recombinant human ACE2 (R&D Systems, Inc., #933-ZN-010) as a positive control, and selectivity of the substrate for hydrolysis was verified by co-incubation with ACE2 inhibitor MLN-4670 (see Figure 2-3). Similar calibration experiments were performed for ACE and TACE activity (data not shown). Control samples were screened to verify that no correction for inner filter effect was required.

Rat brain and serum samples were collected and stored at -80 °C. Samples of rat cerebral cortex and striatum were homogenized and centrifuged in radioimmuno-precipitation assay buffer with phosphatase and protease inhibitors, and the supernatant was assayed to determine protein concentration using the Bradford protein
assay. Brain samples were then diluted (12µg protein per well) in ACE2 buffer (1mol/L NaCl, 75mmol/L Tris HCl, ph 7.5, and 50µmol/L ZnCl$_2$) or TACE buffer (100mmol/L NaCl, 50mmol/L Tris HCl, ph 7.5, 100µmol/L ZnCl$_2$, 10mmol/L CaCl$_2$) and were incubated in black flat-bottomed 96-well plates in 100µl of reaction mixture containing ACE2 buffer, 10µmol/L captopril, and 50µmol/L fluorogenic Mca-YVADAPK(Dnp)-OH ACE2 substrate (R&D Systems, Inc., #ES007), or TACE buffer and 10umol/L fluorogenic TACE substrate (Enzo Life Sciences, #BML-P132-0500), respectively. For the quantification of TACE activity, brain samples were co-incubated with 50µmol/L TAPI-2, a TACE inhibitor, to identify and subtract out non-specific peptidase activity.

Samples of rat serum (6µl per well) were assayed undiluted in reaction mixture containing 25µmol/L Mca-YVADAPK(Dnp)-OH ACE2 substrate. The slope of the fluorescence curves from 30-60 minutes were used to calculate relative fluorescence units (RFU) per minute using a Synergy Mx Microplate Reader (BioTek Instruments, Inc.) with excitation at 320nm and emission at 405nm. All samples were run in duplicate.

For ACE2 activity assays in human serum, samples were first processed to remove endogenous inhibitor of ACE2$^{141}$ by diluting 250µl serum into 1.2 mL of low ionic strength buffer (20mM Tris-HCl, pH 6.5), and mixing for 30 minutes with 200µl ANX Sepharose 4FastFlow binding resin (GE Healthcare, #17-1287-01) that was prewashed in 1mL buffer. After spinning for 10 minutes at 1200rpm, the precipitated resin was washed again with 1.2 mL of buffer, centrifuged again, and protein was eluted by washing with 500uL of high salt buffer (1M NaCl, 20mM Tris-HCl, pH 6.5). Supernatant containing eluted protein was decanted following a final spin-down and
stored at -80°C until activity assay. For ACE2 activity assays, the reaction mixture contained 75uL processed serum, 10µmol/L captopril, and 20µmol/L fluorogenic Mca-APK(Dnp) ACE2 substrate (Enzo Life Sciences, BML-P163-0001) in ACE2 buffer (1mol/L NaCl, 75mmol/L Tris HCl, ph 7.5, and 50µmol/L ZnCl₂). For ACE activity, the reaction mixture contained 0.2uL of unprocessed serum and 10µmol/L Mca-RPPGFSAFK(Dnp)-OH ACE substrate (R&D Systems, #ES005) in ACE2 buffer. For TACE activity, reaction mixture contained 20uL of unprocessed serum and 10umol/L fluorogenic TACE substrate (Enzo Life Sciences, BML-P132-0500) in TACE buffer (100mmol/L NaCl, 50mmol/L Tris HCl, ph 7.5, 100µmol/L ZnCl₂, 10mmol/L CaCl₂). All samples were run in duplicate.

**Chemicals**

Endothelin-1 (ET-1) was purchased from American Peptide Company, Inc. (Sunnyvale, CA, USA). A-779 was from Bachem Bioscience (Torrance, CA, USA), and diminazene aceturate was from Sigma-Aldrich (St Louis, MO, USA) and Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Mouse anti-NeuN was from Millipore (Bedford, MA, USA), and rabbit anti-Ang-(1-7) Mas receptor antibody was from Alomone Labs (Jerusalem, Israel). Chicken anti-GFP and mouse anti-HuC/D were purchased from ThermoFisher Scientific Life Technologies (Grand Island, NY), rabbit anti-Iba-1 from Wako (Osaka, Japan), and rabbit anti-GFAP from Sigma-Aldrich (St. Louis, MO). (Alexa Fluor® donkey anti-rabbit 594 and anti-mouse 488 were from Molecular Probes [Invitrogen] (Carlsbad, CA, USA). Alexa Fluor® donkey anti-rabbit Cy3, donkey anti-mouse Cy3, and donkey anti-chicken 488 were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Vectashield mounting medium with DAPI
was from Vector Laboratories (Burlingame, CA, USA). Unless otherwise noted, all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Data Analyses

Enzyme activity data are expressed as means ± SEM. For animal experiments, sample size determination was based on the primary outcome as specified in each experimental protocol and assumed a standard deviation of 15%, an effect size of 15%, power of 0.8, and alpha < 0.05. Statistical significance was evaluated with the use of the Kruskal-Wallis test, Mann Whitney test, one-way ANOVA for groups of equal variances, or student’s T-test.

For the human observational study, significance was assessed by one-way ANOVA with post-hoc t-test with Welch’s correction for comparisons of enzyme activity level by group or by race, by two-way Mann Whitney tests for comparison of baseline characteristics, by two-way Wilcoxon matched-pairs signed rank test to compare acute versus convalescent enzyme activity levels, and by two-way bivariate correlation analyses of ACE2 activity with indicated variables using Prism (GraphPad, Inc.). Normality was assessed and multiple linear and logistic regression analyses were performed using SAS version 9.3 (SAS Institute). ICH patients were excluded from regression analyses due to low sample size. One outlier from the acute time point for each enzyme activity assay was identified as ≥2.5 times the absolute deviation around the median142 and removed. For correlation analyses of systolic blood pressure (SBP) with activity levels, one outlier ≥2.5 times the standard deviation around the mean was removed from each group. Statistical significance was assumed at α<0.05 for all analyses.
Results

Effect of Stroke on ACE2 in Rat Brain and Serum

Ischemic stroke induction, as described in Experiment 1 in the Methods, resulted in significantly increased ACE2 activity in the cerebral cortex ipsilateral to the stroke when compared to control activity levels from sham-operated rats at 4h, 12h, and 1d after ischemia (Figure 2-4A), along with an increase at 12h in the ipsilateral striatum compared to both shams and contralateral striatum (Figure 2-4B). At 12h, ACE2 activity in the contralateral cortical samples was also significantly increased compared to respective sham levels (Figure 2-4A). ACE2 activity levels in the cerebral cortex and striatum had returned to sham levels by 3d (Figure 2-4A&B). There was not a significant change in ACE2 mRNA levels in the ipsilateral cortex either 1d or 3d following ischemia (Figure 2-4C). Stroke resulted in an initial minor but significant decrease of ACE2 activity in rat serum measured at 4h post-stroke as compared to normalized pre-stroke levels. This was followed by a significant rebound increase three days post-stroke (Figure 2-4D).

Serum Activity of ACE2 and ACE in Human Stroke

We next sought to validate several of these findings from experimental stroke models in an observational study of human stroke. Baseline participant characteristics are presented in Table 2-1. Samples were from 38 patients with ischemic (IS) and 8 with intracranial (ICH). The IS group included 24 large vessel/cardioembolic and 14 small vessel/lacunar strokes, and intravenous tissue plasminogen activator (IV tPA) was administered in 25 IS patients. In addition, serum was obtained from 24 patients with transient ischemic attack or symptoms mimicking stroke (TIA/mimic) and from 20 control participants. The TIA/mimic group, 5 of whom received IV tPA, included patients
presenting with symptoms later determined to be due to TIA (n=5), atypical migraine (n=7), hypertensive encephalopathy (n=1), seizure (n=3), altered mental status (n=3), or idiopathic or other causes (n=5). Variables that were significantly higher in the stroke groups are identified in Table 2-1 and included age, history of hypertension and diabetes, and SBP at presentation.

Our preclinical studies revealed that IS results in initial decreases in serum ACE2 activity when measured four hours after focal ischemia followed by rebound increases over three days (Figure 2-4D). In line with these findings, levels of serum ACE2 activity amongst patients with acute IS measured at an average of 3.6 hours after symptom onset were significantly lower than levels from control participants and from stroke TIA/mimic patients (Figure 2-5A). This decrease in ACE2 activity was reversed by three days post stroke (Figure 2-5A). Activity of serum ACE2 amongst patients with acute ICH was also significantly lower than control levels, but there was no reversal to control values by three days (Figure 2-5A). Serum levels of ACE activity were significantly decreased compared to acute levels at three days after stroke (Figure 2-5B). TACE activity in the serum was not significantly affected as compared to control or TIA/mimic levels amongst IS or ICH patients at either time point (data not shown).

The initially decreased levels of ACE2 activity were significantly correlated with the presence of IS by multiple linear regression (R² = 0.25, p = 0.04, Table 2-2) after controlling for age, sex, race, baseline ACEi therapy, SBP at presentation, and history of type II diabetes or hypertension. Multiple logistic regression revealed that among control, TIA/mimics, and IS patients, those with serum ACE2 activity in the lowest two quartiles had 3.75 fold higher odds ratio (95% CI 1.01-13.9) of having suffered an IS as
compared to those with levels in the top quartile. Analysis of serum ACE2 activity by race revealed significantly higher ACE2 activity levels among African Americans within control and TIA/mimic groups combined (Figure 2-5C). Furthermore, controlling for ACE2 activity levels, age, sex, ACEi therapy, and history of diabetes and hypertension revealed that among all participants, African American race was associated with 10 times increased odds ratio of having suffered an IS as compared to Caucasian race (95% CI 1.22 – 88.44).

**Correlations of Human Serum ACE2 and ACE Activity with Clinical Measures**

To evaluate the relationship of serum ACE2 activity and blood pressure, we performed correlation analyses among several groups. Activity of serum ACE2 among TIA/mimics showed a significant positive correlation with SBP at presentation (Figure 2-6A). This was also true for plasma ACE2 activity among a separate population of healthy young adults (Figure 2-6B). By contrast, SBP and ACE2 activity levels showed no correlation among patients experiencing acute IS (Figure 2-6C). Bivariate analysis of ACE inhibitor (ACEi) therapy at baseline and serum ACE2 activity revealed a significant negative correlation among IS patients indicating lower levels of ACE2 activity acutely among IS patients taking an ACEi despite no difference in SBP at presentation, but not among mimic/TIA patients (Figure 2-6D). Analysis of other clinical variables – including measures of stroke severity, laboratory results, cardioembolic versus small vessel lacunar stroke, baseline demographics, infarct volume by magnetic resonance imaging volumetric analysis, etc. (see Table 2-3) – did not reveal additional associations with acute or three day post stroke ACE2 activity among TIA/mimic or stroke patients.

Acute serum ACE activity was found to be significantly correlated with sex among IS patients, with females exhibiting higher levels of ACE activity during stroke
(Pearson r = 0.36, p = 0.03, Figure 2-7A). Interestingly, this correlation was reversed among control participants, where female sex was significantly associated with lower levels of ACE activity (Pearson r = -0.30, p=0.05, Figure 2-7B). Finally, ACE activity in IS was negatively correlated with modified Rankin scores at discharge from the hospital (Pearson r = -0.36, p = 0.04, Figure 2-7C), indicating that lower levels of ACE activity during acute IS may be associated with worse functional outcomes, at least initially.

**Effect of Stroke on Other RAS and Related Components in Ischemic Cerebral Cortex in Rats**

ACE2 is thought to exert neuroprotective effects in part via conversion of Ang II to Ang-(1-7), which subsequently binds and signals through the Ang-(1-7) receptor Mas. We therefore assessed the impact of stroke on mRNA levels of Mas and also of the neuroprotective AT2R, and did not find significant differences at 1d following MCAO (Figure 2-8A). In addition to these protective arms of the RAS, components of the classical ACE/Ang II/AT1R pathway were evaluated. Compared to sham, there was an increase in ACE mRNA levels in the ipsilateral cortex, but no change in AT1R mRNA (Figure 2-8A). As expected, we also observed increased mRNA levels of LCN2, a marker of astrocyte activation, and CD11b, a marker of activated microglia (Figure 2-8B). We further evaluated the levels of tumor necrosis factor-α-converting enzyme (TACE, also named ADAM17), an enzyme that forms soluble ACE2 by cleaving ACE2 from its membrane-bound form via its sheddase activity, in samples from the ipsilateral cortex, and found a significant increase of TACE mRNA but not activity at 1d post-stroke relative to shams (Figure 2-8C&D). Interestingly, although TACE mRNA expression levels were not significantly different between sham and stroke groups at 3d, TACE activity was significantly decreased in samples from the ischemic cortex at this
time point. Serum TACE activity was not different between sham and stroke groups at 1d post-stroke (data not shown).

**Effect of Stroke on Distribution and Cellular Localization of Brain Mas in Rats and AT2Rs in Mice**

To further characterize the stroke-induced changes in Mas expression in neurons and other brain cells, we employed immunohistochemical staining to label NeuN and Mas immunoreactive cells. Representative fluorescence micrographs (Figure 2-9A) show NeuN and Mas staining within the stroke penumbra from sham and stroke rats 1d post-stroke. Semi-quantitative analysis of immunofluorescent staining showed that the percentage of fractional area of both NeuN and Mas immunopositive staining in the ipsilateral cerebral cortex was significantly decreased compared to sham rat cerebral cortex (Figure 2-9B&C).

Binding of Ang II to AT2Rs promotes neuroprotective effects in stroke, and it is not clearly established what cell subtypes might express this receptor during stroke to mediate these effects, or whether there is altered distribution of these receptors in the brain after stroke. The AT2R reporter mouse, where every cell containing AT2R exhibits green fluorescence, provides an excellent model system for answering these questions, and our group previously demonstrated that in normal mice AT2R are localized exclusively to neurons\(^{132}\). We performed intraluminal temporary MCAO in AT2R reporter mice followed by assessments of AT2R co-localization with astrocytes (GFAP), microglia (Iba-1), and neurons (HuC/D) at 7 days and 14 days after stroke. By qualitative assessment, compared to naïve mice\(^{132}\) or those that underwent sham surgery (Figure 2-10A), distribution of AT2Rs throughout the cerebral cortex and striatum was not altered at 7 days (Figure 2-10B) or 14 days (data not shown) post-
stroke. As expected, stroke induced a profound micro and astro-gliosis in the ipsilateral hemisphere (Figure 2-10B). The cortical regions of highest AT2R concentration, such as the median prefrontal cortex, do not apparently overlap with areas of gliosis, and the lack of detectable AT2R expression in striatum in control mice persisted in the stroked animals up to 2 weeks post-stroke.

We next evaluated the post-stroke cell subtype expression of AT2Rs in the brain, which under baseline conditions has been found to be limited to neurons.\textsuperscript{132} We did not find any co-localization of AT2Rs with the astrocytic marker GFAP (Figure 2-10C-G) or the microglial marker Iba-1 (Figure H-K) in any brain regions either ipsilateral or contralateral the stroke at 7 or 14 days after stroke. Higher power micrographs showed no overlap of staining even in instances of close proximity of GFAP+ (Figure 2-10G) or Iba-1+ (Figure 2-10K) cells to AT2R+ cells. By contrast, co-localization with neuronal marker HuC/D was readily apparent in these AT2R+ cells (Figure 2-10L-N).

**Discussion**

This study aimed to assess *endogenous* post-stroke changes within the RAS, with a focus on ACE2 activity and AT2R expression. We found significant increases of endogenous ACE2 activity, but not ACE2 mRNA expression, within the ischemic cerebral cortex and striatum of rats during the first 24 hours after stroke, while serum ACE2 activity levels were initially decreased, followed by convalescent increases *in both rats and humans*. Among humans, lower ACE2 activity levels were significantly associated with the presence of ischemic stroke. At 1d after stroke, levels of immunoreactive Mas were significantly decreased in the ischemic penumbra, and mRNA levels of the of deleterious RAS component ACE were increased in rat brains.
Expression of AT2R mRNA was not altered at 1 day post-stroke and AT2R distribution and cell-subtype expression were not altered in the weeks following ischemic stroke.

Few studies have examined the effect of stroke on the induction of components of the classical RAS. It has been reported that Ang II levels are increased in the stroke cortex one day after stroke, and others have shown Ang II to up-regulate ACE, AT1R, and TACE, and decrease ACE2 expression, which might account for some of the changes that we have observed one day post-stroke (Figure 2-4C, Figure 2-8A&C). The role of TACE as an ACE2 sheddase has received recent attention as a possible contributing factor to the development of neurogenic hypertension, where it has been hypothesized that TACE-mediated shedding impairs brain ACE2 compensatory activity. Along these lines, evidence from a study in cell culture showed that Ang II-induced proteolytic cleavage of membrane-bound ACE2 was mediated by increased activity of TACE, which was accompanied by an increase in plasma ACE2 activity, presumably as a result of an increase in soluble-ACE2. Our findings that show significantly increased expression of TACE mRNA in the cortex (Figure 2-8C) coinciding with early rebound increases in serum ACE2 activity starting at 1d after stroke (Figure 2-4D) suggest that brain ACE2, once cleaved from the membrane, may move into the circulation after stroke-induced blood brain barrier breakdown. This seems unlikely to completely account for the increased serum ACE2 activity at 3 days after stroke as the levels of brain TACE activity are significantly attenuated by that time (Figure 2-8D), and our data (not shown) did not demonstrate post-stroke changes in serum TACE activity in rats or humans.
Other explanations for the observed post-stroke changes in serum ACE2 activity levels (Figure 2-4D and 2-5A) include alterations in the release of soluble ACE2 from the membrane of endothelial cells. Evidence is not sufficient to positively identify what signaling processes could initiate such alterations in ACE2 shedding following stroke, but it seems plausible that Ang II, which has been shown to increase TACE, is involved. Another explanation incorporates bone marrow-derived hematopoietic cells as a potential source of soluble ACE2. In atherosclerotic plaques, ACE2 is expressed in CD34+ cells, a marker for endothelial or hematopoietic progenitor cells. Evidence suggests that activation of mononuclear cells leads to an increase of ACE2 and of TACE which could result in increased shedding of ACE2 from activated leukocytes. Further, levels of CD34+ cells in peripheral blood following stroke in humans may follow a similar pattern to that observed in serum ACE2 activity (Figure 2-4D & 2-5A) with an initial decrease followed by a rebound increase. Taken together, these findings suggest that ACE2 shedding from brain, endothelial, and/or activated hematopoietic cells may contribute to changes in serum ACE2 activity following stroke. The physiologic relevance of changes in serum ACE2 activity is not clear, but the evidence seems to indicate that it is closely linked with the activity of TACE as regulated by Ang II signaling.

Studies of AT2R expression to date have been limited, in part due limitations of available techniques to localize this receptor, but seem to indicate a time course for early changes in AT2R expression following stroke. An early study exploring the changes in AT2R mRNA expression in rat cortex following transient global ischemia found a large increase in AT2R expression in the first 3 hours after ischemia with a
return to sham expression levels by 1 day and up to 7 days after stroke. Another study employed autoradiographic labeling of AT1Rs and AT2Rs at 1 day after intraluminal MCAO in rats, and found that in comparison to the contralateral hemisphere, AT2Rs were slightly increased and AT1Rs were slightly decreased in the ischemic regions, although a comparison to sham or naïve levels was not performed. A later study found that AT2R expression and distribution at 2 days post-stroke were increased as compared to brains from sham operated rats, without any differences in AT1Rs at this time point. Most recently, it was reported that the number of AT2R+ cells in ischemic regions increased to a peak level at 3 days post-intraluminal MCAO and decreased by 7 days toward sham levels. Additionally, this study reported co-localization of AT2Rs with neuron marker NeuN, a finding that is in line with our observations. Although our study of AT2R mRNA expression did not indicate an increase in AT2R expression at one day post-stroke (Figure 2-8A), our rodent model of endothelin-1 induced stroke for this experiment produces a more focal ischemia that may not have been sufficient to induce AT2R expression changes at the 1 day time point. Our qualitative assessment of AT2R distribution using brains from AT2R reporter mice did not indicate an obvious difference in levels of AT2R immunostaining in ischemic hemispheres compared to either contralateral or sham tissues (Figure 2-10A&B). This non-difference does not disrupt the consensus from earlier studies that describes an early time course of AT2R expressional changes after stroke, since this evaluation was performed at 7 days and then again at 14 days. The presence of AT2Rs on neurons makes plausible several potential mechanisms by which agonists have been shown to induce neuroprotection, which are discussed in more detail in Chapter 4.
The novel findings from the preliminary observational study of decreased ACE2 activity in human serum following stroke, followed by a return to control levels, and of associations with SBP and ACEi therapy add to an increasingly complex picture of ACE2 activity and cardiovascular health. Although it was limited by the lack of a control group for comparison, a previous study found that serum ACE2 levels within the first 24h post-stroke differ slightly by stroke subtype with lacunar strokes exhibiting lower levels than cardioembolic strokes. There was no difference in Ang II or ACE levels between stroke subtypes, and ACE2 levels positively correlated with serum brain natriuretic peptide levels.\textsuperscript{149} Levels of circulating ACE2 activity are increased in patients with chronic heart failure and after myocardial infarction, where they have been shown to predict follow-up ejection fraction.\textsuperscript{150} Higher ACE2 activity levels are also associated with hypertension and with decreased ejection fraction.\textsuperscript{151} These increases in ACE2 activity in chronic conditions are in contrast to the decreased serum ACE2 activity observed in IS (Figure 2-8B). The levels of serum ACE2 activity in IS, even though decreased, may still have prognostic value, as correlation analyses of convalescent ACE2 activity hinted at associations with measures of stroke severity such as NIHSS score at presentation and discharge and mRS at discharge (data not shown), with higher levels of ACE2 activity at 3d post-stroke potentially correlating with worse outcomes. If confirmed in future studies, this would support the suggestion from other studies that higher circulating ACE2 activity may represent a compensatory response to worsening cardiovascular and related diseases.\textsuperscript{149-151}

As we observed among TIA/mimic patients (Figure 2-6A) and healthy young adults (Figure 2-6B), others have reported positive correlations between soluble ACE2
activity and blood pressure. In IS, however, we observed no correlation between SBP and ACE2 activity (Figure 2-6C). The wider range and higher pressures among the IS group as compared to other groups may be an important factor in this difference. The mechanisms responsible for these dynamic changes are unknown and are likely influenced differently during periods of rapid alterations or very high levels of blood pressure, such as during stroke, as compared to chronically altered pressures. Interestingly, no correlation of ACE2 activity and SBP was observed 72h after stroke. Also, the observed changes may have been affected by differences in ACE2 genetic variants, which are known to be associated with blood pressure and cardiovascular function. Our data indicate that differences in baseline activity of ACE and presumably Ang II levels, such as in the case of ACEi therapy, may predispose IS patients to lower levels of ACE2 activity despite experiencing similar blood pressure changes. The use of ACEi’s in the management of permissive hypertension in the initial days post-stroke likely influences endogenous post-stroke changes in the RAS. While it has been shown that ACEi or ARB therapy is effective at prevention of secondary strokes, future studies are needed to assess the potential neuroprotective effects of ACEi or ARB therapy in IS.

The difference in serum ACE2 levels in stroke groups versus both controls and TIA/mimics implies added potential for ACE2 as a stroke biomarker. Temporary ischemia, as in TIA, may not be sufficient to induce the alterations in serum ACE2 activity that is associated with stroke and the accompanying cerebral infarct. A better understanding of the time course over which ACE2 levels are changing after stroke will be useful in assessing further the value of ACE2 as a stroke biomarker.
Differences in the activity of TACE, which cleaves membrane bound ACE2 to form active soluble ACE2, contribute to altered serum ACE2 activity in hypertension,\textsuperscript{145} and may also play a role in stroke.\textsuperscript{154} Although we did not observe a significant change in TACE activity in the serum, a change in vascular or brain TACE activity with resulting alterations in shedding of ACE2 into interstitial and vascular spaces may regulate the initially decreased serum ACE2 in stroke. The decreasing activity of ACE (Figure 2-7B), presumably resulting in decreased Ang II signaling through AT1Rs, may also contribute to the subsequent increase of serum ACE2 activity, as Ang II has been shown to down-regulate ACE2 mRNA and activity in vascular smooth muscle cells\textsuperscript{155} and cardiovascular control centers in the brain.\textsuperscript{143} In the absence of a return to baseline of serum ACE activity and the subsequent inhibitory effects of Ang II, uninhibited increases in ACE2 expression and activity could account for the increasing convalescent levels.

To our knowledge, this is the first report to compare ACE2 activity by race (Figure 2-5C). ACE2 polymorphisms have been demonstrated to have associations with blood pressure in adolescents of European and French Canadian descent,\textsuperscript{156} and they have been explored among Chinese and other populations,\textsuperscript{157} but assessments of ACE2 levels or activity were not performed. The finding that ACE2 activity is significantly increased in African Americans as compared to Caucasians may represent the first evidence correlating race and activity of ACE2. Additionally, the increased odds ratio of IS among African Americans after controlling for relevant factors, including ACE2 activity, highlights potentially important questions about the cause and effect relationship between these elevated ACE2 levels by race and other related stroke risk factors.
Regulation of the renin angiotensin system is known to be influenced by sex hormones, specifically by estrogen which lowers levels of renin and ACE.\textsuperscript{158} Accordingly, among control patients, we observed females to exhibit significantly lower activity of serum ACE than males (Figure 2-7A). The reversal of this correlation among IS patients (Figure 2-7B), wherein females had significantly higher serum ACE activity, is intriguing. The difference in age between the two groups may offer a partial explanation in that IS patients likely had much lower levels of estrogen post-menopause, and thus a lack of ACE inhibition by estrogen.

Our observations in human stroke are preliminary, and have several limitations. First, evaluation of serum enzyme activity alone does not allow for an assessment of the tissue-specific renin angiotensin systems (e.g. renal, vascular, brain) that are also altered during stroke to affect changes in blood pressure and tissue inflammation and damage. Second, the use of enzymatic activity assays to measure system components provides accurate data regarding the levels of functional serum enzymes, but does not allow for a determination of the tissue and cellular sources of these enzymes. ACE2 functions in both membrane-bound and soluble states, and it is unclear which might provide the primary source of serum ACE2 activity in the setting of stroke, where ischemic cell death may be contributing to increased release of both types. Future studies to evaluate the role and effect of ACE2 shedding from various tissues in stroke are needed. Third, our study population was limited in number and exhibited substantial variability in risk factors and stroke mechanisms. The discovery of significant physiological differences within this small heterogeneous population may indicate potential for more substantial findings to be made from larger, more homogeneous
populations. Finally, an evaluation of serum levels of Ang II and Ang-(1-7) could provide potentially valuable information regarding the downstream effects of the enzyme activity changes we have observed. The sample collection and preparation protocol in this study was found to be inadequate to reliably assess these inherently unstable peptide hormones.

The characterization of ACE2-mediated protection in a variety of cardiovascular diseases,\textsuperscript{134} including hypertension\textsuperscript{134} and stroke,\textsuperscript{89} and of AT2R-dependent stroke neuroprotective effects\textsuperscript{98} has opened a promising avenue for development of novel treatments, which will rely on a thorough characterization of pathophysiologic changes in the RAS. In this study, we characterized endogenous changes in this pathway in the hours and days following stroke in rats and in human patients. In an observational human study, we report the novel finding that stroke induces dynamic changes in the serum activity of ACE2, a key regulatory enzyme of the protective arm of the renin-angiotensin system. Initially decreased ACE2 activity during acute IS and ICH, and the convalescent increases in IS, are consistent with the animal studies and show the potential for acute neuroprotective interventions to counteract these changes and improve outcomes. A clearer understanding of the alterations in activity of the renin angiotensin system in stroke, and the consequences of these changes, has great potential to augment the development and testing of clinically viable therapies and diagnostic or prognostic tools. Indeed, the consideration of serum ACE2 and ACE activity levels as a predictor of disease progression and longer-term outcomes (Figure 2-7C) seems to warrant further study. The novel evidence that ACE2 is dynamically altered in humans in a potentially detrimental way during stroke may represent an
important clinical target for future stroke therapies. Future work in this area may deepen our understanding of the cell subtype specific regulation of stroke-induced changes in protective and deleterious RAS components, as well as the physiologic significance of changes in systemic versus central ACE2 activity.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CONT (n=20)</th>
<th>MIMIC (n=24)</th>
<th>IS (n=38)</th>
<th>ICH (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, y, mean ± SD</strong></td>
<td>58.2 ± 13</td>
<td>57.6 ± 15.5</td>
<td>70.6 ± 14.9*†</td>
<td>70.0 ± 16.1‡</td>
</tr>
<tr>
<td><strong>Female / Male</strong></td>
<td>10 / 10</td>
<td>12 / 12</td>
<td>15 / 23</td>
<td>6 / 2</td>
</tr>
<tr>
<td><strong>Race, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>15 (75%)</td>
<td>17 (71%)</td>
<td>28 (74%)</td>
<td>7 (88%)</td>
</tr>
<tr>
<td>African American</td>
<td>2 (10%)</td>
<td>4 (17%)</td>
<td>8 (21%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>2 (10%)</td>
<td>3 (12%)</td>
<td>2 (5%)</td>
<td>1 (12%)</td>
</tr>
<tr>
<td>Indian</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>Hypertension, n (%)</strong></td>
<td>10 (50%)</td>
<td>16 (75%)</td>
<td>29 (76%)‡</td>
<td>7 (88%)</td>
</tr>
<tr>
<td><strong>Diabetes, n (%)</strong></td>
<td>5 (25%)</td>
<td>11 (46%)</td>
<td>20 (53%)‡</td>
<td>3 (38%)</td>
</tr>
<tr>
<td><strong>Hyperlipidemia, n (%)</strong></td>
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<td>13 (54%)</td>
<td>27 (71%)</td>
<td>6 (75%)</td>
</tr>
<tr>
<td><strong>AFib, n (%)</strong></td>
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<td>2 (8%)</td>
<td>8 (21%)</td>
<td>1 (12%)</td>
</tr>
<tr>
<td><strong>Smoking, n (%)</strong></td>
<td>-</td>
<td>10 (42%)</td>
<td>7 (18%)</td>
<td>1 (12%)</td>
</tr>
<tr>
<td><strong>Prior stroke, n (%)</strong></td>
<td>-</td>
<td>10 (42%)</td>
<td>10 (26%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>BMI, kg/m², mean ± SD</strong></td>
<td>-</td>
<td>29.7 ± 5.0</td>
<td>28.9 ± 6.6</td>
<td>32.2 ± 7.9</td>
</tr>
<tr>
<td><strong>SBP, mmHg, mean ± SD</strong></td>
<td>-</td>
<td>138.5 ± 22.4</td>
<td>157.1 ± 26.8†</td>
<td>167.3 ± 31.5§</td>
</tr>
<tr>
<td><strong>DBP, mmHg, mean ± SD</strong></td>
<td>-</td>
<td>75.8 ± 14.1</td>
<td>79.6 ± 16.8</td>
<td>82.5 ± 26.1</td>
</tr>
<tr>
<td><strong>WBC, count*10⁹/L, mean ± SD</strong></td>
<td>-</td>
<td>8.2 ± 3.1</td>
<td>9.5 ± 2.9</td>
<td>12.6 ± 5.7</td>
</tr>
<tr>
<td><strong>Hemoglobin, g/dL, mean±SD</strong></td>
<td>-</td>
<td>13.2 ± 2.0</td>
<td>13.3 ± 2.1</td>
<td>13.8 ± 1.0</td>
</tr>
<tr>
<td><strong>Hematocrit, %, mean ± SD</strong></td>
<td>-</td>
<td>39.2 ± 6.1</td>
<td>39.9 ± 6.2</td>
<td>41.5 ± 3.7</td>
</tr>
<tr>
<td><strong>Sodium, mmol/L, mean ± SD</strong></td>
<td>-</td>
<td>139.5 ± 3.3</td>
<td>139.6 ± 3.1</td>
<td>140.0 ± 2.5</td>
</tr>
<tr>
<td><strong>Potassium, mmol/L, mean ± SD</strong></td>
<td>-</td>
<td>4.0 ± 0.6</td>
<td>4.2 ± 0.5</td>
<td>3.9 ±0.4</td>
</tr>
<tr>
<td><strong>Creatinine, mg/dL, mean ± SD</strong></td>
<td>-</td>
<td>1.4 ± 1.4</td>
<td>1.3 ± 0.6</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td><strong>Glucose, mg/dL, mean ± SD</strong></td>
<td>-</td>
<td>148.6 ± 89.3</td>
<td>133.4 ± 43.1</td>
<td>162.1 ± 66.7</td>
</tr>
<tr>
<td><strong>ProTime, seconds, mean ± SD</strong></td>
<td>-</td>
<td>13.6 ± 1.4</td>
<td>13.6 ± 1.1</td>
<td>13.9 ± 2.8</td>
</tr>
<tr>
<td><strong>INR, ratio, mean ± SD</strong></td>
<td>-</td>
<td>1.05 ± 0.14</td>
<td>1.05 ± 0.10</td>
<td>1.11 ± 0.29</td>
</tr>
<tr>
<td><strong>ACEI, n (%)</strong></td>
<td>-</td>
<td>4 (17%)</td>
<td>7 (18%)</td>
<td>2 (25%)</td>
</tr>
<tr>
<td><strong>ARB, n (%)</strong></td>
<td>-</td>
<td>5 (21%)</td>
<td>4 (11%)</td>
<td>2 (25%)</td>
</tr>
<tr>
<td><strong>tPA treatment, n (%)</strong></td>
<td>-</td>
<td>5 (21%)</td>
<td>25 (66%)†</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>NIHSS, median (IQR)</strong></td>
<td>-</td>
<td>4 (3–6)</td>
<td>6.5 (3–12)</td>
<td>18 (6–25)</td>
</tr>
<tr>
<td><strong>mRS, score, median (IQR)</strong></td>
<td>-</td>
<td>-</td>
<td>2 (1–5)</td>
<td>18 (6.5–26)</td>
</tr>
<tr>
<td><strong>ICH score, median (IQR)</strong></td>
<td>-</td>
<td>-</td>
<td>2 (1–3.5)</td>
<td>4.5 (3.8–5.3)</td>
</tr>
</tbody>
</table>

Abbreviations: ACEI, angiotensin converting enzyme inhibitor previously prescribed; AFib, atrial fibrillation; ARB, angiotensin receptor blocker previously prescribed; BMI, body mass index; DBP, diastolic blood pressure at presentation; CONT, control group; ICH, intracerebral hemorrhage group; IS, ischemic stroke group; Mimic, stroke mimic and transient ischemic attack group; INR, international normalized ratio; IQR, interquartile range; mRS, modified Rankin scale at discharge; NIHSS, National Institutes of Health stroke scale score at presentation (p) or discharge (d); SBP, systolic blood pressure at presentation; SD, standard deviation; tPA, tissue plasminogen activator; WBC, white blood cell count.

* p<0.01 versus Cont.  † p<0.05 versus Cont
‡ p<0.01 versus Mimic.  § p<0.05 versus Mimic.
<table>
<thead>
<tr>
<th>$R^2$ model</th>
<th>Variable</th>
<th>$\beta$ coefficient</th>
<th>95% CI of $\beta$</th>
<th>t</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R^2 = 0.25$</td>
<td>ACE2 activity</td>
<td>-0.00122</td>
<td>-0.00237 to -0.00006</td>
<td>-2.12</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.01089</td>
<td>0.00322 to 0.01857</td>
<td>2.85</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>-0.06104</td>
<td>-0.31209 to 0.19002</td>
<td>-0.49</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>Race</td>
<td>0.00840</td>
<td>-0.19372 to 0.21052</td>
<td>0.08</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>ACEi therapy</td>
<td>0.03187</td>
<td>-0.30063 to 0.36436</td>
<td>0.19</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>SBP at presentation</td>
<td>0.00413</td>
<td>-0.00071 to 0.00897</td>
<td>1.72</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Type II Diabetes</td>
<td>0.02088</td>
<td>-0.25061 to 0.29238</td>
<td>0.15</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>Hypertension</td>
<td>-0.14814</td>
<td>-0.47312 to 0.17684</td>
<td>-0.92</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Abbreviations: ACE2, angiotensin converting enzyme 2; ACEi, angiotensin converting enzyme inhibitor; CI, confidence interval; SBP, systolic blood pressure. Values for groups were assigned as follows: ischemic stroke=1, mimic/TIA=0; female sex=1, male sex=0; race: Indian=3, Hispanic=2, African American=1, Caucasian=0; history of ACEi therapy, type II diabetes, or hypertension=1, negative history=0.
Table 2-3. Bivariate correlations of clinical variables with ACE2 activity among stroke mimic/TIA and acute IS patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>ACE2 in Mimic</th>
<th></th>
<th>ACE2 in IS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P value</td>
<td>r</td>
<td>P value</td>
</tr>
<tr>
<td>Sex</td>
<td>-0.18</td>
<td>0.43</td>
<td>-0.12</td>
<td>0.50</td>
</tr>
<tr>
<td>Age</td>
<td>0.18</td>
<td>0.43</td>
<td>0.23</td>
<td>0.17</td>
</tr>
<tr>
<td>Type II Diabetes</td>
<td>0.13</td>
<td>0.59</td>
<td>0.15</td>
<td>0.38</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.34</td>
<td>0.13</td>
<td>-0.01</td>
<td>0.95</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>0.14</td>
<td>0.54</td>
<td>0.12</td>
<td>0.48</td>
</tr>
<tr>
<td>SBP at presentation</td>
<td>0.47</td>
<td>0.04</td>
<td>-0.16</td>
<td>0.35</td>
</tr>
<tr>
<td>DBP at presentation</td>
<td>0.03</td>
<td>0.90</td>
<td>0.01</td>
<td>0.94</td>
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<tr>
<td>Treatment with IV tPA</td>
<td>0.34</td>
<td>0.14</td>
<td>0.04</td>
<td>0.80</td>
</tr>
<tr>
<td>Length of Hospital Stay</td>
<td>-0.22</td>
<td>0.36</td>
<td>-0.03</td>
<td>0.88</td>
</tr>
<tr>
<td>WBC at presentation</td>
<td>-0.36</td>
<td>0.11</td>
<td>0.09</td>
<td>0.59</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>-0.12</td>
<td>0.60</td>
<td>-0.19</td>
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<tr>
<td>Hematocrit</td>
<td>-0.12</td>
<td>0.59</td>
<td>-0.16</td>
<td>0.33</td>
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<tr>
<td>Sodium</td>
<td>-0.01</td>
<td>0.96</td>
<td>0.05</td>
<td>0.79</td>
</tr>
<tr>
<td>Potassium</td>
<td>-0.21</td>
<td>0.35</td>
<td>0.12</td>
<td>0.48</td>
</tr>
<tr>
<td>Creatinine</td>
<td>-0.27</td>
<td>0.25</td>
<td>0.13</td>
<td>0.44</td>
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<tr>
<td>Glucose</td>
<td>0.11</td>
<td>0.64</td>
<td>0.19</td>
<td>0.26</td>
</tr>
<tr>
<td>Prothrombin Time</td>
<td>0.16</td>
<td>0.46</td>
<td>0.25</td>
<td>0.14</td>
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<tr>
<td>INR</td>
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<td>0.88</td>
<td>0.05</td>
<td>0.78</td>
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<tr>
<td>ACEi prescribed at baseline</td>
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<td>0.30</td>
<td>-0.34</td>
<td>0.04</td>
</tr>
<tr>
<td>ARB prescribed at baseline</td>
<td>-0.20</td>
<td>0.39</td>
<td>0.22</td>
<td>0.19</td>
</tr>
<tr>
<td>ACE activity</td>
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<td>0.93</td>
<td>0.08</td>
<td>0.63</td>
</tr>
<tr>
<td>Mimic class or Stroke subtype</td>
<td>-0.28</td>
<td>0.22</td>
<td>-0.07</td>
<td>0.67</td>
</tr>
<tr>
<td>Sample time from onset</td>
<td>-</td>
<td></td>
<td>-0.13</td>
<td>0.45</td>
</tr>
<tr>
<td>Left versus right</td>
<td>-</td>
<td></td>
<td>0.03</td>
<td>0.87</td>
</tr>
<tr>
<td>Infarct volume by MRI</td>
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<td></td>
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<td>NIHSS score at presentation</td>
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<td>NIHSS score at discharge</td>
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<tr>
<td>mRS at discharge</td>
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<td></td>
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<tr>
<td>mRS at follow-up</td>
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</tr>
<tr>
<td>THRIVE Score</td>
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<td></td>
<td>0.24</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Abbreviations: ACE2, angiotensin converting enzyme 2; ACEi, angiotensin converting enzyme inhibitor; DBP, diastolic blood pressure; INR, international normalized ratio; IS, ischemic stroke; IV tPA, intravenous tissue plasminogen activator; MRI, magnetic resonance imaging; mRS, modified Rankin score; NIHSS, National Institutes of Health stroke scale; WBC, white blood cell count.
Figure 2-1. Determination of optimal fluorogenic substrate concentrations. These curves display ACE2 activity with increasing fluorogenic substrate concentration of each of the four sample types indicated: (A) rat serum; (B) rat cerebral cortex, (C) human serum, (D) rhACE2 positive control. ACE2: angiotensin converting enzyme 2; RFU: relative fluorescence units; rhACE2: human recombinant ACE2.
Figure 2-2. Validation of ACE2 activity assay by assessing activity level trends for increasing enzyme concentrations. These graphs display ACE2 activity levels for each of the four sample types as the concentration of ACE2 enzyme, represented by the amount of sample added, increases. Each of the samples tested yielded a positive, approximately linear correlation of ACE2 activity with enzyme concentration. The panels represent each of the following sample types: A) rat serum; B) rat cerebral cortex; C) human serum; D) rhACE2 positive control. ACE2: angiotensin converting enzyme 2; RFU: relative fluorescence units; rhACE2: human recombinant ACE2.
Figure 2-3. Validation of ACE2 activity assay with addition of MLN-4760, an inhibitor of ACE2. These graphs are representative plots of relative fluorescence units over time, the slope of which yields the ACE2 activity, for each of the four sample types that were run both with and without MLN-4760. The graphs show that all of the sample types were successfully inhibited in the presence of MLN-4760. The panels represent the following sample types: A) rat serum; B) rat cerebral cortex; C) human serum; D) rhACE2 positive control. ACE2: angiotensin converting enzyme 2; RFU: relative fluorescence units; rhACE2: human recombinant ACE2.
Figure 2-4. Activity of ACE2 in brain and serum is altered following stroke in rats. Rats underwent sham surgery or MCAO, followed by serial serum collections, euthanasia, and tissue harvesting at the time points indicated. A&B) Bar graphs are the average percent activity levels of ACE2 post MCAO, in tissue from (A) cerebral cortex or (B) striatum contralateral (Contra) and ipsilateral (Ipsi) to ischemia. Data are normalized to the corresponding sham values for each time point (A: n=4-6 rats at 4h, 12h, and 3d and n=12 rats at 1d time points; B: n=6-8 per time point). C) mRNA levels of ACE2 at 1d and 3d post-stroke. Data are normalized to GAPDH and corresponding sham values (n=17 at 1d and n=4-6 at 3d). D) Bar graphs are the average percent activity levels of ACE2 in serum at the indicated time points post MCAO. Serum was collected from the same sham- or stroked rat where possible. Data are normalized to pre-stroke values for either sham or stroke groups, respectively (n=19 sham and 31 stroke for pre-stroke, n=31 sham and 27 at 4h, n=6 sham and 8 stroke at 12h, n=19 sham and 21 stroke at 1d, n=7 sham and 10 stroke at 2d and 3d time points). Data are means ± SEM. * p< 0.05 vs. respective sham controls; % p<0.05 vs. contralateral stroke values; # p<0.05 vs. respective pre-stroke values (Kruskal-Wallis test with post-hoc Mann-Whitney test for panels A&B; Mann-Whitney test for other panels). ACE2, angiotensin converting enzyme 2; mRNA, messenger ribonucleic acid; rhACE2, human recombinant ACE2. ACE2: angiotensin converting enzyme 2; Contra: contralateral. Ipsi: ipsilateral; mRNA: messenger ribonucleic acid.
Figure 2-5. Activity of ACE2 & ACE in serum is altered following stroke and differs by race. A&B) For human serum, bar graphs are means ± SEM and represent enzyme activity levels of ACE2 (A) and ACE (B) from control participants (white bar), stroke mimic/TIA (black bar), or ischemic (light grey bars) and hemorrhagic (dark grey bars) stroke patients at an average of 3.6 hours (no stripes) and again at three days after stroke (with stripes). * p<0.05 versus control and † p<0.05 versus mimic/TIA. ‡ p<0.05 versus acute ischemic stroke. C) Bar graphs represent average serum ACE2 activity categorized by race from all research participants combined. C=Caucasian, AA=African American, H=Hispanic, I=Indian. * p<0.05 versus Caucasian. ACE: angiotensin converting enzyme; ACE2: angiotensin converting enzyme 2; RFU: relative fluorescence unit.
Figure 2-6. Serum ACE2 activity is significantly correlated with SBP in non-stroke patients and healthy adults, but not IS patients. A-C) Correlation graphs of ACE2 activity and SBP among mimic/TIA patients (A) and healthy young adults (B) as compared to stroke patients (C). Young adult plasma samples from a biorepository established by Wegman et. al. Samples were obtained from research participants undergoing baseline measurements. D) Correlation graph of ACE2 activity and ACEi therapy status among patients experiencing a stroke mimic/TIA (left) or ischemic stroke (right). Also shown are the SBP of these patients at the time of presentation (grey triangles, right y-axis). ACE2: angiotensin converting enzyme 2; ACEi: angiotensin converting enzyme inhibitor; RFU: relative fluorescence unit; SBP: systolic blood pressure.
Figure 2-7. Serum ACE activity is significantly correlated with sex and modified Rankin score at discharge. A&B) Correlation graphs of ACE activity and sex among IS patients at presentation (A) and control + mimic/TIA patients (B). C) Correlation graph of ACE activity and mRS at discharge from hospital among IS patients. ACE: angiotensin converting enzyme; ACEi: angiotensin converting enzyme inhibitor; IS: ischemic stroke; mRS: modified Rankin score; RFU: relative fluorescence unit.
Figure 2-8. Alterations in RAS component mRNAs and TACE mRNA/activity following stroke in rats. Ipsilateral cerebral cortical samples were collected from sham and stroke rats at 1d post-MCAO. A) mRNA levels of Mas, AT2R, ACE, and AT1R (normalized to GAPDH and corresponding sham values). B) mRNA levels of LCN2 and CD11b. C) Levels of TACE mRNA expression following stroke (normalized to GAPDH and corresponding sham values for each time point). D) Levels of ipsilateral cerebral cortical TACE activity (normalized as percent of sham values). Data are means ± SEM from 10-12 rats per time point. *p<0.05 compared to respective sham control (Mann-Whitney test).

AT1R: angiotensin II type 1 receptor; AT2R: angiotensin II type 2 receptor; ACE: angiotensin converting enzyme; CD11b: cluster of differentiation molecule 11B; LCN2: lipocalin 2; mRNA: messenger ribonucleic acid; TACE: tumor necrosis factor alpha converting enzyme.
Figure 2-9. Immunoreactive Mas is decreased in the cerebral cortex following stroke in rats. A) Representative high power fluorescence micrographs from the ipsilateral cerebral cortex of sham (first column, n=6) and stroke (second column, n=4) brains harvested at 1d post-MCAO show immunoreactive NeuN (green, neuronal marker) and Mas (red), and NeuN plus Mas co-localization (merge). B&C) Semi-quantitative comparisons of average % fractional area of (B) NeuN and (C) Mas immunopositive staining are shown. Data are means ± SEM. *p<0.05 compared to sham control (Mann-Whitney test). NeuN: neuron specific protein.
Figure 2-10. Ischemic stroke does not result in a redistribution of AT2Rs, which remain stably expressed on neurons. A&B) Representative immunofluorescent mosaic micrographs show immunoreactive GFP-AT2R, GFAP, Iba-1, or HuC/D, as indicated, in brain slices from mice 7 days after sham surgery (A) or intraluminal MCAO (B). C-K) High power micrographs of GFAP (red) or GFP-AT2R (green) immunopositive cells (C-G) or of Iba-1 (red) or GFP-AT2R (green) immunopositive cells (H-K) do not show overlap. L-M) Overlap is apparent from high power micrographs of HuC/D (red) and GFP-AT2R (green) immunopositive cells. AT2R: angiotensin II type 2 receptor; GFAP, glial fibrillary acidic protein; GFP: green fluorescence protein; HuC/D: human neuronal protein; Iba-1: ionized calcium-binding adapter molecule 1.
Figure 2-10 Continued.
Figure 2-10 Continued.
Figure 2-10 Continued.
CHAPTER 3
THE PROTECTIVE RAS IN ISCHEMIC STROKE AS A TARGET FOR TRANSLATIONAL THERAPIES: NEW APPROACHES FOR AN OLD PROBLEM

Introduction

Stroke, a disease for which hypertension remains the leading modifiable risk factor, is a devastating reality each year for many millions of people worldwide and is a leading cause of death and disability.\(^\text{159}\) The renin angiotensin system (RAS) holds promise as a potential target for novel stroke therapies, especially so with the recent discovery of a counter-regulatory arm of the RAS that exerts opposite effects of AT1R signaling via activation of other receptors, including the angiotensin-(1-7) [\text{Ang-(1-7)}] receptor Mas and the angiotensin II type 2 receptor (AT2R). \text{Ang-(1-7)} is formed from angiotensin II (\text{Ang II}) by the action of angiotensin converting enzyme 2 (ACE2).\(^\text{134}\)

Further characterization of the actions of ACE2 in various pathologies has expanded our view of the therapeutic potential of small molecule activators of this pathway.\(^\text{134}\) Activation of AT2Rs has similarly been shown to induce significant neuroprotective effects in neurological disease and stroke.\(^\text{98, 136}\)

Significant research effort in the field of neuroprotective stroke therapies has been aimed at the discovery of novel treatments that may be administered to salvage penumbral tissue that is uniquely vulnerable to collapse.\(^\text{18}\) The ACE2–\text{Ang-(1-7)}–Mas pathway has been highlighted as a promising target for induction of stroke neuroprotection,\(^\text{97}\) and has proven efficacy in reducing infarct size and improving neurological function in preclinical models of ischemic\(^\text{89, 106, 107, 109, 110, 112, 113}\) and

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hemorrhagic stroke. Stroke neuroprotection has been demonstrated in both young and aged animals, and methods for activating the axis have included direct intracerebroventricular administration of Ang-(1-7), delivery of ACE2-primed endothelial progenitor cells, and neuronal ACE2 overexpression.

Pharmacological activation of this axis has recently become more feasible with the identification of diminazene aceturate, trade name Berenil®, as an activator of ACE2. We have shown that when given by intracerebroventricular infusion before, during, and after stroke, diminazene reduced infarct size in rats, an effect that was reversed by co-administration with Mas antagonist A-779. Other strategies for targeting this axis have also recently been developed, including oral delivery of Ang-(1-7) or ACE2 via novel carrier mechanisms. This includes the development of an oral formulation of Ang-(1-7) that is couched within a cyclodextran ring that functions to prevent degradation of this otherwise unstable compound in the gut until it is released following bacterial digestion of the ring and absorbed into the circulation, where it has been shown to induce anti-thrombotic and cardioprotective effects, as well as salutary effects in various other conditions. Bioencapsulation of ACE2 within plant chloroplasts was recently accomplished to allow for oral delivery of this protein, with protection by this administration method already demonstrated in models of pulmonary hypertension and uveitis.

Targeting of AT2Rs in stroke by pharmacological activation has also reproducibly induced decreases in infarct size and improvements in neurological function. We recently reported that post-stroke activation of AT2Rs with a novel agonist, Compound 21 (C21), was effective at reducing stroke-induced brain damage when
given starting four hours after the onset of ischemic stroke. To strengthen the translational potential of these experimental studies, we sought to incorporate stroke treatment academic industry roundtable (STAIR) criteria that have been suggested for improving preclinical stroke research that include reproducible testing of preclinical treatments in co-morbid animal models, including aged animals, over sustained periods of recovery, using non-invasive administration methods given at appropriate post-stroke time points, and with multiple anatomical and behavioral outcome measurements.

Here, we assessed the hypothesis that post-stroke systemic administration of compounds to activate the protective RAS in rats – including an ACE2 activator, diminazene, orally active Ang-(1-7), bioencapsulated ACE2, or AT2R agonist C21 – results in neuroprotection in animal models of ischemic stroke.

**Methods**

For descriptions of Ethical Approval, Anesthesia, Analgesia, and Euthanasia, Endothelin-1 Induced Middle Cerebral Artery Occlusion, Intraluminal Transient Middle Cerebral Artery Occlusion, Intracerebral Infarct Volume Determination, Randomization and Allocation Concealment, ACE2 Activity Assay, and Chemicals, please see the corresponding section described in the Methods section of Chapter 2.

**Animals and Housing**

For these experiments, we used nine week old male Sprague Dawley rats (n=196, 275-300g) purchased from Charles Rivers Farms (Wilmington, MA, USA) and 18-20 month old male Sprague Dawley rats (700-800g) purchased from Hilltop Laboratories (n=28, Scottsdale, PA, USA). All animals were drug and test naïve prior to inclusion in this study and housed in well-ventilated, specific pathogen-free,
temperature-controlled facilities (24 ± 1 °C; 12–12 h light–dark cycle) with *ad libitum* access to water and standard rodent chow.

**Assessments of Neurological Function**

**Bederson and Garcia exams**

Post-stroke neurological function was assessed using modified Bederson and Garcia scoring scales as we have described previously.\textsuperscript{51, 89, 106, 127} In brief, these exams assign scores that reflects the severity of stroke-induced behaviors. The Bederson exam uses a scale from 0, no deficit, to 3, maximal deficit, which includes asymmetrical forepaw flexion, circling behavior, and decreased resistance to lateral push. The Garcia exam is graded on a scale from 18, no deficit, to 3, maximal deficit, which includes impairments in proprioception, vibrissae sensation, forelimb outstretched, climbing ability, and spontaneous movement.

**Paw adhesive test**

Functional sensorimotor deficits after stroke were sensitively assessed by applying a 1 cm diameter adhesive tape to the contralateral impaired paw and recording the latency removal. Damage to somatosensory cortex slows or eliminates removal by impairing both sensation and motor dexterity.\textsuperscript{173} Times that exceeded two minutes were recorded as 120 seconds. Each session included three trials, and measurements were recorded as the average of the two best trial times per session. Rats underwent two paw adhesive sessions of training before stroke followed by serial post-stroke assessments.

**Rotarod test**

Another test of proprioceptive and motor function involves placing rats on a Rotarod, which consists of a rotating rod and a sensor to measure the amount of time
which rats are able to maintain balance on the rod as it slowly accelerates. Rats were given two sessions of pre-training on this test along with post-stroke measurements. Each session consisted of 3-5 trials in which the rod began rotating at 4 rotations per minute and accelerated up to 40 rotations per minute over the course of several minutes. The two best trial scores were averaged to calculate a score for each time point tested, and these scores were normalized to the baseline scores to obtain a percentage of baseline.

**Measurement of Absolute Cerebral Blood Flow**

For the measurement of absolute cerebral blood flow, we followed published protocol\textsuperscript{174} which utilizes a reference arterial blood sample to which levels of tissue perfusion are normalized. Rats were anesthetized and the abdominal aorta was catheterized for the collection of arterial reference blood, after which a thoracotomy was performed to expose the heart. Instead of radiolabeled spheres, $2 \times 10^5$ fluorescent microspheres (15µm, Triton Technology, Inc., San Diego, CA, USA) in 0.2mL of the manufacturer’s suspension solution were injected over several seconds directly into the left ventricle. One minute of reference blood sampling using a constant withdrawal pump was started 5 seconds prior to microsphere injection and continued 55 seconds afterward for a total sample volume of 1mL (1 mL/min). Animals were immediately euthanized for harvesting of the brain, which was separated into left and right hemispheres. Isolation of microspheres was accomplished by a sedimentation method described in detail in the Manual for Using Fluorescent Microspheres to Measure Regional Organ Perfusion from the Fluorescence Microsphere Resource Center at the University of Washington. This manual is available for free online at [http://fmrc.pulmcc.washington.edu/documents/fmrcman99.pdf](http://fmrc.pulmcc.washington.edu/documents/fmrcman99.pdf). Quantification of isolated
spheres was performed by fluorescence emission detection using black 96-well flat bottomed plates in a Synergy Mx Microplate Reader. We applied an internal standard to each sample using fluorescent microspheres of a different color to normalize for differences in microsphere recovery. The calculation of absolute cerebral blood flow was performed as previously described previously.\textsuperscript{175} Measurement of relative cerebral blood flow by laser Doppler flowmetry (see Figure 3-2A) was performed as described in the \textit{Methods} of Chapter 2.

**Magnetic Resonance Imaging Scans and Image Analysis**

At three weeks after stroke induced by intraluminal transient MCAO, aged rats from \textit{Experiment 6} described below were taken to the University of Florida’s Advanced Magnetic Resonance Imaging and Spectroscopy Facility (AMRIS) for MRI using methods developed by Dr. Marcelo Febo (University of Florida, Department of Psychiatry) and described here. During imaging, rats were anesthetized under 1.5% isoflurane gas, placed in a body tube cradle, and setup in a 96 surface transmit/receive radio frequency coil system used for high-resolution imaging on a Magnex Scientific 4.7 Tesla MR scanner. T2 relaxometry pulse sequences were run on a VnmrJ 3.1 console (Agilent, Palo Alto, CA, USA). Core body temperature and respiratory rates were monitored throughout the experiments. For T2 relaxation, the following parameters were used: echo time (TE) = 37.5 ms, repetition time (TR) = 2000 ms, target b value = 1269.92 s/mm\textsuperscript{2}, field of view 25.6 mm\textsuperscript{2} along the read and phase directions and 1.5 mm along the slice direction, and data matrix of 96 x 96 x 8 slices. Signal averaging was used to increase signal to noise. Images were imported to ImageJ (NIH) for processing of T2 maps. T2 maps were reconstructed from a log linear regression of the multi-TE value datasets. Infarct volume was measured by an investigator blinded to treatment.
conditions. Infarct area was calculated for each slice, and the total volume of infarct for each animal was calculated by multiplying the infarcted area by the thickness of the slice (1mm) and summing the volume of the eight slices. In T2 images, the area of infarct appears lighter than healthy tissue due to the extra free water content from edema.

**Experimental Protocols**

**Experiment 1.** To determine whether post-stroke administration of diminazene exerted protective effects, rats (n = 80) were randomly assigned to undergo either sham surgery or endothelin-1 MCAO followed by post-stroke intraperitoneal injections of 0.3mL drug vehicle (H₂O) or diminazene (0.75 – 15mg/kg) at +4 hours, +1 day, and +2 days, with percent infarct volume assessed as the primary outcome measure and neurological function as a secondary outcome. The effect of intraperitoneal diminazene on baseline blood pressure was assessed via indirect tail cuff method as detailed previously. Several rats also underwent serial collection of serum once daily at +1, +2, and +3 days following stroke. To assess the role of central Mas during diminazene treatment, rats (n = 24) to receive post-stroke diminazene intraperitoneal injections were randomized to treatment centrally for five days before and three days after stroke with the Mas antagonist A-779 (0.87μg/μL infused at a rate of 0.5μL/h) or sterile saline (0.9%) via implantation of Alzet® osmotic mini-pumps and stainless steel intracerebroventricular cannulas at 1.5mm lateral and 1.3mm posterior to bregma. Upon euthanasia at 3 days post-stroke, the brains were immediately sectioned and two 2mm sections from 1 to 5mm rostral to bregma were used for infarct volume analysis.

In a related experiment to evaluate the role of central ACE2 in stroke, randomly assigned rats (n = 16) were treated centrally for five days prior to and three days after stroke with the ACE2 inhibitor MLN-4760 (0.43 μg/μL infused at a rate of 0.5μL/h) or
sterile saline (0.9%) via intracerebroventricular infusion as above. Following endothelin-1 MCAO, neurological function was assessed at +4 hours, +1 day, and +3 days, and brains were harvested at three days post-stroke for infarct volume analysis as above.

**Experiment 2.** To assess the effect of peripherally administered diminazene on relative cerebral blood flow (primary outcome measure) during endothelin-1 MCAO, rats (n = 10) were randomized to injection with vehicle or diminazene (7.5 mg/kg) 30 minutes prior to stroke induction. Cerebral blood flow was recorded by laser Doppler flowmetry starting 10 minutes before MCAO and concluding four hours afterward.

**Experiment 3.** This experiment was used to measure the effect of central intracerebroventricular infusion of diminazene (5μg/μL infused at a rate of 0.5μl/h) or vehicle on levels of absolute cerebral blood flow (primary outcome measure) during baseline conditions. Randomly assigned rats (n = 12) were implanted with Alzet® osmotic mini-pumps and intracerebroventricular cannulas as described in Experiment 2 above. One week later, absolute cerebral blood flow was assessed by the microsphere method described above. The absolute measurements reported are from the left hemispheres only, which were not different from levels in the right hemispheres (data not shown).

**Experiment 4.** The neuroprotective effects of post-stroke gavages of orally active Ang-(1-7) were assessed by randomly assigning rats (n=55) to receive 0.5mL of H₂O (vehicle) or orally active Ang-(1-7) (62.5, 125, or 250 μg/kg) at +4 hours, +1 day, and +2 days after endothelin-1 induced MCAO, with percent infarct volume assessed as the primary outcome measure and neurological function as a secondary outcome. Rats
that underwent evaluation by the paw adhesive test (n=9 control and 9 orally active Ang-(1-7), 125ug/kg) also received an additional gavage at +90 minutes post-stroke.

**Experiment 5.** To test the efficacy of oral delivery of ACE2 bioencapsulated in chloroplasts, 170 rats (n=23) were randomly assigned to receive daily oral gavages of powdered plant leaves (500mg, mixed in phosphate buffered saline) containing control chloroplasts or ACE2 chloroplasts starting three days before endothelin-1 induced MCAO. Assessment of ACE2 production within the transgenic plants has shown that ACE2 expression makes up ~2% of total leaf protein. 170 Infarct volume was assessed as the primary outcome measure and neurological function as a secondary outcome.

**Experiment 6.** To evaluate the long-term effects of post-stroke systemic administration of C21 in aged animals, rats (n=28) were randomly assigned to receive intraperitoneal injections of sterile saline (0.9%) or C21 (0.03mg/kg) at +90 minutes, +1 day, and +2 days following intraluminal transient MCAO. The primary outcome measure was infarct volume as assessed by magnetic resonance scanning at three weeks post-stroke with neurological function as a secondary outcome.

**Inclusion and Exclusion Criteria**

Of the rats that underwent endothelin-1 induced MCAO in Experiments 1-5 described above, a small number were excluded from analysis: three exhibited atypical contralateral or bilateral strokes, confirmed by post-mortem staining; two had malfunctions of the guide cannula during MCAO; six had no signs of stroke on infarct volume analysis; and two exhibited abnormal cerebral blood flow recordings. Four infarct volume outliers from Experiment 4 were identified as ≥2.5 times the absolute deviation around the median 142 and removed.
Among the 28 aged rats that underwent intraluminal transient MCAO, five died in the initial 48 hours post-stroke (4 from control and 1 from C21 group), and an additional five were excluded (3 from control and 2 from C21 group) based on having met both of the following exclusion criteria: 1) No or very limited neurological deficits by behavioral assessments at one day post-stroke; 2) no evidence of infarct on magnetic resonance imaging at three weeks post-stroke.

Data Analyses

Data are expressed as means ± SEM. Sample size determination was based on the primary outcome as specified in each experimental protocol and assumed a standard deviation of 15%, an effect size of 15%, power of 0.8, and alpha < 0.05. Statistical significance was evaluated with the use the Mann-Whitney test, one-way analysis of variance for groups of equal variances, or student’s T-test with Welch’s correction as indicated in the figure legends.

Results

Neuroprotective Effects of Post-stroke Administration of Peripheral Diminazene, an ACE2 Activator

The observed increase in ACE2 activity in the ipsilateral cerebral cortex following stroke represents a promising target for inducing stroke neuroprotection. We have previously reported that pre-stroke activation of central ACE2 using diminazene results in significant neuroprotection when administered by intracerebroventricular infusion prior to, during, and after endothelin-1 MCAO. Here, we tested a more clinically relevant treatment protocol, as described in Experiment 1 of the Methods, which employed post-stroke intraperitoneal injections of drug or vehicle at +4 hours, +1 day, and +2 days...
following MCAO, times that overlap with endogenous stroke-induced changes to ACE2–Ang-(1-7)–Mas axis (Figures 2-4, 2-8, & 2-9).

To identify the maximally efficacious dose of diminazene for post-stroke peripheral administration, we performed a set of dose-response experiments in rats. Stroke resulted in large infarct volumes among vehicle-treated versus sham-operated rats. Compared to vehicle-treated controls, infarct volume was significantly decreased by post-stroke intraperitoneal injections of diminazene at doses of 0.75, 2.5, and 7.5 mg/kg, but not 15 mg/kg (Figure 3-1A). The accompanying stroke-induced neurological deficits were significantly attenuated by 7.5 mg/kg diminazene at +1 day (Figure 3-1B&C) but not at +3 days (data not shown). The data presented in Figure 3-1D demonstrate that post-stroke administration of diminazene (7.5 mg/kg, intraperitoneal) at +4 hours, +1 day, and +2 days resulted in significantly higher levels of serum ACE2 activity at +2 days post-stroke with a similar though not significant increase at +3 days. Based on these collective results and the finding that intraperitoneal administration did not affect levels of baseline blood pressure or cerebral blood flow (Figure 3-2), the 7.5 mg/kg dose was used for all subsequent experiments.

We assessed the Mas-dependency of the neuroprotection induced by post-stroke intraperitoneal diminazene injections by chronically co-administering the Mas antagonist A-779 or 0.9% sterile saline vehicle via the intracerebroventricular route. Blockade of central Mas receptors by infusion of A-779 in rats co-treated with post-stroke peripheral diminazene resulted in significantly larger infarct sizes compared to saline infusion (Figure 3-1E).
To examine the role of endogenous central ACE2 in stroke, we infused MLN-4760, an ACE2 inhibitor, via the intracerebroventricular route for five days before and three days after stroke in the absence of any treatment. This resulted in significantly worse neurological function at +4 hours and +3 days post-stroke without significantly increasing infarct volume (Figure 3-1F&G).

**Effect of Diminazene on Cerebral Blood Flow during Baseline and Stroke**

Ang-(1-7) has vasodilatory actions\(^{134}\) which may potentially contribute to the neuroprotection induced by ACE2 activation. To test its effect on cerebral blood flow, we administered diminazene to rats at neuroprotective doses during endothelin-1 induced MCAO, not post-stroke, as described in *Experiments 2&3* in the Methods. Relative cerebral blood flow was not increased by diminazene during stroke as compared to control as measured by laser Doppler flowmetry and up to 4 hours post-stroke (Figure 3-2A). Also, there was not an observed increase in the baseline levels of absolute cerebral blood flow, as assessed by the microsphere injection method, amongst rats chronically treated by intracerebroventricular infusion with diminazene (Figure 3-2B) as compared to saline-infused rats.

**Neuroprotective Effects of Orally Active Ang-(1-7), Bioencapsulated ACE2**

Building on these findings, we also tested recently development compounds with oral bioavailability to activate the ACE2–Ang-(1-7)–Mas axis and induce neuroprotection in stroke. In a dose response study, rats received post-stroke oral gavages of an orally active Ang-(1-7) formulation or control H\(_2\)O as described in *Experiment 4* in the Methods. Rats receiving gavages of 125ug/kg orally active Ang-(1-7), but not 62.5 or 250ug/kg, exhibited significantly smaller infarct sizes on average compared to H\(_2\)O-treated rats (Figure 3-3A). Addition of a +90 minute treatment time point in a subset of
rats did not afford additional protection against infarct size or neurologic function by Bederson and Garcia exam scores (data not shown). Performance on the paw adhesive test, a sensitive measure of sensorimotor deficit, was significantly improved among treated versus control animals, as demonstrated by decreased time to remove the paw adhesive at +3 days post-endothelin-1 induced MCAO, and this decrease was coupled with large, though non-significant, reductions in time to touch the paw adhesive at both +1 and +3 days post-stroke. (Figure 3-3B&C). As described in Experiment 5 in the Methods, other groups of rats received gavages of bioencapsulated ACE2 (500mg/day) or vehicle solution before and after stroke. Treatment with bioencapsulated ACE2 resulted in significantly smaller infarcts measured +3 days post-stroke (Figure 3-3D), as well as significantly improved behavioral exam scores at +4 hours and +1 day on the Bederson exam (Figure 3-3E) and at +1 day on the Garcia exam (Figure 3-3F).

**Sustained Neuroprotection by Post-stroke AT2R Agonist C21 in Aged Rats**

To build on the experimental evidence that AT2R activation in stroke is neuroprotective in ischemic stroke, we assessed the long-term efficacy of post-stroke injections of C21 in aged rats as described in Experiment 6 of the Methods. This experiment was designed to test whether AT2R-dependent neuroprotection that has been reported in young animals during the initial days after stroke could be durably induced in a model that more closely approximates the population of aged stroke patients. Intraperitoneal injections of C21 (0.03mg/kg) starting at reperfusion after 90 minutes of intraluminal transient MCAO, resulted in sustained reductions in infarct volume as assessed by volumetric analysis of magnetic resonance images taken at three weeks post-stroke (Figure 3-4). While total infarct volume tended to be reduced (p=0.09), comparison of infarct volumes by magnetic resonance imaging slice revealed
a significantly smaller average percent infarct volume within slice 2 in C21 versus control-treated rats (Figure 3-4B). The assessment of neurological function by Bederson and Garcia exams (Figure 3-5A&B), paw adhesive testing (Figure 3-5C&D), and rotarod performance (Figure 3-5E) revealed that C21 resulted in a sustained improvement in function beginning in the first +24 hours after stroke and persisting through at least three weeks post-stroke.

**Discussion**

These experiments were designed to test the neuroprotective efficacy of post-stroke activation of the protective arms of the RAS by systemic administration of several novel compounds. Administration of post-stroke diminazene resulted in significant neuroprotection, as well as increased serum ACE2 activity, and diminazene did not increase cerebral perfusion. Neuroprotective effects were also achieved with treatments targeting the receptor Mas, which included an orally active Ang-(1-7) and bioencapsulated ACE2. Using the specific AT2R agonist C21 among aged rats, we observed durable improvements in infarct volume and neurological function that were sustained for at least three weeks after stroke. The treatment protocols employed in these studies demonstrate elements that bolster the translational potential of the findings, including systemic and non-invasive administration methods, timing starting in the acute post-stroke period, and in the case of C21, effects that were obtained in a clinically-relevant aged rat model of stroke and that were shown to last long-term.

Since the recent indication that it activates ACE2, diminazene has been shown to have efficacy in treating a variety of inflammatory diseases, including stroke. We are the first to show that post-stroke administration of this small molecule activator results in significant neuroprotection (Figure 3-1A-C). Importantly, a recent report
questioned whether diminazene activates ACE2, showing that it had no effect when incubated in vitro with recombinant ACE2 and ex vivo with kidney lysates. We found similar results in unpublished experiments using recombinant human ACE2 and lysates of cerebral cortex. However, our assays of serum ACE2 activity in animals that received systemic diminazene over the course of several days following stroke revealed significant increases in serum ACE2 activity (Figure 3-1E), a finding similar to that from a study of diminazene in myocardial infarction. Diminazene may only increase ACE2 activity in vivo over a period of time and/or under conditions of disease or stress through as yet undiscovered effects on transcription, translation, or protein modification of ACE2 or related molecules. Regardless, the observed increase in ACE2 activity implies an increase in Ang-(1-7) generation and subsequent Mas signaling as a result of post-stroke injections of diminazene, which is further validated by our data showing significantly worse infarct volume with co-administration of A-779 (Figure 3-1D). In addition, the administration of orally active Ang-(1-7) and bioencapsulated ACE2 produced similar neuroprotective effects (Figure 3-3).

The vasodilatory action of Ang-(1-7) has been suggested to contribute to its neuroprotective effects in stroke, but our data do not indicate that diminazene increases regional cerebral blood flow during endothelin-1 MCAO or absolute cerebral blood flow during baseline conditions (Figure 3-1A&B). Nonetheless, Ang-(1-7) may still act to increase perfusion at the level of the microcirculation in such a way that it is not detected by our measures of cerebral blood flow. Other potential non-ACE2 mediated effects of diminazene, specifically those that may independently reduce inflammation, cannot be ruled out as contributing to the neuroprotective effects we have observed. As
these experiments were limited to exploring the neuroprotective benefits in the acute and subacute phase of ischemic stroke, there remains the important question of the impact of treatments targeting the ACE2–Ang-(1-7)–Mas axis on longer-term outcomes and late mortality. We anticipate that future studies in this area will address these and other translationally-relevant questions.

The protective effects of AT2Rs in stroke are becoming well-established and are discussed in detail in Chapter 1, *Angiotensin II Type 2 Receptors and Stroke*. The data showing that similar levels of neuroprotective are achieved by AT2R activation with C21 in aged rats represent an important step on the pathway to translating these findings toward clinical application. The STAIR criteria recommend that preclinical testing of neuroprotective compounds should include experiments in aged animals, be replicated in multiple stroke models, and assess multiple outcome measures over a sustained period of weeks, among other criteria.\(^{172}\) The design of our study showing sustained C21-induced neuroprotective effects in aged rats addresses these specific criteria for the first time in regards to compounds targeting AT2Rs in preclinical studies. Until now, studies were performed in young adult rats, both healthy\(^{127, 130}\) and hypertensive,\(^{118, 124, 129}\) and in mice\(^{125}\) with assessments being taken out to a maximum duration of seven days post-stroke.\(^{130}\) We found that the neuroprotective effects reported by us and these other groups to be reproduced in aged 700-800 gram rats, which are also highly likely to suffer from metabolic syndrome and related disorders (unpublished assessments), and that the benefits of C21 treatment were sustained for at least three weeks after stroke. The observation of significantly improved neurological function before and at three weeks post-stroke despite non-significant reductions in total infarct volume at three
weeks post-stroke might have several implications (Figure 3-4). The neuroprotective effects of activation of AT2Rs, which we found to be exclusively on neurons primarily distributed throughout the cortex in post-stroke AT2R reporter mouse brains (Figure 2-10), may provide for enhanced protection and recovery of functionally-important cortical neurons even within areas of increased stroke-induced edema that are selected for with our magnetic resonance imaging. Infarct volume analysis using these images may therefore be inadequate to assess the subtle, yet functionally important, differences that result from treatments. It is also possible that by the three week time point, once larger differences in infarct volume have now become more diminished as the injury evolves, inflammation and edema begin to resolve, and the measured infarct becomes reduced in size as compared to earlier time points at which volumes have been assessed in previous studies of AT2R-induced neuroprotection in stroke. While the results from these experiments are highly encouraging, several other questions remain to be addressed on the path for C21 or other AT2R agonists to clinical translation, including testing in female animals and in higher order species.

New and innovative approaches continue to demonstrate neuroprotective efficacy of treatments that activate the protective pathways of the RAS. While much work remains to be done, the conclusion that non-invasive systemic administration of such treatments beginning in the hours following stroke result in reduced infarct size and improved neurological function appears to be emerging as a consensus. Treatments targeting these pathways are already appearing on the clinical scene in early phase trials for other indications with positive results,\textsuperscript{79, 179, 180} adding plausibility to the idea of trials for the treatment of stroke patients in the near future.
Figure 3-1. Post-stroke administration of Diminazene, an ACE2 activator, results in decreased infarct volume and improved neurological function.  A) Average infarct volumes at 3d post-MCAO for groups of rats that received intraperitoneal injections after stroke of vehicle (H₂O, n=20) or different doses of diminazene (0.75 mg/kg: n=11; 2.5 mg/kg: n=11; 7.5 mg/kg: n=5; 15 mg/kg: n=6), or of vehicle after sham surgery (n=10). Representative 2,3,5-triphenyltetrazolium chloride (TTC)-stained sections are shown for each treatment condition. B&C) Neurological function was assessed at 1d after stroke using the Bederson (B) and Garcia (C) scales. D) Levels of serum ACE2 activity in serum samples at 1d, 2d, and 3d after stroke from control-treated (n=10) or diminazene-treated (7.5 mg/kg, n=4) stroked rats. E) Average infarct volumes from diminazene-treated rats co-treated with vehicle (NaCl, n=10) or Mas antagonist A-779 (n=11) by continuous intracerebroventricular infusion. F) Average infarct volumes from rats given ACE2 inhibitor MLN-4760 by intracerebroventricular infusion (0.43μg/μL infused at a rate of 0.5μL/h, n=6) for five days before and three days after ET-1 MCAO as compared to NaCl infusion (n=7). G) Neurological function was assessed at 4h and 3d post-stroke from MLN-4760 or NaCl infused rats. Data are means ± SEM. *p<0.05 compared to respective controls. (one-way analysis of variance with post-hoc student’s T test for panel A-C, Mann-Whitney test for panels D-G; DIZE=diminazene; ICV=intracerebroventricular; IP=intraperitoneal).
Figure 3-2. Neuroprotective doses of diminazene administered systemically or centrally do not increase cerebral blood flow. A) Averaged laser Doppler flow recordings from rats undergoing endothelin-1 MCAO that received a single intraperitoneal injection of vehicle (H₂O, n=5) or diminazene (7.5 mg/kg, n=5) thirty minutes prior to stroke induction; black arrow indicates MCAO. B) Levels of relative cerebral blood flow during baseline conditions as measured by injection of fluorescent microspheres following 5 days of intracerebroventricular infusion of vehicle (0.9% NaCl, n=6) or diminazene (5μg/μL infused at a rate of 0.5μl/h, n=5; DIZE= diminazene; ICV=intracerebroventricular; IP=intraperitoneal). Data are means ± SEM.
Peripheral administration of activators of the ACE2–Ang-(1-7)–Mas result in neuroprotective effects. A-C) Rats underwent endothelin-1 induced MCAO and received post-stroke oral gavages of H2O (vehicle, n=21) or different doses of orally active Ang-(1-7) (62.5 μg/kg, n=9; 125 μg/kg, n=19; or 250 μg/kg, n=8). Infarct volume (A) was measured as a percentage of the contralateral hemisphere and neurological function was assessed in a subset of these rats (n=9 H2O and 9 orally active Ang-(1-7), 125μg/kg) at the time points indicated by measuring time to touch (B) and time to remove (C) a paw adhesive. D-F) A separate group of rats treated with pre- and post-stroke oral gavages of ACE2 bioencapsulated in chloroplasts (n=8) or control (vehicle, n=9). Graphs represent infarct volume (D) and neurological function assessed by the Bederson (E) and Garcia (F) behavioral exams. Data are means ± SEM. *p<0.05 versus control by Mann-Whitney test for comparisons of infarct volume in (A) and (D) and by student’s T test with Welch’s correction for comparisons of neurological function in all other panels. ACE2: angiotensin converting enzyme 2; Ang-(1-7): angiotensin-(1-7).
Figure 3-4. Post stroke intraperitoneal injections of AT2R agonist C21 result in long-term reductions in infarct volume in aged rats. A&B) Magnetic resonance images were obtained three weeks after induction of ischemic stroke by 90 minute monofilament occlusion of the middle cerebral artery with post-stroke treatment with NaCl (grey triangles, n=7) or C21 (blue circles, n=10), and infarct volume was computed and summarized as (A) the composite infarct volume of all MRI slices combined or as (B) volume across individual slices. C&D) Representative magnetic resonance images from animals treated with NaCl (C) or C21 (D) are shown. Data are means ± SEM. *p<0.05 versus respective control by Mann-Whitney test.
Figure 3-5. Post stroke C21 results in durable improvements in neurological function in aged rats. A-E) Behavioral tests were performed at the time points indicated after stroke in rats treated with intraperitoneal injections of NaCl (n=8) or C21 (0.03mg/kg, n=10), and included the (A) Bederson exam, (B) Garcia exam, paw adhesive time to (C) touch or (D) removal, and (E) rotarod performance time (n=3 NaCl and 4 C21 for rotarod). Data are means ± SEM. * p<0.05 versus control for respective time points by Mann-Whitney test.
CHAPTER 4
SUMMARY, MECHANISMS, AND FUTURE DIRECTIONS

Summary

Specific Aim 1

To assess whether ischemic stroke results in changes to the endogenous activity and expression of components of the classical and protective arms of the RAS, we performed experiments in experimental rodent stroke models and assessed enzyme activity function from the serum of stroke patients in an observational human study. The findings from these studies paint a striking picture of dynamic alterations in the acute period of ischemic stroke. Within the cerebral cortex of rats that underwent transient focal ischemia by endothelin-1 induced MCAO, activity of ACE2 was increased in the first hours and days followed by a return to sham levels by three days following stroke. The activity of serum ACE2 was initially decreased, and then rebounded to levels that were significantly higher than pre-stroke by three days. In humans, the alterations in serum ACE2 activity in both ischemic and hemorrhagic stroke followed a similar pattern of initial decline by comparison to activity levels from groups of control patients, with a convalescent increase by three days among ischemic stroke patients. Lower ACE2 activity was significantly correlated with ischemic stroke. Among control patients, ACE2 activity correlated significantly with systolic blood pressure, with higher pressures being...
associated with higher levels of ACE2 activity. Human serum ACE activity was correlated with female sex oppositely among control patients versus ischemic stroke patients, and in stroke, was negatively associated with modified Rankin scores at discharge. Assessments of components of the classical RAS in rat cortical samples indicated an increase of mRNA expression of ACE as well as TACE one day after stroke. Expression of the Ang-(1-7) receptor Mas, on the other hand, was decreased at one day post-stroke, as assessed by mRNA and immunostaining analysis. From experiments in AT2R reporter mice, it did not appear that ischemic stroke by intraluminal transient MCAO induced changes in the distribution or cellular subtype expression of AT2Rs at one or two weeks after stroke, with AT2R expression observed on neurons only. The picture of a fluctuating RAS, in which protective serum components are initially decreased and brain protective components increased, followed by returns to baseline levels, provides an intriguing background for the development and testing of neuroprotective therapies to intervene during the early minutes and hours following stroke onset.

**Specific Aim 2**

Having observed both stroke-induced changes in the RAS and neuroprotective potential for treatments to activate these components, we designed experiments to explore the potential to induce neuroprotection via this axis using translational treatment protocols. These included giving treatments systemically, by intraperitoneal injection or by oral gavage, and giving them starting hours after stroke onset, in a time course that more closely mimics the clinical course of patients who seek care only after symptom onset. We found that post-stroke injections of an ACE2 activator resulted in significantly reduced infarct volume at three days post-stroke, with accompanying improvements in
neurological function. Inhibition of ACE2 within the brain worsened stroke damage. Similar neuroprotection was induced by oral gavage with a formulation of Ang-(1-7) or ACE2 bioencapsulated in plant chloroplasts. Administration of the AT2R agonist C21 post-stroke by intraperitoneal injections in aged rats recapitulated the neuroprotective effects seen previously only among young rats. Importantly, the protective effects on infarct size and on neurological function were sustained for several weeks following stroke and were apparent using a variety of behavioral assessments. We feel that the consensus among these experiments and previously published reports of reproducible neuroprotection by the protective pathways of the RAS provides a strong foundation for the additional studies, including those in higher order species, and the potential design of clinical trials oriented for the use of such compounds in the treatment of ischemic stroke patients.

**Mechanisms of Neuroprotection by the RAS**

In assessing the clinical potential for therapeutics that activate the protective RAS, consideration should also be given to the mechanisms by which these treatments may be inducing neuroprotection. The beneficial effects of Ang-(1-7)/Mas signaling extend beyond stroke, and have been demonstrated in a variety of inflammation-related disease models including arthritis, hypertensive kidney disease, atherosclerosis, asthma, and acute respiratory distress syndrome.\(^{181-185}\) Similarly, the ACE2–Ang-(1-7)–Mas axis has recently been examined for its potential to be manipulated as a therapy for cardiovascular disease, where its activation has been demonstrated to have therapeutic potential for hypertension and related pathologies, myocardial infarction, heart failure, as well as several types of cancer\(^ {75-82}\) and other diseases.\(^ {43}\) The mechanisms of protection in these varied disease pathologies are likely to overlap, as many tissues,
including the brain, express tissue-specific RAS components. Mas receptors are present on neurons, microglia, and endothelial cells, and consistent with this, studies have indicated that the neuroprotective actions of Ang-(1-7) occur via multiple mechanism. In the section, we review such studies that have focused on the mechanisms of Ang-(1-7)-induced protection in stroke, and we supplement these data with conclusions drawn from studies in other inflammatory and related disorders to propose a multi-faceted mechanistic hypothesis for the neuroprotective actions of the ACE2–Ang-(1-7)–Mas pathway in stroke (see Figure 4-1).

**Anti-inflammation and Anti-oxidation**

Many studies, including several in stroke, have explored the specific hypothesis that Mas activation by Ang-(1-7) has anti-inflammatory and anti-oxidative effects. Our group demonstrated that central administration of Ang-(1-7) during ischemic stroke attenuated the increased levels of pro-inflammatory markers within the cerebral cortex. Activation of the Ang-(1-7) axis may also decrease oxidative stress and thus limit neuronal cell death, as the levels of inducible nitric oxide synthase, a pro-oxidant molecule that is increased in stroke, were also reduced by this peptide. In an animal model of permanent cerebral ischemia, Mas activation exerted similar changes, decreasing oxidative stress and suppressing NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activity and consequent tumor necrosis factor alpha (TNF-α) signaling. NF-κB inhibition by Ang-(1-7) also occurs in other animal disease models, including hypertensive kidney disease, pulmonary fibrosis, and fatty-liver disease. Inhibition of nuclear translocation of NF-κB by Ang-(1-7) has also been shown to prevent the upregulation of vascular cell adhesion protein-1 by Ang II, as well as reduced leukocyte chemotactic signaling, rolling, and adhesion. These effects
may be particularly relevant in the sub-acute period of stroke to limit excessive inflammatory cell infiltration across the compromised blood-brain barrier. In addition to reducing stroke infarct size, ACE2 overexpression in neurons of mice engineered to over-produce Ang II resulted in attenuated levels of reactive oxygen species following stroke in vivo\(^\text{190}\) in vitro,\(^\text{111}\) and in aged mice,\(^\text{112}\) likely secondary to the observed reduction in nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase levels. While it remains to be more clearly demonstrated, it is possible that the augmented activity of neuronal ACE2 in these mice may be exerting anti-oxidative effects indirectly through alterations in neuron-to-microglia signaling, possibly in a way that limits the activation of inflammatory microglia. In support of this possibility, we have observed in rats that administration of Ang-(1-7) in stroke resulted in attenuated cerebral cortical expression of markers of activated microglia and neuronal-derived chemokines,\(^\text{89}\) as well as decreased numbers of activated microglia.\(^\text{115}\) In hypertension, administration of Ang-(1-7) to SHRs lowered oxidative stress, neuronal autophagy and apoptosis, levels of NADPH-oxidase and inducible nitric oxide synthase, and expression of the Ang II/AT1R axis while improving endogenous antioxidant function,\(^\text{108}\) effects that were at least partially reversible by Mas antagonism. The anti-inflammatory/anti-oxidant effects of Ang-(1-7) in stroke as well as other disease states are becoming well-established,\(^\text{97, 191, 192}\) and future work will help to clarify the specific signaling mechanisms and cell subtypes through which this peptide may be acting.

**Vascular Effects and Angiogenesis**

There is abundant evidence which indicates that Ang-(1-7) may have a vasodilatory effect to increase regional blood flow to certain tissues, including cerebral blood flow, which might contribute to its neuroprotective efficacy.\(^\text{98}\) This hypothesis is
supported by findings from multiple groups that axis activation resulted in upregulation of endothelial nitric oxide synthase and nitric oxide production in stroke, as well as improved endothelial function in spSHRs. It was very recently demonstrated that application of Ang-(1-7) into the RVLM resulted in complete attenuation of the detrimental stroke-induced pressor response as well as preventing increased heart rate. In addition to a vasodilatory role, the vasoprotection conferred to the endothelium by activation of the ACE2–Ang-(1-7)–Mas axis has been abundantly demonstrated in the context of exercise, aging, pulmonary hypertension, atherosclerosis, and vascular remodeling, to name a few. In line with these findings, several recent lines of evidence indicate an angiogenic role for the Ang-(1-7)/Mas system in stroke. The priming of endothelial progenitor cells with ACE2 enhanced their angiogenic effect within the peri-infarct region of the mouse cerebral cortex following ischemic stroke. Infusion with Ang-(1-7) by the intracerebroventricular route resulted in significantly increased brain capillary density, and ACE2 overexpression within neurons increased angiogenic cytokines in stroke in addition to improving levels of cerebral blood flow. It is clear that Ang-(1-7)-induced increases in perfusion and angiogenesis would result in great benefit in the setting of cerebral ischemia, and evidence to date points to this as one likely mechanism of protective action.

**Anti-AT1R Effects via Altered Kinase/phosphatase Signaling**

Many of the effects mediated by Ang-(1-7) described above are in direct opposition to the action of Ang II binding to AT1Rs. Indeed, Mas can hetero-oligomerize with AT1Rs and could thus act as a direct antagonist, which is feasible within the cells of the brain as both Mas and AT1Rs are expressed in neurons, microglia, and endothelial cells. Increases in Ang II levels in the brain, as occurs during
stroke, are associated with disease pathophysiology, including enhanced vascular contraction, endothelial damage, neuronal apoptosis and inflammation, and stimulation of reactive oxygen species, effects which are each counteracted by Ang-(1-7)/Mas signaling. We now turn our consideration to recent findings that specifically point toward a role for Ang-(1-7)/Mas in regulating kinase signaling to counter deleterious effects of Ang II binding to the AT1R within any or all of the cellular subpopulations of the brain that could account for many of its other observed beneficial effects (see Figure 4-1). Although inhibition of AT1R-coupled signaling will be the focus of this discussion, such Ang-(1-7)-induced phosphatase activity may also mediate its other beneficial effects by additionally regulating cytokine and growth factor signaling, such as by disrupting the NF-κB pathway as described earlier. Activation of the AT1R by Ang II initiates intracellular kinase signaling, including mitogen activated protein kinase (MAPK)-associated pathways, to induce regulatory effects on blood pressure and fluid balance. Importantly, the AT1R-extracellular signal-regulated kinase (ERK)/p38 MAPK pathway has been shown to induce expressional upregulation of ACE and down-regulation of ACE2, with further suppression by Ang II-induced ACE2 shedding. Considering the importance of kinase signaling in the signal transduction that results from AT1R activation, mechanisms of anti-AT1R action that involve kinase inhibition seem particularly plausible. Along these lines, it has recently been reported that the administration of an ACE2 activator, diminazene aceturate (Berenil®), resulted in significantly down-regulated phosphorylation of MAPKs, including ERK, p38 and c-Jun N-terminal kinases, as well as the NF-κB p65 subunit in mice. We have shown that administration of diminazene aceturate significantly reduces infarct size in rat ischemic
stroke,\textsuperscript{106} which may be the result of such reductions in inflammation, oxidative stress, and apoptosis secondary to the decreased phosphorylation of these pro-inflammatory mediators. In a study using vascular smooth muscle cells of the rat thoracic aorta, Ang-(1-7), via Mas activation, was shown to counteract Ang II-induced decreases in ACE2 expression. Importantly, the effect of Ang-(1-7) was dependent on the activity of MAPK phosphatases, suggesting that Mas signaling may involve activation of phosphatase activity that directly counters AT1R-regulated kinase signaling.\textsuperscript{155}

Further evidence for the role of Ang-(1-7) in activating phosphatases was reported in rat primary astrocytes subjected to radiation-induced inflammation, a setting in which Ang-(1-7) treatment inhibits MAPK activation and reduces markers of inflammation in addition to increasing levels of dual specificity phosphatase 1 (DUSP-1), which is a negative regulator of the MAPK pathway.\textsuperscript{205} These latter findings are of particular interest in the setting of stroke, where inflammation also plays a key role in the pathophysiology of the disease. Similar enhanced negative regulation of Ang II-associated MAPK signaling by Ang-(1-7) is known to occur in a variety of other tissues in both animals and humans. Interestingly, Ang-(1-7) attenuates the increased levels of ACE, AT1Rs, and Ang II in addition to improving hemodynamic parameters in a rat model of vascular calcification.\textsuperscript{206} In the setting of stroke where Ang II and AT1R levels may be increased in the brain,\textsuperscript{99} we and others believe it is likely that the beneficial action of Ang-(1-7) may result, at least in part, from phosphatase activation and subsequent down-regulation of Ang II-induced intracellular kinase signaling,\textsuperscript{207} resulting in a decrease in the deleterious Ang II/AT1R effects as well as disinhibiton of ACE2 to further increase activity of the protective ACE2–Ang-(1-7)–Mas axis.
Interactions with Bradykinin and AT2Rs

Several lines of evidence suggest that in addition to the intracellular modifications of kinase signaling, and perhaps as a precursor to such changes, the Ang-(1-7) receptor Mas may interact with other receptors during its activation, specifically the ubiquitously expressed bradykinin receptors and the AT2R, which we briefly discuss in turn here. The interplay of the RAS and the kinin-kallikrein system has been an area of research and clinical interest in the decades since the discovery that ACE acts to cleave and inactivate bradykinin. Although the precise nature of the interaction between the important vasodilatory systems is not entirely clear, Mas signaling appears to play an essential role, as the vasorelaxant effects of both bradykinin and Ang-(1-7) were completely inhibited by antagonists of the Mas receptor in human umbilical vein endothelial cells, and were absent in Mas-deficient murine microvessels. These effects appear to be mediated by altered phosphorylation of nitric oxide synthase. Central infusion of Ang-(1-7) during stroke resulted in significantly increased levels of bradykinin, and at higher doses, upregulated expression of the bradykinin B1 and B2 receptors. Additionally, the effects of bradykinin to dilate porcine arteries were potentiated by Ang-(1-7). Current evidence seems to indicate that activation of Ang-(1-7)/Mas augments the vasodilatory actions of bradykinin signaling by increasing expression of kinin system components, potentiating its effects, and preventing its metabolism, but future research will be helpful in clarifying more precisely the interaction of these systems.

Mechanisms of AT2R-induced Neuroprotection

Studies of neuroprotection by activation of AT2Rs include evidence for several potential mechanisms by which this receptor may be signaling to reduce stroke
damage, some of which overlap with mechanisms of Ang-(1-7)/Mas-induced protection described above. Consideration of these mechanisms according to the cell types that may be mediating the effects may be helpful: neurons, glial cells, and vascular cells. To begin with neurons, treatment with AT2R agonists in ischemic stroke models have been shown to reproducibly reduce markers of apoptosis or increase neuron survival in vitro\textsuperscript{118, 125} and in vivo.\textsuperscript{129, 130} A number of studies have also demonstrated a robust antioxidative effect of AT2R activation in ischemic brain tissue,\textsuperscript{118, 127, 128, 130} with treatments resulting in lower levels of nitric oxide synthase enzymes and superoxide and nitrative free radicals that are damaging to neurons. Activation of AT2Rs appears also to be linked to the production/release of neurotropic factors such as BDNF\textsuperscript{129, 130} and the anti-inflammatory chemokine IL-10,\textsuperscript{130} while simultaneously decreasing the expression of TNF-\textgreek{a} and certain potentially deleterious chemoattractant factors, such as monocyte chemoattractant protein-1 and C-C chemokine receptor type 2\textsuperscript{127, 128} that could induce excessive migration of inflammatory cells to areas of vulnerable tissue.

Regarding glial cells, AT2R activation after stroke induction appears not to have a profound effect on the activation status of astrocytes and microglia/monocytes since several studies showed no significant effects on markers of their activation.\textsuperscript{127, 129, 130} One of these studies did show an increased number of activated microglia in response to pre- and post-stroke treatments with an AT2R agonist, but not post-stroke only treatments.\textsuperscript{129} An earlier study from this group also showed that post-stroke treatments with AT2R agonist CGP42112 led to increased activation of microglia in the core, but not penumbra.\textsuperscript{124} The presence of a healing and repair oriented population of microglia
within the core\textsuperscript{25} could be beneficially affected by activation of AT2Rs to reduce neuroinflammation in and around this area.

Vascular cells, such as endothelial cells, pericytes, and smooth muscle cells, may also play a role in the protection afforded by AT2R signaling via effects on both perfusion and angiogenesis. AT2R activation has been found in several studies to increase cerebral blood flow during periods of reperfusion\textsuperscript{125} and of recovery in the days following ischemia,\textsuperscript{128} although data from our group did not uncover an acute effect of AT2R activation by intraperitoneal injection of C21 on cerebral blood flow under conditions of baseline or during endothelin-1 induced MCAO.\textsuperscript{127} At high enough doses, C21 has been shown to induce vasorelaxation in isolated rat basilar arteries.\textsuperscript{129} Interestingly, a vasoregulatory peptide called vasoconstriction-inhibiting factor was recently discovered which is reported to attenuate the action of Ang II by activation of AT2Rs,\textsuperscript{210} although the potential for interaction between this peptide as a cofactor with agonists such as C21 has not been explored. Treatment with C21 initiated at the time of reperfusion in mice has also found to decrease the permeability of the BBB,\textsuperscript{128} although the mechanisms remain unclear. Finally, AT2R activation by C21 in the hours after stroke may also result in increased vessel growth in ischemic penumbral areas and subsequent tissue salvage, as it was shown that the number of vascular profiles was increased in the rat brain by treatment and that the migration of human cerebromicrovascular endothelial cells was increased by C21 in a BDNF-dependant manner.\textsuperscript{130}

The areas of mechanistic overlap between AT2R and Ang-(1-7)/Mas-induced protection, such as decreased oxidative stress (e.g. decreased expression of inducible
nitric oxide synthase) and increased angiogenesis and tissue perfusion in response to either treatment, are in contrast to the areas of mechanistic differences between the pathways. Divergent effects include the observation that AT2R-induced neuroprotection in post-stroke treatment protocols does not apparently involve alterations in the activation of inflammatory cells, such as GFAP+ astrocytes and CD11b+ or CD68+ microglia/monocytes, whereas treatment with Ang-(1-7) has been shown to reduce these markers of inflammatory activation. This difference in effect is likely mediated by the cellular localization of these receptors, with Mas expression on neurons\(^\text{115, 200}\), and microglia\(^\text{115, 201}\) while AT2Rs appear limited to neurons (Figure 2-10). Hence, anti-inflammatory effects of AT2R agonists may result secondarily to modifications in chemokine and cytokine signaling from neurons that affect a more tolerable milieu for survival and repair.

**Mas and AT2R Cross-talk**

Accumulating evidence also suggests the possibility of an interaction between Mas and the neuroprotective AT2R which may contribute to the protective effects of Ang-(1-7) in stroke as illustrated in Figure 4-1. The involvement of AT2Rs in the stimulation of Ang-(1-7) effects has been demonstrated in numerous studies through their inhibition or attenuation by AT2R antagonists such as PD 123319 and PD 123177.\(^\text{211-216}\) For example, Ang-(1-7) mediated prostaglandin synthesis,\(^\text{217}\) vasodilation of coronary arteries,\(^\text{218}\) and reduction of mean arterial pressure\(^\text{219}\) were all significantly halted by administration of PD 123319. Importantly, the cerebroprotective effects of Ang-(1-7) in stroke can be blocked by AT2R antagonism, as can the protection conferred by administration of AT2R agonist C21 by Mas antagonism.\(^\text{220}\) Many studies in other disease models have demonstrated cross-inhibition of these receptors through
their respective antagonists within Ang-(1-7) signaling pathways, providing additional evidence to indicate the potential formation of Mas/AT2R heterodimer complexes. It should be noted that many of these results might be explained by a lack of specificity of these antagonists for their respective receptors. Dimerization of other RAS and related receptors have been reported, including interactions of AT1R with Mas, AT2R, bradykinin B2 receptor, and AT1R homodimerization, to name just a few. Indeed, a proposed mechanism of increasing nitric oxide production involves the dimerization of AT2R to the bradykinin B2 receptor. Through direct binding, the AT2R may act as an antagonist of the AT1R by inhibiting G-protein activation and subsequent signaling. Similarly, Mas serves as an AT1R antagonist through heterodimer complex formation which attenuates Ang II actions. These studies illustrate the common occurrence of oligomeric complexes within the RAS, making plausible the idea of Mas/AT2R heterodimerization. Future studies in this area are anticipated to clarify the nature and the functional implications of these intriguing interactions.

In discussing the potential linkages between the Mas and AT2R signaling pathways, it is important to consider the likely localization for a direct Mas/AT2R interaction. It is expected that an interaction between Mas and AT2R would be within shared expression sites. As stated earlier, Mas expression in the brain has been reported in microglia and neurons, as well as endothelial cells, while AT2R is found primarily within neurons and endothelial cells, with some evidence for its expression in cultured microglia. Our data from AT2R reporter mice do not indicate that microglia or astrocytes begin expressing AT2Rs in the weeks following
stroke (Figure 2-10). Following cerebral ischemia, there is an excessive activation of pro-inflammatory cells, including resident brain microglia. Additionally, there are reports of an enhancement of AT2R levels in the tissue surrounding the infarcted region.⁷⁰, ¹²⁰ One consequence of this upregulation may be an increased potential for Mas/AT2R interactions within neurons with subsequent anti-inflammatory cytokine and chemokine release (e.g. interleukin-10) to modulate the activation status of microglia from an inflammatory to a healing and repair subtype.

Along with the evidence suggesting a direct Mas/AT2R interaction, there are several important findings that indicate their roles as independent protective pathways. Two studies have shown Ang-(1-7) induced effects in AT2R knockout mice,²³², ²³³ and another report demonstrated successful vasodilation by an AT2R agonist in Mas-deficient mice.²³⁴ Additionally, multiple studies have reported AT2R antagonists having no influence on Ang-(1-7) effects, suggesting that certain Mas-mediated actions may be signaled through AT2R-independent cascades.¹⁰⁷, ²³⁵-²³⁸ The reasons for these different findings remain to be clarified, but could be explained by differences in species, animal models of disease, timing of drug administration, or age,²²² or possibly by tissue type as formation of receptor dimers has been shown to be tissue-specific.²³⁹ Considering the currently available evidence, a direct Mas/AT2R interaction or dimerization seems plausible and may contribute to the protective effects in studies of stroke resulting from activation of either AT2Rs or the ACE2–Ang-(1-7)–Mas axis.
Future Directions

Translation from the Bench to the Bedside

An increasing number of studies indicate that activation of alternative pathways within the RAS is neuroprotective in stroke. While these studies provide an essential foundation, there are several limitations that our studies help to address.

First, changes in expression and activity of the endogenous components of these protective pathways in the brain after stroke warranted further investigation. The recent demonstration of increases in levels of ACE2, Ang-(1-7), and Mas in the 48h after stroke in the ischemic cerebral cortex of rats was an important first look. A second study of transient MCAO in rats that examined axis components in the rostral ventrolateral medulla (RVLM) showed slightly different findings in that region, with decreases in Ang-(1-7) and Mas at 1d following stroke. In light of these findings, our results, which show an increase in ACE2 activity in the ischemic cortex (Figure 2-4A) but a decrease in Mas immunoreactivity (Figure 2-9C), are intriguing. The reductions in immunoreactive Mas observed in penumbral regions of the ischemic cortex at 1d after stroke agreed with findings from the RVLM and may be due to an overall decrease in penumbral Mas+ cortical neurons, as immunostaining of neuronal nuclear marker NeuN was decreased at this time point (Figure 2-9B&C). Further work is needed to clarify the various changes we and others have observed. It is likely that the difference in the stroke models, transient versus permanent MCAO, plays a distinctive role in the induction of RAS components. Our data showing increased ACE2 activity in the cerebral cortex support the overall consensus that stroke activates the protective axis.

Second, in all but one of the previous studies, the treatment conditions were started before stroke onset, either by genetic modification or pre-stroke administration of
activating compounds. Using a more clinically-relevant protocol, Chen and colleagues were the first to utilize a post-stroke treatment protocol to study ACE2-mediated neuroprotection by delivering endothelial progenitor cells, with or without ACE2 priming, by tail vein injection starting 2 hours after stroke in mice.\textsuperscript{113} As these cells were protective even in the absence of ACE2 priming, and since ACE2 enhanced their beneficial effects, the isolated effects of activating the ACE2–Ang-(1-7)–Mas pathway after stroke onset were unclear. Here, we administered an ACE2 activator by peripheral injection starting at 4h after stroke onset. This allowed for assessment of therapeutic efficacy in a setting that overlaps favorably with the 4.5 hour treatment window for the delivery of tissue plasminogen activator to human stroke victims, as well as the endogenous stroke-induced changes to the RAS. It is also relevant that \textit{systemic} as well as central\textsuperscript{51} administration of diminazene demonstrated neuroprotective efficacy, as intravenous drug administration is a preferred route in humans in emergent settings. There is evidence to indicate that peripheral diminazene can cross the intact blood brain barrier,\textsuperscript{178} but in the setting of stroke, well-characterized barrier leakiness allows many compounds to cross.\textsuperscript{98} 

A third limitation of previously published studies that we can now begin to address involves the scarcity of data describing the Ang II/AT2R and ACE2–Ang-(1-7)–Mas axes in human stroke. Higher levels of ACE2 were found among patients who experienced cardioembolic stroke versus other stroke subtypes,\textsuperscript{149} although baseline or control ACE2 levels were not assessed for comparison. It has also been shown that ACE2 gene polymorphisms may be associated with increased risk for stroke.\textsuperscript{240} Serum measures of protective RAS components are limited, but direct assessment of human
serum ACE2 activity holds promise as a clinical marker in stroke. We were the first to assess ACE2 enzymatic activity in animal serum following stroke (see Figure 2-4D). It is encouraging for the translational potential of the animal data that the changes following stroke in ACE2 activity in rat serum show a similar pattern to changes we have observed in our observation human study using human serum obtained from stroke patients as compared to controls (see Figure 2-7B). Although human studies of treatments that target the Ang-(1-7)/Mas axis have not been performed in stroke, it has been shown that application of recombinant human ACE2 in healthy volunteers is well-tolerated, making plausible the idea of future studies in stroke patients. We also explored the distribution and cellular localization of AT2Rs in the brain of AT2R reporter mice, assessing the effects of stroke on this receptor for the first time using this powerful method (Figure 2-10).

Future Studies and Considerations

The quantity and breadth of preclinical evidence centered on the protective arms of the RAS as targets for neuroprotective therapies has placed researchers in an exciting position to carry forward studies designed to translate these results into efficacious, clinically testable stroke treatments. Salient problems to be addressed in the near term include the necessity of replicating many of the findings described above in female rodent models, and then in higher order models, which are recommended to include gyrencephalic species. In addition, although much promising work has been done to elucidate the mechanisms of action of compounds such as Ang-(1-7) and C21, additional future studies may shed more light on their roles in modulating the complicated balance between populations of inflammatory versus healing and repair monocytes/macrophages in the stroke brain. Experiments along these lines will likely
require techniques for cell sorting with enough sensitivity to differentiate between monocyte versus microglial-derived populations as well as anti-versus pro-inflammatory status. It will also be of value to design experiments that can determine how the modulation of these cellular populations that likely occur in response to our experimental treatments is affected: is it by changes in the permeability of the BBB, alterations in neuronally-derived chemokine signaling, or possibly by altering the proliferation of resident microglia in response to ischemic insult?

Another area yet to be explored includes the assessment of protective RAS treatments on processes related to neurogenesis and migration of new-born neurons. Although it is difficult to conceive of a process by which new-born neurons could completely replace the anatomical and functional connections lost when neurons die in regions affected by ischemic stroke, the idea of recruiting young migrating neurons whose role is to help re-establish the homeostatic environment in which brain cells normally function best seems more plausible. Ischemic injury results in an increased proliferation of neural progenitor cells within specific brain areas such as the subventricular zone and migration of these cells to damaged brain areas, but the concurrent increased expression of repressors to neurogenesis are thought to limit their development. The activation of AT2Rs present on neurons in and around ischemic areas, or potentially on the progenitor cells themselves, may counteract these repressor signals or stimulate the newly-formed neurons to survive and migrate to areas of damage. It has been demonstrated that cell proliferation results from activation of AT2Rs by Ang II in rat hippocampal neuronal stem cells, and that stimulation of AT2Rs on bone marrow mononuclear cells increases their therapeutic efficacy and
enhances the mobilization and homing of these stem cells when they are exogenously applied.\textsuperscript{245, 246} Through anti-inflammatory pro-survival signaling and subsequent maturation into oligodendrocytes, GABAergic neurons, or glutamatergic spiny neurons, progenitor cells that become activated by AT2R-mediated cascades may function to limit neuronal death and dysfunction following stroke and foster an environment more favorable for plasticity and repair during recovery.

In tandem with the endeavor to more fully uncover basic mechanisms, efforts to design and conduct early phase clinical trials may also be warranted. Some of these trials need not be postponed due to concerns over the treatment compounds themselves, as safe and well-established drugs acting on the RAS are already readily available and in clinical use: ACEi’s and ARBs. To our knowledge, no prospective clinical trials have been conducted to assess the neuroprotective efficacy of early initiation of ACEi or ARB therapy in the setting of acute ischemic stroke. Additionally, as early clinical testing continues to establish safety and efficacy of activators of the protective arms of the RAS,\textsuperscript{79, 179, 180, 241} specifically AT2R agonists and activators of ACE2–Ang-(1-7)–Mas, conducting prospective clinical trials of such compounds in stroke in the near future is becoming increasingly plausible.

**Conclusion**

Through the use of several experimental stroke models, novel treatment paradigms, and careful observational study of a human population, the work reported here adds key information to the unfolding narrative centered on the potential for neuroprotective roles of the AT2R and the ACE2–Ang-(1-7)–Mas axis in ischemic stroke. The endogenous alterations of the RAS observed in rodents were recapitulated in our observations of human serum RAS activity. Rodents treated with activators of
these pathways, such as ACE2 activator diminazene, an orally active formulation of Ang-(1-7), and AT2R agonist C21, experienced significantly reduced infarct sizes and better function outcomes as a result of receiving therapy initiated hours after the onset of ischemia. Stroke does and will continue to exist as a scourge in the lives of tens of millions of people worldwide, one for which most have little or no hope of receiving treatments. Nearly every person knows someone who has or has themselves experienced a stroke, many to devastating effect. The process of translating promising therapies from the laboratory bench to the bedside of patients is a long and sometimes complicated one, and the successful completion of each step along the path has meaning. While it is true that previous efforts have yet to produce effective neuroprotective treatments for patients suffering from ischemic stroke, the encouraging results from these studies, which carry forward the positive results from previous others, should offer hope for the future.
Figure 4-1. Mechanisms of neuroprotection by Ang-(1-7). The neuroprotective effects that result from Mas activation by Ang-(1-7) are summarized here. We propose a mechanism of action in which phosphatase activation by Mas signaling leads to dephosphorylation of essential elements of the Ang II/AT1R-induced kinase signaling cascade, thus inhibiting its deleterious effects. Also summarized are the beneficial actions of Mas activation that may be induced independently from Ang II/AT1R signaling, with potential contributions from signaling in neurons, microglia, endothelial cells, and vasculature. Abbreviations: Ang II, angiotensin II; Ang-(1-7), angiotensin-(1-7); AT1R, angiotensin type 1 receptor; AT2R, angiotensin type 2 receptor; MAPK, mitogen activated protein kinase; DUSP-1, dual specificity phosphatase 1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TNF-α, tumor necrosis factor alpha; PIC, pro-inflammatory cytokine; VCAM-1, vascular cell adhesion protein 1; infl. cell, inflammatory cell; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; BK R, bradykinin receptor; CBF, cerebral blood flow; iNOS, inducible nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate oxidase; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.
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BIOGRAPHICAL SKETCH

Douglas Martin Bennion was born in Salt Lake City, UT, and graduated from Flower Mound High School near Dallas, TX, in 2004. From 2005 to 2007, he served as a volunteer Hmong-speaking missionary in Anchorage, AK, for the Church of Jesus Christ of Latter-day Saints. He met and married his sweetheart, Jennifer, in 2008 while they were attending Brigham Young University in Provo, UT. He performed three years of undergraduate honors thesis research on receptor-mediated mechanisms of learning and memory in the hippocampus in the laboratory of Dr. Jeffrey G. Edwards, PhD. Doug graduated with honors from BYU as valedictorian of Neuroscience with a Bachelor of Science. They moved to Gainesville, FL, in 2010, after he was accepted into the combined MD-PhD Training Program at the University of Florida College of Medicine. He completed the first two years of medical school before beginning his PhD research in 2012 in the laboratory of Colin Sumners, PhD. In the lab, Doug has mentored and trained fifteen undergraduate students, optimized several enzyme activity protocols, and published multiple first-author manuscripts, including one book chapter. For this work, he has been the recipient of multiple awards at the college, university, state, and national levels, has given multiple media interviews, and has been invited to present at national and international conferences.

In the community, Doug is actively engaged in his fifth year as the president of a men’s service organization with the aim of providing for the physical and spiritual well-being of ~75 Gainesville families. Doug enjoys music and performs regularly with a string trio, plays the piano, organ, and violin at church, and sings regularly with his family in a variety of settings. His other hobbies include fly fishing, basketball, hiking, and do-it-yourself home improvement projects. Upon completion of his PhD, Doug looks
forward to rejoining the medical school class of 2018. Doug aspires to be a 
compassionate and effective physician scientist and a successful husband and father. 
Doug and Jenn are the happy parents of 3 children - Amanda, age 6, Donovan, age 3, 
and Martin, age 1 year. They are the light of his life.