MECHANISMS OF PROTECTION BY ANGIOTENSIN II RECEPTOR ANTAGONISTS AFTER STROKE: ROLE OF VEGF-A AND B

by

SAHAR AHMED SOLIMAN

(Under the Direction of Susan C. Fagan)

ABSTRACT

Stroke remains to be a major health problem and one of the most common and undertreated disorders worldwide. With the limited treatment options, recovery is limited to the plasticity of the brain tissue and its ability to remodel after injury. Angiogenesis is a mediator of recovery and switching on angiogenesis can be achieved by the use of safe and FDA-approved medications. Experimentally, angiotensin II receptor blockers (ARBs) promoted cerebral angiogenesis with or without ischemic injury. Candesartan, a member of the ARB family, enhanced expression of vascular endothelial growth factors-A and B (VEGF-A and B) when administered after the onset of focal cerebral ischemia. In clinical trials, ARBs have demonstrated benefit in stroke prevention. However, the effect of ARB treatment in the acute phase of stroke has been complicated by its blood pressure lowering effect. In this study, we investigated the role of VEGF-A and B in mediating the benefits of ARB treatment after focal cerebral ischemia. In addition, we studied the effect of low-dose candesartan, to avoid blood pressure reduction, in combination with the anti-inflammatory and anti-apoptotic agent, minocycline, on recovery.
Our findings include induction of a prolonged proangiogenic state in the brain tissue and cerebrospinal fluid after a single candesartan treatment. In addition, we demonstrated an autocrine proangiogenic and a paracrine neuroprotective effect of candesartan treatment *in vitro*, mediated through VEGF-A and B upregulation. The effect of candesartan treatment appeared to be a class effect of ARBs due to a similar proangiogenic response elicited by losartan treatment, another member of the ARB family. Little is known about the relatively new isoform, VEGF-B. To assess its role in mediating candesartan’s protective effect in acute injury, VEGF-B expression was silenced using shRNA technique. The ability of candesartan treatment to ameliorate acute injury was measured with or without VEGF-B knockdown. Lastly, we optimized ARB proangiogenic therapy by the rational sequential combination of the anti-inflammatory and MMP-inhibitory agent, minocycline. Timing of the combination treatment was optimized to avoid potential antagonistic interactions.

INDEX WORDS: Stroke, Candesartan, Angiotensin II Receptor Blockers (ARBs), Minocycline, Angiogenesis, Matrix Metalloproteinases (MMPs), Neurovascular Coupling, Neuroprotection, Vascular Protection.
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SAHAR AHMED SOLIMAN

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by

SAHAR AHMED SOLIMAN

Major Professor: Susan C. Fagan
Committee: Azza B. El-Remessy
Adviye Ergul
Somanath Shenoy
Anilkumar Pillai

Electronic Version Approved:
Maureen Grasso
Dean of the Graduate School
The University of Georgia
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Stroke; Why Is Immediate Attention Needed?

Stroke, or brain attack, occurs when blood flow is interrupted to parts of, or less commonly the entire brain (Lo et al., 2003). Although it can go unnoticed, it usually results in sudden-onset reversible or irreversible neurological symptoms, ranging from hemiplegia to sensory deficits (Bradberry et al., 2004).

Stroke Prevalence, Incidence, Mortality and Economic Burden

According to the American Heart Association 2014 updates (Go et al., 2014), it is estimated that 6.8 million people suffer from stroke in the US alone. The number is expected to rise to 10.2 million by 2030. Each year ≈ 795,000 stroke cases occur, of which 610,000 are new, while 185,000 are recurrent. Accordingly, someone suffers a stroke every 40 seconds.

Stroke is the fourth leading cause of death after coronary artery disease, cancer and chronic obstructive respiratory diseases. It accounts for 1 in every 19 deaths in the US and 1 death every 4 minutes. The annual direct and indirect cost of stroke is estimated to be $36.5 billion (Go et al., 2014). With such alarming statistics, it is of utmost importance to study the barriers to stroke care, the shortcomings of the currently available treatment options and to develop new stroke therapeutics.
Pathophysiology of Stroke

Ischemic stroke accounts for 87% of stroke cases, while intracerebral and subarachnoid hemorrhages account for 10 and 3%, respectively (Go et al., 2014). As the name implies, hemorrhagic strokes occur secondary to vascular rupture, subsequent bleeding and hematoma formation within the brain tissue. Ischemic strokes, on the other hand, result from a reduction in blood supply. Atherosclerotic injury, plaque formation and vascular stenosis within cerebral or non-cerebral vessels are usually involved. Thrombosis occurs when an atherosclerotic plaque is detached, migrates and occludes narrower vessels. In other cases, blood clots are formed in the heart (as in atrial fibrillation for example) and carried with the circulation to block one or more of the cerebral arteries and arterioles (Bradberry et al., 2004). A diagram depicting different stroke types is shown in Figure 1.1

![Figure 1.1: Diagrammatic illustration of different types of stroke. ICH; Intracerebral Hemorrhage; SAH: Subarachnoid Hemorrhage. Adapted from: Stroke Pathophysiology, Diagnosis and Management, 5th Edition (Mohr)
Ischemic Stroke: Treatments and Challenges

Almost two decades have passed since the successful completion of the NINDS Stroke trial that proved the efficacy of recombinant human tissue plasminogen activator (tPA) for stroke thrombolysis. However, tPA remains the only Food and Drug Administration (FDA)-approved agent for this indication. Even more alarming, only 2-3% of patients receive tPA, due to the strict eligibility criteria and the risk of bleeding, making ischemic stroke one of the most undertreated conditions (Ishrat et al., 2012).

Indeed, decades of experimental research have led to the discovery of an enormous number of promising stroke therapies, only to fail in clinical trials (Chacon et al., 2008; Drummond et al., 2000; Kahle and Bix, 2012; Kidwell et al., 2001). A total of 178 controlled trials that recruited more than 73,000 participants, have been conducted in the second half of the twentieth century and offered nothing but one FDA-approved agent, tPA (Kidwell et al., 2001). Figure 1.2 illustrates different classes of drugs that have been studied in acute ischemic stroke clinical trials.
Translational Gap in Ischemic Stroke Research; Lessons Learned from Failure

Continuous failure of translating stroke research from bench to bedside has led leaders of the field to analyze possible reasons behind the current translational gap. A summary of the most common pitfalls in experimental and clinical trial design is shown in Figure 1.3. In this chapter, we discuss common design limitations as well as the steps taken to address them in order to improve the translational potential of this work.

Figure 1.2: Graphical representation of clinical trials conducted in the second half of the 20th century, subdivided by the agent mechanism of action (Kidwell et al., 2001).
Points to consider in a preclinical study

**Time Window for Treatment:**

While most of preclinical studies administer tested drugs at or shortly after reperfusion, this is not the case in a clinical setting. Treatment windows are usually longer and more variable than preclinical trials. The median time to administer drugs after stroke in a clinical setting was 14 hours, with a range of patient admission of 4 hours to 12 days.
(Kidwell et al., 2001). Indeed, the NINDS-rtPA study screened more than 17,000 patients in order to recruit 624 eligible patients meeting the 3-hour time window criterion (Gladstone et al., 2002). Drug administration in experimental studies occurs, however, in shorter and less variable time windows, and this could be one of the reasons behind failure of translation. It is, therefore, imperative to study drugs in different and longer time windows before considering a drug as a potential clinical trial candidate. In this study, we investigate the mechanisms of protection by angiotensin II receptor blockers (ARBs), a class of antihypertensive medications. Starting ARB treatment 3 hours after focal cerebral ischemia decreased infarct size and improved neurobehavioral outcome (Brdon et al., 2007). In addition, ARB administration at 3 hours after experimental cerebral ischemia increased the safety of late tPA treatment (Ishrat et al., 2013). Although administration of ARBs at a 3-hour window might not be clinically feasible due to blood pressure concerns, preclinical success of a relatively late ARB treatment justifies further studies on the mechanisms of protection by ARBs as well as optimization of ARB treatment.

**Optimal Duration of Treatment and Timing of Outcome Assessment:**

Studies have demonstrated neuronal loss and infarct progression for days after ischemic stroke (Bullock et al., 1995; Castillo et al., 1997). It has been reported that an infarct acquires its final form 12-42 days after the onset of an ischemic insult (Persson et al., 1989). Most preclinical studies, however, administer the tested drug as a single post-stroke treatment and assess only early outcomes. This is especially problematic with drugs that only delay but do not stop progression of cell death cascades (Valtysson et al., 1994). Isoflurane decreased cortical and subcortical infarcts 2 days after an ischemic insult. The effect, however, was not maintained at 14 days (Kawaguchi et al., 2000). Accordingly, the
The use of a multiple treatment regimen might warrant optimizing concentration and/or timing of the treatment. In **Chapter 5**, we discuss a multiple treatment regimen with candesartan, an antihypertensive drug belonging to the ARB class. Several studies demonstrated the angiogenic effects of the ARB family (Alhusban et al., 2013; Forder et al., 2005; Kozak et al., 2009; Munzenmaier and Greene, 2006; Soliman et al., 2014), a predictor of favorable outcome and a mediator of recovery after stroke (Krupinski et al., 1994; Szpak et al., 1999; Xiong et al., 2010). However, candesartan’s blood pressure lowering effect, especially in a multiple dosage regimen, could result in decreased cerebral perfusion, possibly negating its beneficial effect (He et al., 2014; Jauch et al., 2013; Sandset et al., 2011). In order to harness the positive pro-recovery effects of candesartan, employing a low dose candesartan treatment was considered. Moreover, we assessed recovery at different time points (1, 3, 7 and 14 days) after focal cerebral ischemia.

**Selection of Outcome Measures:**

While most preclinical experiments measure infarct size as an indicator of drug efficacy, clinical studies rely on behavioral and functional outcome for assessment of therapeutic success. It is, therefore, recommended to measure different aspects of neurobehavioral recovery in animal models as a more sensitive outcome measure (Corbett and Nurse, 1998; Hunter et al., 1998). Several animal tests have been designed to study cognitive, sensory and motor recovery. Tests, however, differ in their sensitivity, reliability and predictive value (Hunter et al., 2000). In our studies, we used a battery of
functional outcome tests, conducted in a blinded fashion, in order to accurately assess our candidate drugs.

**Homogeneous Stroke Models:**

One major difference between strokes in a clinical versus experimental setting is the variability among stroke subjects. In a preclinical experiment, one stroke type is induced with very controlled physiological parameters. Most importantly, the animals used are usually young and healthy. In a clinical environment, however, strokes are more variable, usually affecting older patients with other co-morbidities (Alonso de Lecinana et al., 2001). In order to get a real assessment of the candidate drug’s therapeutic efficacy, it is important to study the drug in different stroke models and in different co-morbidities. ARB treatment reduced infarct size and improved neurobehavioral outcome in temporary (Kozak et al., 2009) and permanent filament (Guan et al., 2011a) as well as embolic (Ishrat et al., 2013) models of stroke. In addition, ARB treatment improved neurobehavioral function, decreased infarct size, edema and hemoglobin content in hypertensive rats (Kozak et al., 2008).

**Points to consider in a clinical study design**

**Location of Infarct:**

While the location of the infarct is consistent in a preclinical setting, it is highly variable among stroke patients. Most preclinical studies have demonstrated the protection of cortical grey matter. Including different location infarcts in human studies might dilute the benefits seen in a subgroup of patients with cortical infarcts (Gladstone et al., 2002).
**Size of Penumbra:**

The size of the penumbra, and hence the potentially salvageable tissue, is an important determinant of intervention success (Hakim, 1998). If there is insufficient volume of reversibly damaged tissue, treatment cannot be expected to yield a positive outcome. In fact, it has been argued that some drug treatments have been prematurely aborted in clinical trials due to incorrect patient selection, in terms of having insufficient salvageable tissue. Current expert opinion emphasizes the importance of considering penumbra volume in the entry criteria (Fisher, 1997; Gladstone et al., 2002), which can be identified by neuroimaging techniques (Davis et al., 2012).

Indeed, the Stroke Therapy Academic Industry Roundtable (STAIR) published a group of recommendations in order to improve the translational potential of promising preclinical drugs and to decrease the likelihood of failure (Kahle and Bix, 2012). STAIR criteria include: experimentally testing drugs in a “clinically-relevant” time window, assessment of neurobehavioral outcome rather than infarct size as a more reliable indicator of recovery as well as confirming results in multiple stroke models by different independent investigators and in larger species before testing the candidate drug in a clinical trial ((STAIR). 1999; Fisher et al., 2009)

**The Neurovascular Unit: An Alternative Approach to Ischemic Stroke Therapy**

A growing body of evidence indicates interaction and communication of different cell types within the brain, leading to the emergence of the concept of the “neurovascular unit” (Bushnell et al., 2006). The unit consists of microvessels (including endothelial cells, basal lamina, astrocytic end-feet and pericytes), in addition to astrocytes, neurons and other glial cells (oligodendrocytes and microglia) (del Zoppo, 2010). A major flaw in the
design of preclinical and clinical studies was the focus on a single cell type, usually neurons, as a therapeutic target, overlooking possible interactions with other cell types (Guo et al., 2008; Lo et al., 2005). Continuous failure of neuroprotectant clinical trials, among other drug classes, has directed interest towards studying dynamic interactions between different cell types in health and disease (Guo et al., 2008).

Neurovascular cross-talk plays important roles in neural and vascular development, maintenance of normal function and homeostasis and in response to injury (Quaegebeur et al., 2011).

*Role of Neurovascular Cross-talk in Cerebral Development:*

During cerebral development, microvessel and neuron co-alignment along the extracellular matrix paths reflects bidirectional communication (Engvall et al., 1986; Grant and Kleinman, 1997; Herken et al., 1990; Liesi, 1985). In fact, angiogenesis and neurogenesis are two tightly related processes. The commonalities between the two processes include shared growth factors and molecular cues (Lok et al., 2007). Vascular endothelial growth factor (VEGF), a prototypical angiogenic molecule, promotes neuronal survival and axonal growth (Jin et al., 2002; Jin et al., 2000; Matsuzaki et al., 2001). Arteries have been shown to attract their own innervation by secreting VEGF, artemin and endothelin-3 (Ruiz de Almodovar et al., 2009). Fibroblast growth factor-2 (FGF-2) is another potent angiogenic factor that has been shown to exert a mitogenic effect on neural stem cells (Ciccolini and Svendsen, 1998; Gritti et al., 1996; Kilpatrick and Bartlett, 1995). Similarly, neural growth factors have been shown to exert an angiogenic effect, including ephrins, netrins, semaphorins and slits (Iadecola, 2004; Lok et al., 2007). Neuron-secreted semaphorin 3E (Sema3E) guides vessel growth and inhibits vascular
navigation to unwanted territories. Netrins, slits and ephrin ligands were all identified as guidance cues as well controlling vessel formation process (Quaegebeur et al., 2011).

**Role of Neurovascular Cross-talk in Homeostasis:**

Normal functioning of blood vessels requires establishment of blood brain barrier (BBB) properties. The interdependence of different brain cell types to establish BBB is another example of communication within the neurovascular unit. Neural progenitor cell-secreted Wnt induces BBB characteristics in endothelial cells. Endothelial cells secrete placental-derived growth factor-B (PDGF-B) to recruit pericytes, which in turn stimulate tight junction formation via angiopeitioin-1 (Ang-1) and induce astrocyte attachment and polarization (Quaegebeur et al., 2011).

Maintenance of cerebrovascular tone is a result of communication between neurons, astrocytes, endothelial cells and smooth muscle cells (Iadecola, 2004). Endothelial cells are equipped with the machinery necessary to synthesize vasodilator as well as vasoconstrictor substances (Faraci and Heistad, 1998). Chemical signals released from endothelial cells are translated into changes in intracellular calcium levels within smooth muscle cells (Somlyo et al., 1999). On the other hand, astrocytic end-feet completely surround blood vessels and are enriched in potassium channels, purinergic receptors as well as water channel protein aquaporin-4, indicating a role in vascular permeability (Simard et al., 2003). Moreover, astrocytes play a role in neuronal energy metabolism (Pellerin and Magistretti, 2003) and synaptic function (Newman, 2003). Neuronal processes, in turn, innervate blood vessels and communicate via neurotransmitter release (Iadecola, 2004). Communication between components of the neurovascular unit is, therefore, multidirectional and necessary to maintain homeostasis within the cerebral
microenvironment. An elegant study conducted by Carmaliet group demonstrated a new role of VEGF in regulating resistance arteries. Low VEGF levels resulted in structural and functional defects in neuroeffector junctions as well as impaired smooth muscle cell phenotype (Storkebaum et al., 2010).

**Role of Neurovascular Cross-talk in Response to Injury:**

Communication between components of neurovascular unit is especially important in the context of cerebral injury. Recent reports have documented coupling between neurogenesis and angiogenesis in the brain, both leading to improved functional recovery after an ischemic insult. Mediators of neurovascular cross-talk have been identified, including VEGF, brain-derived neurotrophic factor (BDNF), stromal-derived factor-1 (SDF-1), Ang-1 and nitric oxide (NO) (Madri, 2009).

In the normal brain, there is a continuous loss of neuronal cells in some brain areas, including granule neurons in the dentate gyrus and olfactory bulb cells. To replace dead cells, neural stem cells (NSCs) migrate from subgranular and subventricular zones (SGZ and SVZ, respectively), the areas of their residence, to the areas of neuronal death along fixed migratory pathways, the rostral migratory stream (Xiong et al., 2010). The presence of a focal ischemic insult, however, redirects the NSCs to the areas of injury (Greenberg, 2007). Blood vessels upregulate stromal-derived factor-1 (SDF-1) and angiopoietin-1 (Ang-1) in response to an ischemic insult. These factors bind to CXCR-4 and Tie-2 receptors (receptors for SDF-1 and Ang-1, respectively) on NSCs, recruiting them to the site of injury (Ohab et al., 2006). Moreover, erythropoietin- activated endothelial cells secrete matrix metalloproteinases-2 and-9 (MMP-2 and MMP-9, respectively), that digest the extracellular matrix, allowing for unimpeded migration of neural progenitor cells
In addition to facilitating migration, MMP activation allows for axonal extension and recovery after injury (Larsen et al., 2003; Reeves et al., 2003).

The communication between NSCs and endothelial cells is bidirectional. In stroke, angiogenic factors are upregulated in NSCs, including VEGF-B/VEGFR-1, angiopoietin-2 and prostaglandin-enoperoxidase synthase 1, suggesting a role of NSCs in stimulating angiogenesis (Liu et al., 2007). Teng et al. demonstrated a bidirectional communication between ischemic endothelial cells and NSCs and the involvement of VEGF/VEGFR2 signaling. Ischemic endothelial cells, isolated from rat brains 7 days after embolic middle cerebral artery occlusion, upregulate VEGF expression which in turn binds to VEGFR2 on NSCs. NSC proliferate and differentiate in response to VEGFR2 signaling and secrete more VEGF, thereby promoting a proangiogenic state (Teng et al., 2008).

Li et al. demonstrated the bidirectional communication and the involvement of multiple growth factors in the dynamic interaction between NSCs and endothelial cells (Li et al., 2006). NSC-produced nitric oxide (NO) enhances endothelial expression of VEGF and BDNF. These factors act in an autocrine fashion, creating a proangiogenic response and in a paracrine fashion on NSCs to increase their proliferation and production of NO. The persistent production of NO and the subsequent growth factor production create a positive-feedback loop that maintains NSC self-renewal and stabilization of endothelial cell tube-like structures. The role of BDNF in mediating endothelial cell-neuronal cross-talk was confirmed by Guo et al (Guo et al., 2008). Conditioned media from endothelial cells protected neurons against a variety of apoptotic stimuli, an effect that was lost by neutralizing the BDNF receptor, TrkB. Indeed, the correlation between angiogenesis and neurogenesis in an ischemic setting has also been proven by examining the ultrastructure
of the proliferating clusters in the SGZ. It was found that they consist of neuronal, glial and endothelial precursors, further supporting the cross-talk idea (Lok et al., 2007).

Whether angiogenesis precedes neurogenesis or the other way around is not very clear. However, there is evidence supporting that angiogenesis leads to neurogenesis in adult canaries brains, an effect that is mediated via an interplay of VEGF-A, BDNF and MMP-2 (Chen et al., 2013; Louissaint et al., 2002).

Reparative Angiogenesis: A Target for Stroke Therapy

Switching on angiogenesis is a known response to cerebral ischemia (Navaratna et al., 2009). Angiogenic genes are upregulated within minutes of stroke onset (Hayashi et al., 2003). Animal studies have demonstrated endothelial cell proliferation as early as 12 hours after experimental stroke that was maintained for several weeks thereafter (Hayashi et al., 2003). Human studies provided evidence of active angiogenesis 3-4 days after stroke. It is not clear, however, if the angiogenic response to cerebral ischemia leads to development of fully functional vessels (Ergul et al., 2012). Nonetheless, postmortem human samples have shown a positive correlation between survival time and total vascular density (Krupinski et al., 1994). It is now believed that angiogenesis plays a role beyond revascularization of ischemic tissue in the course of recovery after ischemic stroke (Navaratna et al., 2009). The clean-up hypothesis proposed by Lyden group provided better insight into the neovascular-mediated recovery. Accumulation of macrophages in the vicinity of the newly formed vessels suggests a “clean-up” role played by the neovasculature and thereby promoting recovery (Manoonkitiwongsa et al., 2001). Establishing the concept of the neurovascular unit and the tight association of angiogenesis and neurogenesis has led to developing stroke therapies that aim at
promoting a proangiogenic state. Erythropoietin (Wang et al., 2004), lithium (Guo et al., 2009) and resveratrol (Simao et al., 2012) improved recovery after experimental cerebral ischemia, an effect that was explained by their ability to promote angiogenesis.

Neurovascular coupling represents a viable target for stroke therapies, since it takes into account the interaction of neurons with their surrounding environment. Mediators of this interaction, however, remain to be elucidated. In Chapters 3 and 4, we identify VEGF-A and B as mediators of neurovascular communication and assess the role of the relatively understudied isoform, VEGF-B, in ameliorating acute ischemic injury.

**Angiotensin System in the Brain and Ischemic Damage**

Circulating and local angiotensin II exerts its physiological and pathological roles through two main classes of receptors; Angiotensin II receptor type-1 (AT1) and type-2 (AT2). AT1 receptor stimulation increases vascular tone and controls cerebral blood flow, in addition to its role in vascular remodeling through stimulation of cell growth and inflammation. The AT2 receptor, on the other hand, is poorly expressed in adulthood under normal conditions. Insults, such as cerebral ischemia, upregulate AT2 receptor expression (Gallo-Payet et al., 2011). A summary of the actions mediated by AT1 and AT2 receptor stimulation is illustrated in Figure 1.4. The detrimental role played by AT1 receptor stimulation in an ischemic stroke setting has been documented. AT1-deficient mice had smaller infarct volumes as compared to their wild type counterparts (Walther et al., 2002). Moreover, chronic AT1 receptor blockade by daily candesartan dosing before experimental stroke reduced infarct size and edema (Nishimura et al., 2000). Whether the protective benefits of ARB treatment were dependent on their blood pressure lowering effect needed to be elucidated. Ito et al. demonstrated the superiority of candesartan
pretreatment over an equally hypotensive dose of nicardipine, an antihypertensive medication of the calcium channel blocker family (Ito et al., 2002). In an independent study, a non-hypotensive dose of valsartan, another member of the ARB family, significantly reduced ischemic lesion and neurological deficit (Iwai et al., 2004). Unopposed AT2 receptor stimulation provides an alternative plausible explanation for the benefits of ARBs and has been supported by the significant reduction of valsartan benefit in AT2 receptor-deficient mice (Iwai et al., 2004).

Figure 1.4: Actions of angiotensin II in the brain mediated via its two main receptor subtypes; angiotensin II receptor type-1 (AT1) and angiotensin II receptor type-2 (AT2) (Gallo-Payet et al., 2011).
Due to the limited therapeutic utility of pretreatment, post-stroke ARB treatment was attempted (Engelhorn et al., 2004). Candesartan treatment improved functional outcome and reduced infarct. Even more, administration of candesartan 3 hours after experimental stroke was still neuroprotective (Brdon et al., 2007).

**ARBs and the Angiogenic/Protective Paradox**

The ability of candesartan to reduce neurovascular deficits after experimental stroke was accompanied by a vascular protective effect, as demonstrated by reduced blood brain barrier leakage (Kozak et al., 2009). Paradoxically, candesartan administration activated matrix metalloproteinases (MMPs), a group of matrix degrading enzymes, at 24 hours and increased total vascular density at 7 days, as evident by enhanced laminin staining (Kozak et al., 2009). Oral losartan treatment, another member of the ARB family, increased vascular density at 14 days with or without ischemic injury (Forder et al., 2005; Munzenmaier and Greene, 2006). The study of angiogenic growth factor expression in the ischemic brain in response to ARB administration was conducted. Vascular endothelial growth factor-A (VEGF-A), a prototypical angiogenic molecule, was elevated in the contralesional hemisphere at 24 hours following 3-hour MCAO. Interestingly, a relatively new VEGF isoform, VEGF-B, was rescued in the ipsilesional hemisphere following candesartan administration in the same model (Guan et al., 2011c). In Chapters 3 and 4, we further study these VEGF isoforms, with an emphasis on their temporal pattern, cellular source as well as establishing their role in neurovascular coupling.

**ARBs in a clinical setting and the need to optimize proangiogenic treatment**

Several clinical trials investigating the safety and efficacy of ARBs in stroke have yielded promising results (Devereux et al., 2003; Diener, 2009; Schrader et al., 2005).
However, ARB treatment in the acute phase after stroke is complicated by their blood pressure lowering effect. Whether acute blood pressure lowering should be attempted has been debatable (Fischer and Rothwell, 2011). However, the Scandinavian Candesartan Acute Stroke Trial (SCAST) demonstrated a lack of benefit of moderate blood pressure lowering when candesartan was administered in the acute stroke period (Sandset et al., 2011). A more recent study, the China Antihypertensive Trial in Acute Ischemic Stroke (CATIS), reported lack of benefit of blood pressure lowering after stroke using angiotensin converting enzyme inhibitors, diuretics, calcium channel blockers or a combination of more than one class of medications (He et al., 2014). Current treatment guidelines by the American Heart Association recommends against blood pressure lowering within the first 24 hours (Jauch et al., 2013). In order to harness the benefits of ARBs without the accompanying blood pressure lowering effect, delayed subhypotensive doses should be attempted and studied for preclinical efficacy.

Due to the complexity and dynamics of stroke pathophysiology, rational sequential combination therapy represents a promising approach towards successful treatment (Gladstone et al., 2002). In Chapter 5 of this study, we propose the combination of a subhypotensive dose of candesartan with minocycline, a drug with a solid history of neuroprotection in different CNS injury models. We also optimize treatment timing to avoid possible antagonistic interactions.


professionals from the American Heart Association/American Stroke Association.

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CHAPTER 2

PROBLEM STATEMENT AND SPECIFIC AIMS

Stroke remains a major health problem worldwide. Nationally, it is the fourth leading cause of death and the number one cause of adult long-term disability. Of the more than 700,000 acute ischemic strokes occurring annually in the United States, 98% of patients receive no immediate therapy. Long-term recovery is limited by the brain’s inherent plasticity and ability to actively remodel after injury.

The brain vasculature is undoubtedly a key component of recovery. Therapeutic strategies that are vascular protective are likely to promote better function. In addition, reparative angiogenesis in the penumbra is one of the major mechanisms contributing to recovery after stroke. Studies from our lab have revealed that administration of the angiotensin II type 1 receptor blocker (ARB), candesartan, can reduce infarct size and enhance recovery following experimental cerebral ischemia. This effect was accompanied by a higher vascular density, reduced vascular permeability and enhanced expression of VEGF-A and B in the brains of experimental animals. However, the involvement of VEGF-A and B in mediating a proangiogenic response and ameliorating acute injury after ARB treatment is not clear. The objective of this study was to assess the role of VEGF-A and B in mediating benefits of ARB treatment after focal cerebral ischemia. Once the mechanisms of angiogenesis and neurovascular protection by ARB treatment after experimental stroke are known, it will be possible to optimize the proangiogenic therapy
for promoting recovery in acute ischemic stroke. We accomplished the overall objectives of this study through the following specific aims:

**Specific Aim #1: Determine the involvement of VEGF-A and B in the proangiogenic response to ARB treatment.**

To accomplish this aim, we studied the proangiogenic time course after a single ARB treatment in an *in vivo* model of acute ischemic stroke. In addition, the effect of treatment in promoting different steps of angiogenesis was assessed *in vitro* using human cerebral microvascular endothelial cells. Under this sub-aim, we demonstrated a class effect of ARBs. Lastly, we determined the contribution of VEGF-A and B to the proangiogenic and neuroprotective effects of ARB treatment.

**Specific Aim #2: Determine the role of VEGF-B in ameliorating acute injury after ARB treatment.**

To accomplish this specific aim, brain-specific silencing of VEGF-B expression was attempted using bilateral intracerebroventricular injection of lentiviral particles containing shRNA against VEGF-B. In this animal model, we determined the benefit of ARB treatment in the acute phase after focal cerebral ischemia.

**Specific Aim #3: Optimize proangiogenic therapy with ARB treatment after focal cerebral ischemia.**

To address this specific aim, we attempted a rational sequential combination of minocycline and candesartan and assessed the long-term effect of combination treatment on recovery. In addition, we optimized combination treatment timing *in vitro* using human brain microvascular endothelial cells.
CHAPTER 3

CANDESARTAN INDUCES A PROLONGED PROANGIOGENIC EFFECT AND AUGMENTS ENDOTHELium-MEDIATED NEUROPROTECTION AFTER OXYGEN AND GLUCOSE DEPRIVATION: ROLE OF VEGF-A AND B


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Abstract

Angiogenesis is a key component of recovery after stroke. Angiotensin II receptor blocker (ARB) treatment improves neurobehavioral outcome and is associated with enhanced angiogenesis after stroke. The purpose of this study is to investigate the temporal pattern of the ARB proangiogenic effect in the ischemic brain and its association with vascular endothelial growth factors A (VEGF-A) and B (VEGF-B). Wistar rats were exposed to 90-minute middle cerebral artery occlusion (MCAO) and treated with candesartan (1 mg/kg) at reperfusion. The proangiogenic potential of the cerebrospinal fluid (CSF) was determined at 8, 24, 48 and 72 hours using in vitro Matrigel™ tube formation assay. In addition, the expression of VEGF-A and B was measured in brain homogenates using Western blotting at the same time points. A single candesartan dose induced a prolonged proangiogenic effect and a prolonged upregulation of VEGF-A and B in vivo. In the ischemic hemisphere, candesartan treatment was associated with stabilization of hypoxia inducible factor-1alpha (HIF-1α) and preservation of angiopoietin-1 (Ang-1). The effect of ARB treatment on endothelial cells was studied in vitro. Our results identified brain endothelial cells as one target for the action of ARBs and a source of the upregulated VEGF-A and B, which exerted an autocrine angiogenic response, in addition to a paracrine neuroprotective effect. Taken together, this study highlights the potential usefulness of augmenting the endogenous restorative capacity of the brain through the administration of ARBs.
Introduction

Angiogenesis has been linked to a better recovery after stroke (Krupinski et al., 1994; Manoonkitiwongsa et al., 2001; Navaratna et al., 2009). The role of angiogenesis, however, is not limited to creating a “conduit” to restore oxygen and nutrient delivery. It is now believed that angiogenesis is coupled to neurorestorative processes including neurogenesis and synaptogenesis (Beck and Plate, 2009; Teng et al., 2008; Xiong et al., 2010). It has been demonstrated that these vessels provide neurotrophic support to the newly formed neurons, thereby, improving functional recovery following an ischemic stroke (Navaratna et al., 2009).

There is extensive preclinical evidence that ARB treatment after ischemic stroke improves neurobehavioral outcome and enhances recovery (Engelhorn et al., 2004; Guan et al., 2011a; Guan et al., 2011b; Guan et al., 2011c; Hosomi et al., 2005; Ishrat et al., 2013; Kozak et al., 2008). In contrast to reports from other vascular beds where inhibition of angiogenesis has been documented (Willis et al., 2011), ARB treatment has been associated with increased vascular density in the brain (Munzenmaier and Greene, 2006). Chronic pretreatment with losartan (Forder et al., 2005) or valsartan (Li et al., 2008a) increased vascular density and reduced infarct volume after ischemic injury.

The prototypical angiogenic molecule, VEGF-A, has a well-established role in physiological as well as pathological angiogenesis, mediated mostly by VEGF receptor-2 (VEGFR2) (Ferrara, 1995; Shibuya, 2013; Zhang et al., 2000). In addition to its angiogenic potential, VEGF-A is involved in learning and memory, stimulates neurogenesis and exerts a neuroprotective effect (Cao et al., 2004; Jin et al., 2002; Jin et al., 2000; Matsuzaki et al., 2001). Stabilization of HIF-1 under ischemic conditions...
induces VEGF-A expression, possibly by binding to its promoter region (Huang et al., 1996; Levy et al., 1995; Wenger, 2002). Less is known, however, about the relatively new VEGF family member, VEGF-B. Although its proangiogenic effect is controversial, VEGF-B exerts vascular and neuroprotective effects against a wide range of apoptotic stimuli via VEGF receptor-1 (VEGFR1) (Li et al., 2009; Li et al., 2008b; Sun et al., 2006; Zhang et al., 2009). In a rodent model of focal cerebral ischemia, VEGF-B knockout animals showed 50% larger infarcts as compared to their wild type counterparts (Li et al., 2008b). Angiopoietins, another group of vascular-specific growth factors, play a prominent role in angiogenesis as well as in vessel maturation and function (Davis and Yancopoulos, 1999; Thurston et al., 2000; Thurston et al., 1999). Ang-1 expression falls dramatically after focal cerebral ischemia, resulting in a leaky blood brain barrier. Increases in Ang-1 levels, however, preserve vascular integrity after focal ischemia and/or VEGF administration (Tao et al., 2011; Thurston et al., 2000; Zhang and Chopp, 2002; Zhang et al., 2002). The use of the aforementioned angiogenic and vascular protective factors might seem to be a promising treatment for acute ischemic stroke. However, the challenging route of administration limits the benefit of such a treatment. An attempt to augment the endogenous production of these growth factors represents an attractive alternative.

The present study was designed to investigate the temporal pattern of candesartan’s proangiogenic effect as well as VEGF-A and B upregulation in response to a single treatment. In addition, we show for the first time the involvement of the vascular endothelium in candesartan’s neuroprotective effect.
**Materials and Methods**

All experimental protocols were approved by the Care of Experimental Animal Committee of Georgia Regents University/ Institutional Animal Care and Use Committee (IACUC) of the Veterans Affairs Medical Center.

**Experimental cerebral ischemia:** A total of 30 adult male Wistar rats (Charles River Breeding Company, Wilmington, MA), weighing between 280–300 grams, were subjected to 90-minute (MCAO) using intraluminal suture model, as described previously (Kozak et al., 2009). Successful MCAO was confirmed by the presence of hemiparesis prior to reperfusion. Animals with Bederson score < 3 were excluded from the study together with animals that are obtunded or unable to move. At reperfusion, animals received either saline or 1 mg/kg of candesartan (a gift from AstraZeneca) via tail vein injection and were randomized into 4 different groups (8, 24, 48 and 72-hour groups). All animals were singly housed before and after surgery, with free access to food and water. At the aforementioned time points, CSF and brain tissues were collected and snap-frozen.

**Cell culture and treatments:** The human cerebral microvascular endothelial cell line (hCMEC/D3) was a kind gift from Dr. Jason Zastre (University of Georgia, Athens, GA). Endothelial cells were grown in MCDB-131 complete medium (VEC Technologies, Rensselaer, NY). Cells were serum starved overnight prior to treatment with candesartan (0.1, 1, 10 µg/ml), or losartan potassium (0.05 µg/ml, Sigma-Aldrich, St. Louis, MO) in serum-free Eagle’s Minimum Essential Medium (EMEM- ATCC, Manassas, VA) and compared to untreated controls. Clinically relevant concentrations of candesartan (0.1 µg/ml) and losartan (0.05 µg/ml) were determined according to the published pharmacokinetic data (Schulz and Schmolldt, 2003). For neutralization experiments,
VEGF-A neutralizing antibody, VEGF-B neutralizing antibody or both (2 µg/ml- R&D systems, Minneapolis, MN) were added to the conditioned media collected from endothelial cells 30 minutes prior to neuronal treatment. Normal goat IgG (2 µg/ml- R&D systems, Minneapolis, MN) was used as a control. Mouse primary cerebral cortical neuronal cultures were isolated from embryonic day 17 fetuses (E17) of CD1 mice (Charles River Laboratories, Wilmington, MA) as described previously (Paxinos and Franklin, 2001; Pillai et al., 2008). The experimental protocol is approved by Georgia Regents University, Committee on Animal Use for Research (Pillai). Isolated neurons were cultured in Neurobasal medium (Life Technologies, Grand Island, NY), supplemented with B27 (Life Technologies, Grand Island, NY), 2 mM L-glutamine (Life Technologies, Grand Island, NY) and antibiotics (Cellgro Manassas, VA). Neurons were used for experiments between days 5 and 7 in vitro.

**Oxygen and glucose deprivation (OGD):** To mimic ischemic conditions that occur during stroke, neuronal and endothelial cells were incubated in a hypoxia chamber (Biospherix Proox Model C21, Lacona, NY) at O2 concentration <1 % and 5% CO2 at 37°C. Culture medium was changed to the glucose-free Neurobasal-A medium (Life Technologies, Grand Island, NY). After 2 hours of OGD, cells were reoxygenated and received serum-free EMEM with or without treatment.

**Tube formation:** The ability of endothelial cells to align into tube-like structures was measured using Matrigel™ tube formation assay as described previously (Kozak et al., 2009). To measure the proangiogenic potential of CSF, confluent cells were serum starved overnight, harvested and suspended in a mixture of serum-free EMEM and growth factor-reduced Matrigel™ (BD Biosciences, San Jose, CA) in a ratio of 70: 30. The mixture was
quickly transferred to a 96-well plate (100µl/well). Approximately $5 \times 10^4$ cells in a volume of 100 µl were seeded into each quadruplicate well. CSF (50 µl) was added and the mixture is allowed to solidify. Images were captured for the center of each well after 24 hours using Zeiss Axiovert microscope at an objective lens magnification of 10x. Tube-like structures were counted in a blinded fashion. Tubes were defined as endothelial cells that had aligned to form > 90% closed structures. To measure the proangiogenic effect of candesartan treatment, confluent endothelial cells were exposed to either normoxic or 2-hour OGD conditions as explained previously. The cells were then treated with candesartan or losartan. At 24 hours, cells were harvested and the same procedure was followed. Additional images were captured and analyzed at 24, 48 and 72 hours.

**Cell proliferation:** Endothelial cell proliferation was assessed by BrdU colorimetric assay kit (Roche Indianapolis, IN) according to the manufacturer’s protocol. Cells were plated at a density of $5 \times 10^3$ cells/well in 96-well plate and left overnight to attach. Cells were serum starved, exposed to normoxic or 2-hour OGD followed by treatment with different doses of candesartan. BrdU labeling solution was then added. At 24 hours, absorbance was measured at 450 nm.

**Cell migration:** Endothelial cell migration was assessed by the *in vitro* wound healing assay as described previously (Kochuparambil et al.). Images were taken at 0, 18 and 24 hours using phase contrast microscopy on an inverted microscope at an objective lens magnification of 5x. The width of the scratch was measured at 20 fixed points in each well and the average was calculated. The percent migration was presented as fold-increase relative to the control.
Western blotting: Protein expression was measured by western blotting as described previously (Guan et al., 2011c). Non-specific binding was blocked by incubating the membranes in 5% milk in TBST for 60 minutes prior to overnight incubation with primary antibodies against VEGF-A, phospho-VEGFR-1 (Tyr 1213) (Millipore, Billerica, MA), VEGF-B (Abcam, Cambridge, MA), HIF-1α, Angiopoietin-1 (SantaCruz, Dallas, TX), total VEGFR-1, phospho-VEGFR-2 (Tyr 996), total VEGFR-2, GAPDH and cleaved caspase-3 (Cell Signaling, Danvers, MA). β-Actin (Sigma-Aldrich, St. Louis, MO) were used as an endogenous loading control. Densitometric measurements were done using ImageJ software and results were represented as fold-increase relative to the control group.

Slot blot: Detection of nitrotyrosine (NY) and 4-hydorxynonenal (4-HNE) was done using slot blot technique as described previously (Abdelsaid et al., 2010). Briefly, tissue or cell lysate were immobilized on nitrocellulose membrane using Whatman Minifold slot blot system (GE Healthcare Bio-Sciences, Pittsburgh, PA). Membranes were blocked then incubated with primary anti-nitrotyrosine antibody (Millipore, Billerica, MA) or anti- 4-HNE (Alpha Diagnostic Intl, San Antonio, TX), followed by peroxidase labeled goat anti-mouse IgG (EMD Chemicals, San Diego, CA). Densitometric measurements were done using ImageJ software and results were represented as fold-increase relative to the control group.

Angiotensin II determination: Angiotensin II release into the cell culture media was quantified using angiotensin II enzyme immunoassay kit (SPI-BIO). The cell supernatant was collected 24 hours after treatment and centrifuged at 13,000 rpm for 5 minutes to get
rid of any cells. The supernatant was then used for the assay according to the manufacturer’s instructions.

**Statistical Analysis:** All statistics were carried out using NCSS 8 software. Results were expressed as mean ± standard error of the mean (±SEM). Data were statistically analyzed using two-sample unpaired Student’s t-test for single comparisons. One Way Analysis of Variance (ANOVA) was used for multiple comparisons and followed by Dunnett’s two-sided multiple comparison test. Neutralizing antibody experiments were analyzed by two-way ANOVA. Data from CSF proangiogenic effect experiment were analyzed using linear regression. Results were considered statistically significant at \( P < 0.05 \).

**Results**

*Candesartan treatment induces a prolonged proangiogenic state.* CSF collected from candesartan- treated animals induced a prolonged proangiogenic response as evident by profound increases in tube formation in brain endothelial cells. Candesartan treatment enhanced the proangiogenic potential of CSF as compared to saline treatment, an effect that was maintained throughout the measured time points. The mean slope for the candesartan group was 10- fold higher than the saline group (0.24±/-0.03 vs. 0.02±/-0.01; \( P<0.05 \)) (Fig 3.1 A- C).

*A single candesartan dose induces a prolonged upregulation of VEGF-A and B expression in vivo.* In the contralateral hemisphere, candesartan enhanced VEGF-A and B expression at all the studied time points. In comparison to saline treatment, VEGF-A expression increased by 40-50% as early as 8 hours and continued till 48 hours. At 72 hours, Candesartan increased VEGF-A expression 2-fold. In the same hemisphere, VEGF-B expression was elevated by 20-40% in the first 48 hours and by over 50% at 72 hours,
as compared to the saline group (Fig 3.2 A, C). In the ipsilateral hemisphere, however, candesartan treatment induced an early rise in the expression of both VEGF-A and B, an effect that was blunted at later time points. At 8 and 24 hours, VEGF-A expression was ~20% higher in the candesartan than the saline group. VEGF-B elevation, however, was more dramatic. Candesartan resulted in over 85% increase in VEGF-B levels at 8 hours that came down to 30% at 24 hours, as compared to the saline levels. (Fig 3.2 B, D).

**Candesartan treatment stabilizes HIF-1α, exerts an antinitrative effect and preserves Ang-1 expression in the ischemic hemisphere.** In saline-treated animals, HIF-1α stabilization was observed as early as 8 hours after focal cerebral ischemia. Ipsilateral hemisphere had 60% higher HIF-1α levels than contralateral hemisphere. Such stabilization was blunted at 24 hours after stroke. Candesartan treatment enhanced both the extent and duration of HIF-1α stabilization. HIF-1α levels were 2.3-fold higher in ipsilateral as compared to the contralateral hemisphere at 8 hours. This stabilization continued to be observed at 24 hours, where HIF-1α levels were 30% higher in ipsilateral versus contralateral hemispheres (Fig 3.3 A).

In saline-treated animals, a 67% elevation in the nitrative stress marker nitrotyrosine (NY) was detected in the ipsilateral as compared to the contralateral side. Candesartan treatment reduced NY levels back to control levels (Fig 3.3 B). Similarly, direct treatment of endothelial cells with peroxynitrite (ONOO-) increased NY level by 84%. Treatment with Candesartan reduced the NY level to 25% (Supplemental Figure 3.S1 A). Moreover, candesartan exerted an antioxidant effect following treatment with either hydrogen peroxide (H₂O₂) or ONOO-. Either treatment increased the level of 4-hydroxynonenal (4-
HNE), an oxidative stress marker, by 40%. Candesartan treatment reduced its level back to control levels. ((Supplemental Figure 3.S1 B, C).

We further investigated if candesartan treatment affects Ang-1 levels after stroke. In saline- treated group, we observed a 40% reduction of Ang-1 levels in ipsilateral as compared to the contralateral hemisphere. Candesartan preserved Ang-1 levels in ipsilateral hemisphere at 24 hours, resulting in only 8% reduction in Ang-1, as compared to the contralateral hemisphere (Fig 3.3 C).

**Candesartan enhances endothelial VEGF-A and B expression as well as their receptor activation in vitro.** Under normoxic conditions, VEGF-A expression was enhanced by 31, 36 and 25% in groups treated with 0.1, 1 and 10 µg/ml, respectively (Fig 3.4 A). The induction of VEGF-A expression was more pronounced under OGD conditions, resulting in 36, 57 and 60% increase in the same treatment groups, respectively (Fig 3.4 B). VEGF-B followed the same pattern with an increase of 30-35% under normoxic conditions in all treatment groups (Fig 3.4 C). However, in OGD, VEGF-B increased by 30, 85 and 90% in the same treatment groups, respectively (Fig 3.4 D). We studied whether the upregulated VEGF-A and B could exert an autocrine effect on endothelial cells. Therefore, we measured the phosphorylation of VEGFR1 and 2 as an indicator of VEGF-B and A functions, respectively. Candesartan enhanced phosphorylation of VEGFR1 by 25 and 65% and VEGFR2 by 30 and 65% in normoxic and OGD conditions, respectively (Fig 3.4 E- H). Taken together, candesartan enhances expression of endothelial VEGF-A and B as well as their receptor phosphorylation irrespective of the oxygenation status of the cell.
Candesartan enhances endothelial cell proliferation, migration and alignment into tube-like structures in a dose-dependent manner. At 24 hours, candesartan increased tube formation by 3, 3.5 and 4.5-fold in groups treated with 0.1, 1 and 10 µg/ml of candesartan, respectively, displaying a dose-dependent effect (Fig 3.5A). We conducted a 3-day time course under both normoxic and OGD conditions, using one candesartan dose (1 µg/ml). Candesartan treatment resulted in a 5 to 11-fold and a 3 to 4-fold increase in tube-like structures under normoxic and OGD conditions, respectively (Fig 3.5B-E). Similarly, endothelial cell proliferation increased under normoxic conditions by 13, 37 and 46% in the groups treated with 0.1, 1 and 10 µg/ml of candesartan, respectively, displaying a dose-response relationship (Fig 3.5F). Candesartan treatment under OGD conditions resulted in 27, 57 and 55% increase in BrdU incorporation in the aforementioned candesartan concentrations, respectively (Fig 3.5G). In agreement with the proliferation and tube formation data, 0.1 and 1 µg/ml of candesartan enhanced cell migration by 58% and 82%, respectively at 18 hours and by 63 and 73%, respectively at 24 hours under normoxic conditions (Fig 3.5H-L). Under OGD conditions, candesartan enhanced cell migration by 33 and 37% at 18 hours and by 38 and 57% at 24 hours in the tested concentrations, respectively (Fig 3.5M).

Losartan treatment promotes a proangiogenic state and increases VEGF-A and B expression in vitro. We conducted in vitro experiments to test whether candesartan proangiogenic effect can be exerted by other ARBs. Losartan treatment increased VEGF-A and B expression by 1.8 and 2.5-fold, 24 hours after treatment (Fig 3.6A). Phosphorylation of VEGFR1 and 2 were increased by 2.5 and 3-fold at the same time point (Fig 3.6B, C). We quantified tube formation and migration after losartan treatment.
Losartan enhanced endothelial tube formation by 2.5-fold (Fig 3.6 D-F). Similarly, endothelial cell migration increased by 45 and 70% at 18 and 24 hours following treatment (Fig 3.6 G).

**Both VEGF-A and B are required for the proangiogenic effect of candesartan.** Consistent with the controversial angiogenic role of VEGF-B, we observed a modest reduction in endothelial cell migration following its neutralization. A more pronounced reduction was observed following VEGF neutralization, consistent with its well-documented angiogenic role. Neutralization of both growth factors exerted a synergistic – rather than an additive- inhibitory effect on endothelial cell migration at 24 hours (Fig 3.7 A-G).

**Candesartan induces a paracrine neuroprotective effect via endothelial VEGF-A and B.** Conditioned media collected from candesartan-treated endothelial cells decreased neuronal death by 40%, as documented by decreased cleaved caspase-3 expression. There was a trend of increasing levels of cleaved caspase-3 with the blockade of either growth factor. The increase in cell death was not significant, however, until both growth factors were blocked simultaneously (Fig 3.8 A).

**Discussion**

The main findings of this study include a prolonged angiogenic effect, associated with enhanced VEGF-A and B expression *in vivo* and *in vitro*, in response to a single dose of candesartan treatment. These effects were associated with stabilization of HIF-1α, preservation of Ang-1 and reduction in tyrosine nitration at 24 hours. In addition, we identified endothelial cells as one of the cellular sources of the enhanced production of
VEGF-A and B that induced an autocrine angiogenic response as well as a paracrine neuroprotective effect.

While several studies have demonstrated the proangiogenic effect of ARBs, the temporal pattern and the molecular mechanisms involved in the angiogenic response remain to be fully elucidated. Here, we show that the CSF collected after a single post-stroke candesartan administration stimulated a proangiogenic response as early as 8 hours and lasted for up to 72 hours. This effect was evident by the ability of the CSF, collected at different time points, to transform brain endothelial cells into tube-like structures resembling blood vessels in vitro. Our previous work has linked candesartan’s beneficial effects to enhanced production of VEGF-A, the prototypical angiogenic molecule, as well as the relatively new VEGF isoform, VEGF-B (Guan et al., 2011c). The exact function of VEGF-B is still controversial. Although its neuroprotective and antiapoptotic functions are proven in several models (Li et al., 2008b), there is conflicting evidence regarding its angiogenic potential (Bhardwaj et al., 2003; Mould et al., 2005; Rissanen et al., 2003; Silvestre et al., 2003; Wright, 2002). In a recent study, VEGF-B was found to be upregulated in the ischemic border zone, following temporary MCAO (Xie et al., 2013). We sought to examine the temporal pattern of VEGF-A and B upregulation in both hemispheres after a single candesartan administration. Our findings show an increased expression of both isoforms in the ipsilateral as well as the contralateral hemispheres. Nevertheless, VEGF-A upregulation was more pronounced and lasted longer in the contralateral hemisphere, suggesting a role of this hemisphere in the process of recovery (Guan et al., 2011c). The increase in VEGF-B, on the other hand, was most pronounced at 8 hours in the ipsilateral hemisphere, consistent with its main role as a prosurvival factor.
We sought to investigate the upstream signaling molecules that lead to the upregulation of VEGF-A in both hemispheres. HIF-1 regulates the expression of multiple genes involved in hypoxia-related adaptations, including VEGF-A (Wenger, 2002; Youn et al., 2011). We report an enhanced HIF-1a stabilization by the treatment which might explain, at least partly, the upregulation of VEGF-A expression. In the contralateral hemisphere, however, other mechanisms could be involved in VEGF-A upregulation in response to treatment. In a retinopathy of prematurity model, candesartan treatment upregulated the expression of hemeoxygenase-1 (HO-1) and VEGF-A under normoxic conditions (El-Remessy et al., 2013). Further studies are warranted, however, to extrapolate these findings to the contralateral hemisphere in our stroke model.

Previous studies have demonstrated the inactivation of VEGF-A signaling by oxidative stress via PI3K tyrosine nitration (Abdelsaid et al., 2010; el-Remessy et al., 2005). In this study, we show an antioxidant and antinitrative effect of candesartan treatment. In vitro, candesartan decreased 4-HNE level, a marker of lipid peroxidation, after exposure to oxidizing conditions. Similarly, candesartan reduced NY levels in response to ONOO- treatment in the same model. We, thereby, postulate that candesartan, by the virtue of its antioxidant and antinitrative effects, reduces peroxynitrite production and protein nitration, respectively. In vivo, we report decreased protein nitration in the ipsilateral hemisphere 24 hours after ischemia/reperfusion, a condition characterized by massive production of oxidizing and nitrating species. This finding suggests the possibility of improved VEGF-A signaling due to reduced protein nitration.

VEGF-A is a known vascular permeability factor and high serum VEGF levels have been associated with cerebral microbleeds after acute ischemic stroke (Dassan et al., 2012). We
have previously shown a preservation of barrier function by candesartan treatment following an ischemic insult. We sought to explain the dilemma of the improved barrier function in spite of the elevated VEGF-A levels. In our current study, we report a preservation of Ang-1 expression in the stroke hemisphere 24 hours after treatment. Ang-1 exerts a barrier protective function as well as a synergistic angiogenic effect with VEGF-A after stroke (Valable et al., 2005). A diagram that depicts the proposed mechanisms of action of candesartan is shown in Fig 3.8 B.

To study the contribution of endothelial cells to the observed effect of candesartan, we measured VEGF-A and B expression in human brain microvascular endothelial cells. Our results have shown increased VEGF-A and B production in a dose-dependent fashion irrespective of the oxygenation status. Yet, the increase was more pronounced under OGD than normoxic conditions, suggesting a role of hypoxia-induced adaptations in the function of candesartan. Indeed, candesartan treatment enhanced different steps of angiogenesis in vitro in microvascular endothelial cells, including cell proliferation, migration as well as tube formation in a dose-dependent manner. Interestingly, losartan showed a proangiogenic action in vitro similar to that of candesartan treatment (Fig 3.6 A-G), in spite of the different functional inhibitory characteristics as well as different lipophilicities, among other differences between the two ARBs. However, studies using other ARBs are needed to confirm the existence of a drug class effect.

In order to assess the contribution of VEGF-A and B to the proangiogenic effects of candesartan, we neutralized either or both growth factors in vitro. Neutralization of either VEGF-A or B reduced the angiogenic potential of candesartan as evident by reduced endothelial cell migration. Nevertheless, a sharp reduction of candesartan angiogenic
effect was observed following neutralization of both growth factors simultaneously. This supports the role of VEGF-B as an angiogenic factor, either directly or indirectly via increasing cell survival. We have previously shown an enhanced proangiogenic state in vitro by candesartan treatment with or without exogenous angiotensin II treatment. Our recent work identified BDNF as a mediator of this effect secondary to unopposed AT2 receptor stimulation (Alhusban et al., 2013). The current study further supports our previous findings, since VEGF-A and BDNF expression is interrelated (Chen et al., 2005; Li et al., 2006). In this study, since no exogenous angiotensin II was added, we tested whether brain endothelial cells can produce and secrete angiotensin II locally. Angiotensin II was detectable in cell culture supernatant under normoxic and OGD conditions (Supplemental Figure 3.S2 A). There was a trend of increasing angiotensin II with OGD conditions that did not reach significance in the tested sample size. Candesartan treatment enhanced endothelial cell secretion of angiotensin II in a dose-dependent fashion (Supplemental Figure 3.S2 B), possibly due to the interrupted negative feedback system secondary to angiotensin receptor blockade. It has been previously shown that locally produced angiotensin II is regulated by an autocrine negative feedback mechanism, operating independently of the systemic renin angiotensin system (Gigante et al., 1997), lending further support to our findings.

While in vivo studies are limited to be correlative and hard to prove the causal relationship between VEGF-A and B upregulation and neurovascular protection, we attempted to examine that concept in vitro. We neutralized either or both growth factors in the conditioned media collected from endothelial cells. Neutralization of either isoform reduced the protective effect of the conditioned media on primary neurons, as evident by
the higher expression of cleaved caspase-3, a marker of apoptosis. However, neuroprotection was significantly minimized when both isoforms were blocked simultaneously. This key figure demonstrates, for the first time, that neuroprotection due to candesartan is mediated, at least partly, through augmenting the endothelial cell-secreted growth factors, VEGF-A and B. The failure of direct neuroprotection in stroke therapy has promoted a more holistic approach to treatment, taking into consideration the communication between different brain cell types and especially considering endothelial cells as a “neuroprotective organ” (Guo et al., 2008). In agreement, a recent study has demonstrated the involvement of VEGF-A in endothelium-mediated neuroprotection after stroke (Ishikawa et al., 2013).

In summary, our findings provide new insights on the benefits of candesartan and that they are mediated through orchestrated actions of multiple players, including endothelial VEGF-A and B. The study further points to the potential usefulness of augmenting the endogenous reparative capacity of the brain for the management of acute ischemic stroke.

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Authorship Contributions

*Participated in research design*: Soliman, El-Remessy, Pillai, Somanath, Ergul, and Fagan.

*Conducted experiments*: Soliman and Ishrat.

*Contributed new reagent or analytic tools*: Pillai.

*Performed data analysis*: Soliman.

*Wrote or contributed to the writing of the manuscript*: Soliman, El-Remessy, and Fagan.

Disclosures

The authors have nothing to disclose. The contents do not represent the views of the funding agencies Department of Veterans Affairs or the United States government.

Footnotes:

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Bibliography


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**Figure 3.8 B: Schematic representation of the mechanisms involved in candesartan proangiogenic, vascular, and neuroprotective effects.** Candesartan treatment induces neuroprotective and proangiogenic effects via an integrated action of VEGF-A and B. In addition, candesartan’s antioxidant and antinitrative actions suggest an improved VEGF-A signaling. Preservation of Ang-1 might contribute to the synergistic angiogenic response, while exerting a simultaneous anti-permeabilizing effect, hence the preservation of barrier function after candesartan treatment.
Figure 3.8 A: Candesartan-induced VEGF-A and B upregulation exerts a paracrine neuroprotective effect. B: Schematic representation of the mechanisms involved in candesartan proangiogenic, vascular, and neuroprotective effects.
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Supplemental Figure 3.S2: OGD conditions and candesartan treatment enhance brain endothelial angiotensin II release in vitro

A: Determination of angiotensin II in brain endothelial cell supernatant under normoxic and OGD conditions (2-hour OGD and 24-hour reoxygenation). There was a trend of increasing angiotensin II release but the increase was not significant with our sample size. B: Determination of angiotensin II in brain endothelial cell supernatant after 2-hour OGD followed by 24 hours with or without candesartan (0.1, 1 and 10 µg/ml) treatment (n= 3, *P<0.05).
Supplemental Figure 3.S2: OGD conditions and candesartan treatment enhance brain endothelial angiotensin II release in vitro.
CHAPTER 4

SILENCING VEGF-B DIMINISHES THE NEUROPROTECTIVE EFFECT OF CANDESARTAN TREATMENT AFTER EXPERIMENTAL FOCAL CEREBRAL ISCHEMIA

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Abstract

**Objective**- The pro-survival effect of VEGF-B has been documented in different in vivo and in vitro models. We have previously shown an enhanced VEGF-B expression in response to candesartan treatment after focal cerebral ischemia. In this study, we aim to silence VEGF-B expression to assess its contribution to candesartan-induced recovery.

**Methods**- Silencing VEGF-B expression was achieved by bilateral intracerebroventricular injection of lentiviral particles containing short hairpin RNA against VEGF-B. Middle cerebral artery was occluded for 90 minutes. At reperfusion, animals received either intravenous saline or candesartan. Neurobehavioral outcome was assessed 24, 48 and 72 hours after the insult and infarct volume was measured at 72 hours. **Results**- Candesartan treatment improved neurobehavioral and motor functions and decreased infarct volume only in the presence of VEGF-B. Silencing VEGF-B expression decreased candesartan’s neuroprotective effect. **Conclusion**- VEGF-B is a mediator of candesartan neuroprotective function after focal cerebral ischemia and represents a druggable target to improve stroke outcome.
Introduction

Stroke is the fourth cause of death and a leading cause of adult disability. More than 700,000 stroke cases occur every year (Go et al., 2014). With the very limited treatment options available, it is of utmost importance to develop new therapies for acute ischemic stroke management.

Candesartan is an antihypertensive medication of the angiotensin II receptor blocker (ARB) family. Preclinical studies have shown a beneficial role of ARBs, including candesartan, when administered after the incidence of acute ischemic stroke (Brdon et al., 2007; Engelhorn et al., 2004; Kozak et al., 2009). The effect of treatment is, however, complicated by its blood pressure lowering effect. Recently, clinical trials have demonstrated a lack of benefit by acute blood pressure reduction after stroke (He et al., 2014; Sandset et al., 2011). However, mediators of recovery by ARBs remain to be a druggable target. It is, therefore, important to identify the mechanisms of neurovascular protection by ARBs in order to develop new therapies for acute ischemic stroke.

Vascular endothelial growth factor- B (VEGF-B) is a relatively new member of the VEGF family. The exact role played by VEGF-B remained elusive for a long time. VEGF-B null mice were healthy and fertile, suggesting a redundant role of this molecule (Li et al., 2009). Further studies, however, demonstrated a potent pro-survival effect of VEGF-B. It was found to be neurovascular protective against a wide variety of apoptotic stimuli (Li et al., 2008b; Zhang et al., 2009). VEGF-B administration after stroke decreased infarct size (Li et al., 2008b) and VEGF-B knockout animals exhibited larger infarcts as compared to their wild type counterparts (Sun et al., 2004). Moreover, VEGF-B expression was upregulated in the ischemic border zone after 90-minute focal cerebral
ischemia, further supporting its involvement in the endogenous protective/ reparative response to ischemic insult (Xie et al., 2013).

We have previously shown an enhanced VEGF-B production in brain tissue and brain endothelial cells by candesartan treatment (Guan et al., 2011c; Soliman et al., 2014). In this study, we demonstrate a causal relationship between VEGF-B upregulation and improved neurobehavioral outcome after candesartan treatment. We hypothesize that silencing VEGF-B diminishes candesartan’s neuroprotective effects after focal cerebral ischemic injury.

Materials and Methods

All experimental protocols were approved by the Care of Experimental Animal Committee of Georgia Regents University/ Institutional Animal Care and Use Committee (IACUC) of the Veterans Affairs Medical Center.

**Intracerebroventricular Injections:** Adult male Wistar rats (Charles River Breeding Company, Wilmington, MA, USA), weighing 180-200 grams. To inject lentiviral particles into the lateral ventricles, rats were anaesthetized with 2-5% isoflurane inhalation and placed in a stereotaxic device. A midline incision was made to expose the skull and a hole was drilled at either side of the brain using the coordinates: anterioposterior (AP)= 0.8mm; mediolateral (ML)= +/- 1.2 mm; dorsoventral (DV)= 3.8 mm with respect to the bregma. Lentiviral particles containing VEGF-B shRNA or a non-targeting control were injected using a Hamilton syringe (5X10^6 transduction units/ hemisphere at a rate of 1 µl/ min). The microsyringe was removed slowly over 5 minutes. The incision was sutured and animals were allowed to recover on a 37ºC heated pad.
Experimental Cerebral Ischemia and Candesartan Treatment: Two weeks after ICV injection of lentiviral particles, animals weighing between 280–300 grams were subjected to 90-minute middle cerebral artery occlusion (MCAO) using intraluminal suture model as described previously (Guan et al., 2011c). Briefly, animals were anesthetized using 2-5 % isoflurane inhalation. Temporary middle cerebral artery occlusion (MCAO) was achieved using 4–0 silicon-coated nylon suture (Doccol, Sharon, MA), advanced into the internal carotid artery to block the origin of the middle cerebral artery. After 90 minutes, animals were re-anaesthetized and sutures were removed to allow reperfusion of ischemic brain areas. At reperfusion, animals received intravenous injection of either saline or candesartan (1 mg/ Kg). Candesartan was a kind gift from AstraZeneca Pharmaceuticals (Wilmington, DE). All animals were singly housed before and after surgery, with free access to food and water.

Neurobehavioral Testing: Neurobehavioral assessment was done 1, 2 and 3 days after the onset of MCAO in a blinded fashion using modified Bederson score, beam walk, paw grasp and grip strength tests.

Modified Bederson score: Animals were assigned a score from 0-3, with lower scores indicating better performance. The animal was given one point for each of the following: forelimb flexion; diminished resistance to lateral push; and contralateral circling (Bederson et al., 1986).

Beam walk: Animals were placed on a beam (60 cm long and 4.5 cm wide) for 60 seconds and assigned a score from 0 to 6 as follows: balances on the beam with a steady posture = 0; grasps side of the beam = 1; hugs the beam with 1 limb falling = 2; hugs the beam with 2 limbs falling =3; falls off the beam within 40 to 60 seconds = 4; falls off the beam within
20 to 40 seconds = 5; falls off the beam in less than 20 seconds = 6 (Watanabe et al., 2004).

**Paw grasp:** Animal were suspended by tail and allowed to grasp a vertical pole. Animals are assigned a score from 1-3 as follows: grasping by both forelimbs= 1; grasping with only one forelimb= 2; failure to grasp= 3 (Machado et al., 2009).

**Grip strength:** Grip Strength Meter (Columbus instruments, Columbus, OH) was used to measure baseline and post-stroke neuromuscular function. Three readings were recorded for each animal and averaged. Results are expressed as percentage of baseline grip strength.

**Assessment of Infarct Volume:** On day 3 post-stroke, animals were deeply anaesthetized with ketamine/ xylazine mixture (85% and 15%, respectively) and transcardially perfused with ice cold PBS. Animals were then decapitated and their brains collected and sliced into seven 2-mm thick coronal sections. Sections were stained with 2% TTC solution (2,3,5-triphenyltetrazolium chloride- Sigma-Aldrich, St. Louis, MO) for 15–20 min and scanned. Infarction areas were measured blindly using Image J software, corrected for edema and expressed as percentage of the contralesional hemisphere using the formula: 100 X [contralateral – (ipsilateral – infarct)]/contralateral.

**Western blotting:** Brain slices B- E were collected and snap frozen. Brain tissue was then homogenized and protein expression was measured as described previously (Guan et al., 2011c). Non-specific binding was blocked by incubating the membranes in 5% milk in TBST for 60 minutes prior to overnight incubation with primary antibody against VEGF-B (Abcam, Cambridge, MA). β-Actin (Sigma-Aldrich, St. Louis, MO) was used as an
endogenous loading control. Densitometric measurements were done using ImageJ software (NIH).

**Statistical Analysis:** All statistics were carried out using NCSS2007 software. Results are expressed as mean ± standard error of the mean (±SEM). Data were statistically analyzed using One Way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparison post-hoc test. A value of $P< 0.05$ was considered statistically significant.

**Results**

**Diminished VEGF-B expression in both hemispheres 17 days after bilateral ICV injection of VEGF-B shRNA lentiviral particles.**

To knock down VEGF-B protein expression, bilateral intracerebroventricular injection of lentiviral particles encoding short hairpin RNA against VEGF-B (VEGF-B shRNA). As shown in **Fig 4.1**, VEGF-B expression was reduced by 45 and 59% in contralateral and ipsilateral hemispheres, respectively, 17 days after injection of VEGF-B shRNA as compared to the injection of the non-targeting control (NTC). Candesartan treatment enhanced VEGF-B expression by 28 and 53% in the contralateral and ipsilateral hemispheres, respectively, only in the NTC group. In the VEGF-B knockdown group (KD), candesartan treatment was still capable of enhancing VEGF-B expression, especially in the ipsilateral hemisphere. However, VEGF-B level in both hemispheres was 50% less in the KD-candesartan group as compared to their respective hemispheres in the NTC-candesartan group.
Silencing VEGF-B expression does not upregulate compensatory VEGF expression or signaling.

We further investigated if VEGF-B silencing elicits a compensatory upregulation of other growth factors such as VEGF. We, therefore, determined the expression of VEGF as well as the phosphorylation of its receptor, VEGF receptor-2, 17 days after intracerebroventricular injection of either NTC or VEGF-B shRNA lentiviral particles. VEGF expression was significantly increased in the NTC group by candesartan treatment in both hemispheres (Figure 4.2). This response was, however, abolished by VEGF-B silencing in the KD group. In addition, there was a trend of decreasing VEGF expression in the saline-treated KD group as compared to their NTC counterparts. Similarly, VEGF receptor-2 phosphorylation was enhanced by candesartan treatment in both hemispheres in the NTC group. Such a response was abolished after silencing of VEGF-B in the KD group.

Silencing VEGF-B decreased candesartan-induced improvement of neurobehavioral function after experimental stroke.

The role played by VEGF-B in mediating candesartan’s neuroprotective effect was determined by measuring neurobehavioral outcome 1, 2 and 3 days after MCAO and infarct size after 3 days. Bederson score shows an improved performance as early as 24 hours in the NTC-candesartan animals as compared to their saline-treated counterparts. In the knockdown group, however, candesartan treatment had a tendency to slightly improve neurobehavioral outcome on days 2 and 3 post-stroke. The effect, however, was not significantly different from the KD-saline group (Fig 4.3 A). Beam walk and paw grasp tests followed the same pattern (Fig 4.3 B, C). Candesartan improved animal performance.
in the NTC group but not in the KD group. Although there was a tendency to improve grip strength by candesartan treatment in the first 48 hours in both NTC and KD groups, the improvement was not significant until 72 hours and was observed in the NTC-candesartan group only (Fig 4.3 D). It is worth mentioning that saline-treated animals in both NTC and KD groups showed comparable neurobehavioral outcome throughout all the measured time points.

Reduction of infarct volume by candesartan treatment is mediated by VEGF-B:

Infarct size analysis 3 days after MCAO shows 57% reduction in stroke volume by candesartan treatment in the NTC group only (Fig 4.4 A, B). Knocking down VEGF-B diminished candesartan’s ability to reduce infarct size. Similar to neurobehavioral tests, no increase in infarct size was observed at 72 hours between the saline-treated NTC and KD.

Discussion

Although a full understanding of the biological functions of VEGF-B is far from complete, it has been recognized as a potent apoptosis inhibitor in different organs and animal models (Li et al., 2008b). We have previously shown an enhanced expression of VEGF-B in response to candesartan treatment in the brain tissue and in endothelial cells (Guan et al., 2011c; Soliman et al., 2014). Neutralization of VEGF-B in endothelial cell conditioned media had a tendency to decrease its neuroprotective effect, as demonstrated by increased expression of the apoptotic marker, cleaved caspase-3, after 2-hour OGD (Soliman et al., 2014). In this study, we demonstrate the role played by VEGF-B in mediating candesartan’s neuroprotective effect in an acute ischemic stroke animal model.

The findings of this study include an improved functional recovery after candesartan treatment in the animals expressing VEGF-B. Silencing VEGF-B significantly reduced
candesartan’s effect on neurobehavioral recovery. Similarly, infarct size was reduced in response to candesartan treatment, an effect that was abrogated in the VEGF-B knockdown animals. The results of this study demonstrate the contribution of VEGF-B to the protective effects of candesartan treatment in experimental ischemic stroke.

Previous studies have shown the neuroprotective effect of VEGF-B in a stroke model. Recombinant human VEGF-B protein treatment reduced infarct size by 32% in wild-type animals (Li et al., 2008b). VEGF-B knockout animals demonstrated 50% larger infarcts in a permanent occlusion model (Sun et al., 2004). Nonetheless, we have not observed worsening of the functional outcome or infarct volume in the saline-treated KD animals as compared to their NTC counterparts. A possible explanation of such a discrepancy is the use of VEGF-B knockout model versus silencing using lentiviral particle injection, which did not abolish VEGF-B expression completely. Another plausible explanation is the experimental limitation. Due to the tight scale of outcome measures, further worsening by knocking down VEGF-B could not be observed. Our results, however, suggest a beneficial but expendable role of VEGF-B in recovery after MCAO.

We have previously demonstrated the involvement of other growth factors in mediating candesartan actions after an ischemic insult. Brain-derived neurotrophic factor (BDNF) (Alhusban et al., 2013) and vascular endothelial growth factor-A (VEGF-A) (Guan et al., 2011c; Soliman et al., 2014) were upregulated in response to candesartan treatment. Neutralization of either growth factor significantly reduced candesartan-mediated proangiogenic effect in vitro (Alhusban et al., 2013; Soliman et al., 2014). Since angiogenesis and neurorestorative processes are coupled in the ischemic brain (Xiong et
al., 2010), we expect the involvement of BDNF and VEGF in candesartan-mediated neurobehavioral recovery after experimental stroke. We, therefore, do not exclude the involvement of other growth factors besides VEGF-B in mediating neurobehavioral recovery after candesartan treatment. Current evidence suggests that candesartan works by enhancing endogenous reparative mechanisms in the brain and the benefits of treatment are possibly mediated through an orchestrated action of several growth factors.

We have not observed a compensatory upregulation of VEGF expression in response to silencing VEGF-B. Interestingly, candesartan failed to upregulate VEGF expression or to enhance phosphorylation of VEGF receptor-2 in the VEGF-B knockdown animals, suggesting a cross-talk between the two growth factors. Our results are in harmony with the recent findings on VEGF-B that demonstrate an enhanced VEGF receptor-2 signaling in transgenic mice overexpressing VEGF-B.

In conclusion, our results suggest an important role played by VEGF-B in mediating candesartan’s neuroprotective effect after stroke. Identifying growth factors that mediate recovery after ischemic stroke presents possible targets for stroke therapeutics.

**Highlights**

- Silencing VEGF-B reduces candesartan-induced functional improvement after stroke.
- Silencing VEGF-B reduces candesartan-induced infarct size reduction.
- VEGF-B is a mediator of candesartan’s neuroprotective effect.

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Disclosures

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Bibliography


neurologic outcome and reduces infarct volume after transient focal cerebral
ischemia in rats, Journal of cerebral blood flow and metabolism: official journal of
the International Society of Cerebral Blood Flow and Metabolism 24 (2004) 1205-
1213.
17. Y. Xiong, A. Mahmood, M. Chopp, Angiogenesis, neurogenesis and brain
recovery of function following injury, Curr Opin Investig Drugs 11 (2010) 298-
308.
18. F. Zhang, Z. Tang, X. Hou, J. Lennartsson, Y. Li, A.W. Koch, P. Scotney, C. Lee,
P. Arjunan, L. Dong, A. Kumar, T.T. Rissanen, B. Wang, N. Nagai, P. Fons, R.
Fariss, Y. Zhang, E. Wawrousek, G. Tansey, J. Raber, G.H. Fong, H. Ding, D.A.
Greenberg, K.G. Becker, J.M. Herbert, A. Nash, S. Yla-Herttuala, Y. Cao, R.J.
Watts, X. Li, VEGF-B is dispensable for blood vessel growth but critical for their
survival, and VEGF-B targeting inhibits pathological angiogenesis, Proc Natl Acad
**Figure 4.1: Assessment of VEGF-B expression 17 days after bilateral intracerebroventricular injection of VEGF-B shRNA lentiviral particles.** Quantification of VEGF-B expression in the contralateral and ipsilateral hemispheres 17 days after bilateral ICV injection of lentiviral particles containing non targeting control (NTC) or VEGF-B shRNA to knockdown protein expression (KD). Animals were exposed, 72 hours before sacrifice, to 90-minute MCAO and received saline or candesartan (1 mg/ Kg) at reperfusion (n=4/ group, * significantly different from NTC-saline in the same hemisphere, # significantly different from NTC-cand in the same hemisphere, P<0.05).
Figure 4.1: Assessment of VEGF-B expression 17 days after bilateral intracerebroventricular injection of VEGF-B shRNA lentiviral particles.
Figure 4.2: Determination of VEGF expression and VEGF receptor-2 phosphorylation 17 days after bilateral intracerebroventricular injection of VEGF-B shRNA lentiviral particles. Quantification of VEGF expression (A) and VEGF receptor-2 phosphorylation (B) in the contralateral and ipsilateral hemispheres 17 days after bilateral ICV injection of lentiviral particles containing non targeting control (NTC) or VEGF-B shRNA lentiviral particles. Animals were exposed to 90-minute MCAO and received saline or candesartan (1 mg/ Kg) at reperfusion. At 72 hours post MCAO, animals were sacrificed and brain tissue was collected. (n=4/ group, ψ significantly different from NTC-candesartan in the same hemisphere, P<0.05).
Figure 4.2: Determination of VEGF expression and VEGF receptor-2 phosphorylation 17 days after bilateral intracerebroventricular injection of VEGF-B shRNA lentiviral particles.
Figure 4.3: Silencing VEGF-B diminishes candesartan-induced neuroprotective effects after focal cerebral ischemia. A-D: Assessment of neurobehavioral and motor functions 1, 2 and 3 days after MCAO using modified Bederson score (A), beam balance (B), paw grasp (C) and grip strength tests (D) in saline and candesartan (1 mg/Kg)-treated animals. * significantly different from all three groups, ð significantly different from saline-treated groups only, \( P<0.05 \)
Figure 4.3: Silencing VEGF-B diminishes candesartan-induced neuroprotective effects after focal cerebral ischemia.
Figure 4.3: Silencing VEGF-B diminishes candesartan-induced neuroprotective effects after focal cerebral ischemia.
Figure 4.4: Silencing VEGF-B abrogates candesartan-induced reduction of lesion volume. A: Representative images of TTC-stained coronal sections 72 hours after MCAO and treatment with saline or 1 mg/Kg candesartan at reperfusion. B: Quantification of infarct volume 72 hours after MCAO and treatment with saline or candesartan (n=4-5/group, * significantly different from NTC-saline, P<0.05)
Figure 4.4: Silencing VEGF-B abrogates candesartan-induced reduction of lesion volume.
CHAPTER 5

SEQUENTIAL THERAPY WITH MINOCYCLINE AND CANDESARTAN IMPROVES LONG TERM RECOVERY AFTER EXPERIMENTAL STROKE

Abstract

Background- Minocycline and candesartan have both shown promise as candidate therapeutics in ischemic stroke, with multiple, and somewhat contrasting, molecular mechanisms. Minocycline is an anti-inflammatory, antioxidant and anti-apoptotic agent and a known inhibitor of matrix metalloproteinases (MMPs). Yet, minocycline exerts antiangiogenic effects both in vivo and in vitro. Candesartan promotes angiogenesis and activates MMPs. Aligning these therapies with the dynamic processes of injury and repair after ischemia is likely to improve success of treatment. Objective- In this study, we propose sequential administration of minocycline and candesartan as a potential treatment for ischemic stroke. We hypothesize that early minocycline administration, to reduce neurovascular injury, followed by 7-day treatment with candesartan, to stimulate angiogenesis, yields a better outcome than either alone. Secondly, opposing actions of both agents on angiogenesis, when administered simultaneously, will reduce the benefit of candesartan treatment. Methods- Wistar rats subjected to 90-minute middle cerebral artery occlusion (MCAO) were randomized into 4 groups: saline, candesartan, minocycline and sequential combination of minocycline and candesartan. Neurobehavioral tests were performed 1, 3, 7 and 14 days after stroke. Brain tissue was collected on day 14 for assessment of infarct size, vascular density and vascular endothelial growth factor (VEGF) expression. VEGF expression was measured in vitro after simultaneous and sequential treatment regimens. In vitro angiogenesis was assessed using endothelial cell proliferation, migration and tube formation assays. Results- Sequential minocycline and candesartan treatment improved neurobehavioral outcome and reduced infarct volume as compared to saline or individual treatments. When added after minocycline, candesartan...
was effective at stimulating angiogenesis. Simultaneous treatment, however, abolished candesartan-induced VEGF upregulation and decreased its proangiogenic effect in vitro.

**Conclusion** - Sequential therapy with minocycline and candesartan improves long term recovery more than either alone. Sequential, rather than simultaneous treatment maintains candesartan’s proangiogenic potential.
Introduction

Stroke is one of the most common and undertreated conditions worldwide. Each year, more than 700,000 stroke cases occur in the United States alone, resulting in significant disability and mortality as well as an estimated cost of $36 billion (Go et al., 2014). Tissue plasminogen activator (tPA) remains the only FDA-approved pharmacologic/biologic treatment option (Ramaiah and Yan, 2013). With only 2-3% of patients receiving tPA nationwide (Ishrat et al., 2012), there is an urgent need to develop new therapies for acute ischemic stroke.

Cerebral ischemia promotes an angiogenic response in the brain (Navaratna et al., 2009) that is considered to be a key component of recovery (Chopp et al., 2007; Xiong et al., 2010). Angiogenic genes are upregulated within minutes of the incidence of an ischemic insult. Endothelial cell proliferation starts as early as 12 hours and continues for several weeks in a mouse model of focal cerebral ischemia (Hayashi et al., 2003). In humans, evidence of active angiogenesis was detected 3-4 days after stroke (Krupinski et al., 1993, 1994). The role played by angiogenesis in mediating recovery goes beyond creating a shunt to reperfuse ischemic tissues (Brumm and Carmichael, 2012). Newly formed vessels help clean up the ischemic site in preparation for neurovascular remodeling (Manoonkitiwongsa et al., 2001). In addition, blood vessels upregulate neuron guidance cues to recruit neural stem cells to the site of injury (Ohab et al., 2006). Due to the tight relationship between angiogenesis and neurogenesis in addition to the dramatic failure of neuroprotectants in stroke clinical trials, therapies that aim at promoting angiogenesis are now being attempted in experimental models of focal cerebral ischemia (Guo et al., 2009; Simao et al., 2012; Wang et al., 2004).
Decades of preclinical research have led to improved understanding of the timing and molecular mediators of injury and repair after ischemic brain insult (Fagan et al., 2004; Hermann and Zechariah, 2009). It has been acknowledged that the mediators of initial injury (eg. Matrix metalloproteinases- MMPs and vascular endothelial growth factor - VEGF) contribute to the repair and remodeling process in the long-term (Hermann and Zechariah, 2009). Sequential therapy, although extensively studied in oncology (Westhoff et al., 2013) and gastroenterology (De Francesco et al., 2014), has been understudied in stroke.

Minocycline, a tetracycline antibiotic with anti-inflammatory, anti-apoptotic, antioxidant and MMP-inhibitory effects, has shown promising results in stroke preclinical studies. Our group, as well as others, have reported reduced infarct size and intracerebral hemorrhage, together with improved neurobehavioral outcome in response to minocycline treatment [reviewed by (Liao et al., 2013)]. Minocycline treatment, however, suppressed in vivo and in vitro angiogenesis via MMP-dependent and independent mechanisms (Gilbertson-Beadling et al., 1995; Jung et al., 2014; Li et al., 2014; Tamargo et al., 1991; Yao et al., 2004). Therefore, combining the benefits of minocycline with a proangiogenic approach represents an attractive therapeutic option.

Candesartan, an angiotensin II type-1 receptor blocker, has long been studied in experimental stroke settings. Candesartan treatment improved neurobehavioral outcome, reduced infarct size (Brdon et al., 2007; Engelhorn et al., 2004; Guan et al., 2011c; Kozak et al., 2009) and increased cerebrovascular density, an effect that was associated with enhanced MMP activity, especially MMP-2 (Kozak et al., 2009). Clinical utility is, however, complicated by candesartan’s blood pressure lowering effect. Recent studies
have shown a lack of benefit, if not a deleterious effect, of blood pressure lowering in the acute phase after ischemic stroke (He et al., 2014; Sandset et al., 2011) and current guidelines advise against blood pressure lowering within the first 24 hours (Jauch et al., 2013). In this study, we propose a combination of early minocycline treatment followed by 7-day low-dose candesartan treatment to improve functional recovery after focal cerebral ischemia. Due to opposite actions of minocycline and candesartan on angiogenesis, we employ a sequential approach designed to preserve candesartan’s proangiogenic effect.

**Materials and Methods**

All experimental protocols were approved by the Care of Experimental Animal Committee of Georgia Regents University/Institutional Animal Care and Use Committee (IACUC) of the Veterans Affairs Medical Center.

**Experimental Cerebral Ischemia and Treatments:** Adult (9-10 week old) male Wistar rats (Charles River Breeding Company, Wilmington, MA, USA), weighing between 280–300 grams, were subjected to 90-minute middle cerebral artery occlusion (MCAO) using intraluminal suture model as described previously (Guan et al., 2011c). Briefly, animals were anesthetized using 2-5% isoflurane inhalation. Temporary middle cerebral artery occlusion (MCAO) was achieved using 4–0 silicon-coated nylon suture (Doccol, Sharon, MA), advanced into the internal carotid artery to block the origin of the middle cerebral artery. After 90 minutes, animals were re-anesthetized and sutures were removed to allow reperfusion of ischemic brain areas. Animals were randomly divided into 4 treatment groups: saline, minocycline only (Mino), candesartan only (Cand) and the sequential combination of minocycline and candesartan (Sequential Comb) and received treatments
as shown in Table 5.1. The dose of minocycline (20 mg/kg) was shown to exert neuroprotective effects in vivo (Matsukawa et al., 2009) and the dose of candesartan (0.3 mg/kg) was determined to be subhypotensive in our pilot experiments (unpublished). The timing of intervention was controlled such that, in all treatment groups, the protective therapy was initiated at reperfusion. Candesartan was a kind gift from AstraZeneca Pharmaceuticals (Wilmington, DE). Minocycline was purchased from Sigma-Aldrich (St. Louis, MO). All animals were singly housed before and after surgery, with free access to food and water.

**Neurobehavioral Testing:** Neurobehavioral assessment was done 1, 3, 7 and 14 days after the onset of MCAO in a blinded fashion using modified Bederson score, beam walk, rotarod performance and grip strength tests.

*Modified Bederson score:* Animals were assigned a score from 0-3, with lower scores indicating better performance. The animal was given one point for each of the following: forelimb flexion; diminished resistance to lateral push; and contralateral circling (Bederson et al., 1986).

*Beam walk:* Animals were placed on a beam (60 cm long and 4.5 cm wide) for 60 seconds and assigned a score from 0 to 6 as follows: balances on the beam with a steady posture = 0; grasps side of the beam = 1; hugs the beam with 1 limb falling = 2; hugs the beam with 2 limbs falling = 3; falls off the beam within 40 to 60 seconds = 4; falls off the beam within 20 to 40 seconds = 5; falls off the beam in less than 20 seconds = 6 (Watanabe et al., 2004).
**Rotarod test:** Three days prior to MCAO, animals were trained to stay on the accelerating rod as follows: On day 1, animals were habituated to stay on the rod rotating at a fixed speed of 2 rpm for 5 minutes. On day 2, animals perform three 5-minute running sessions and allowed to rest for 10 minutes between sessions. Animals run at a basal speed of 2 rpm that was accelerated by 1 rpm every 10 seconds to a final speed of 15 rpm. On day 3, animals follow the same training schedule as day 2 but reaching a final speed of 30 rpm. Baseline performance was recorded before MCAO surgery. Animals were then tested 1, 3, 7 and 14 days after MCAO. Maximum speed reached before the animal falls off the rod was recorded for the 3 sessions and averaged. Rotarod performance was calculated as percentage of baseline performance (Chen et al., 2001).

**Grip strength:** Grip Strength Meter (Columbus instruments, Columbus, OH) was used to measure baseline and post-stroke neuromuscular function. Three readings were recorded for each animal and averaged. Results are expressed as percentage of baseline grip strength.

**Assessment of Infarct Volume and Immunohistochemistry:** On day 14 post-stroke, animals were deeply anaesthetized with ketamine/ xylazine mixture (85% and 15%, respectively) and transcardially perfused with ice cold PBS followed by 10% formalin (Thermofisher Scientific, Waltham, CA). Animals were then decapitated and their brain tissue collected and stored in 10% formalin overnight prior to freezing and cutting into 12 µm sections. Sections were stained with cresyl violet and infarction areas were measured blindly using Image J software, corrected for edema, and expressed as percentage of the contralateral hemisphere using the formula: 100 X [contralateral – (ipsilateral – infarct)]/contralateral.
Frozen 10 µm sections were immunostained for laminin (Dakocytomation, Carpinteria, CA) or Ki67 (Abcam, Cambridge, MA) and CD-31 (BD Pharmingen, San Diego, CA). Briefly, brain sections were incubated with primary antibodies overnight at 4°C at the dilution of 1/50, followed by 2-hour incubation with 1/300 fluorescent secondary antibodies. Slides were then coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and viewed using Zeiss Axio Observer Z1 fluorescent microscope. Micrographs were taken at magnification 20x from four different fields in the ischemic cortex, ischemic border zone and contralateral hemisphere. Micrographs were quantified in a blinded fashion using ImageJ software (NIH).

**MMP Zymography:** MMP gelatinolytic activity was measured using zymography, as described previously (Machado et al., 2006). Briefly, volume containing 100 µg total protein was loaded into 10% zymogram gel and electrophoretically separated under non-reducing conditions. The gel was then incubated for 48 hours with Zymogram Development Buffer at 37°C. Zymogram gel was stained with Coomassie Blue R-250 (Bio-Rad, Hercules, CA) and destained with Destain Solution (Bio-Rad, Hercules, CA). The gelatinolytic bands were analyzed with ImageJ software (NIH).

**Human cerebral microvascular endothelial cell line (hCMECs):** Cells were provided by Dr. Jason Zastre (University of Georgia, Athens, GA). The cells were grown in MCDB-131 complete medium (VEC technologies, Rensselaer, NY). Before treatments, endothelial cells were serum-starved overnight in serum-free EMEM (ATCC, Manassas, VA).

**Oxygen and Glucose Deprivation (OGD) and treatments:** To mimic ischemic conditions that occur during stroke, hCMECs were incubated in a hypoxia chamber (Bioshperix
Proox Model C21, Lacona, NY) at 0.1 % O2 and 5% CO2 at 37°C and switched to glucose-free Neurobasal-A medium (Life Technologies, Carlsbad, CA). After 2 hours, cells were returned to normoxic conditions and switched to serum-free medium with or without different treatments. For functional assays, cells were treated with either minocycline (6 µg/ ml) or candesartan (0.1, 1 and 10 µg/ ml) for individual treatment groups, or a combination of minocycline and candesartan at the same concentrations. For VEGF determination, cells were treated with minocycline (6 µg/ml) at reoxygenation for 6 hours, followed by 24-hour candesartan treatment (10 µg/ml) with or without a second dose of candesartan (10 µg/ ml) administered 6 hours prior to cell harvesting in the sequential treatment groups. Alternatively, cells were treated with both minocycline (6 µg/ml) and candesartan (10 µg/ml) simultaneously at reoxygenation in the co-treatment group. As experimental controls, cells were treated with either minocycline or candesartan (single or double dosing) for the same treatment duration. The tested concentration of minocycline (6 µg/ml) is the peak serum concentration achieved in humans after intravenous administration of the FDA –approved dose of 200 mg (Saivin and Houin, 1988). Candesartan concentrations have been shown to exert an angiogenic effect in vitro on human brain microvascular endothelial cells (Soliman et al., 2014).

**Western blotting:** Brain tissue was collected from a separate set of animals 14 days after the onset of MCAO. From each hemisphere, slices B-E were collected and snap frozen. Brain tissue was then homogenized and protein expression was measured as described previously (Guan et al., 2011c). Non-specific binding was blocked by incubating the membranes in 5% milk in TBST for 60 minutes prior to overnight incubation with primary antibody against VEGF (Millipore, Billerica, MA). β-Actin (Sigma-Aldrich, St. Louis,
MO) was used as an endogenous loading control. Densitometric measurements were done using ImageJ software (NIH). To assess VEGF expression in vitro, human brain endothelial cells were harvested in RIPA buffer. Protein expression and electrophoresis were carried out as described.

**Cell Proliferation:** In vitro, endothelial cell proliferation was assessed by BrdU colorimetric assay kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s protocol. Cells were plated in density 5000 cells/well in 96-well plate and kept overnight in complete medium. The cells were serum starved, exposed to 2-hour OGD. Upon reoxygenation, cells were treated with candesartan (0.1, 1 or 10 µg/ml), minocycline (6 µg/ml) or the combination of minocycline and different concentrations of candesartan. The BrdU labeling solution was then added. At 24 hours, absorbance was measured at 450 nm.

**Cell Migration:** Cell migration was assessed by the in vitro wound healing assay as described previously (Soliman et al., 2014). Cells were seeded at high density in 6-well plates. Confluent cells were serum starved overnight and 2 perpendicular scratches were made using a 1000 ul tip (Thermofisher Scientific, Waltham, MA). Treatments or serum-free medium were added and 5x micrographs were taken at 0, 18 and 24 hours using phase contrast microscopy on an inverted microscope. Width of the scratch was measured at 20 points (5 points/ arm) and the average width was calculated for each well using the formula: Percent migration= [(average width at 0 hours- average width at x hours)/ average width at 0 hours]*100.

**Tube Formation:** The ability of cells to form tubes was measured by Matrigel tube formation assay (Castellon et al., 2001). Confluent cells were serum starved overnight.
Cells were subjected to serum free medium with or without treatment for 24 hours. Cells were then harvested and resuspended in serum-free medium: matrigel (BD Biosciences, Franklin Lakes, NJ) in ratio 70:30. The mixture was quickly transferred to 96-well plate (100 µl/well) and matrigel is allowed to solidify. Approximately 5 X 10^4 cells in 100 µl were seeded into each quadruplicate well. At 24 hours, 10x micrographs were taken from the center of each well and tube-like structures were quantified in a blinded fashion.

**Statistical Analysis:** All statistics were carried out using NCSS2007 software. Results are expressed as mean ± standard error of the mean (±SEM). Data were statistically analyzed using One Way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparison post-hoc test. Tests of interaction were done using Two Way ANOVA. A value of P< 0.05 was considered statistically significant.

**Results**

*Sequential minocycline and candesartan treatment improves outcome compared to individual treatments.*

To assess the neuroprotective effect of individual versus sequential minocycline and candesartan treatment, we measured infarct size 14 days after stroke. Infarct size was significantly reduced by 35% and 64% in the minocycline only and the candesartan only treatment groups, respectively, as compared to the saline treatment (Fig 5.1 A, B). There was a trend of increased neuroprotective effect with the sequential combination treatment, as evident by a 78% reduction in lesion volume. Further, we tested whether the reduction in infarct size was accompanied by improved functional recovery. In Bederson test, there was a trend towards enhanced recovery with individual treatments that became significant only in the candesartan group at 14 days. However, sequential treatment resulted in
significant improvement starting at day 7, as compared to the saline-treated group (Fig 5.2A). Assessment of motor function and balance by the beam walk test revealed a trend of improvement as early as day 1 in the minocycline only group that continued slowly thereafter. Candesartan only treatment resulted in continuous and significant improvement starting day 3, as compared to saline treatment. Improvement with the sequential combination treatment followed the same pattern as the candesartan only treatment during the first week but became significantly superior to minocycline treatment at day 14 (Fig 5.2B). We observed an early and profound enhancement of Grip strength in all treatment groups, as compared to the saline group (Fig 5.2C). However, this effect plateaued at days 3 and 7 in the minocycline only and candesartan only treatment groups, respectively. At 14 days, grip strength in the sequential combination group was significantly enhanced as compared to saline as well as individual treatment groups. Similar to grip strength test, rotarod performance showed an early and significant improvement starting day 1 in all treatment groups, as compared to saline treatment (Fig 5.2D). Nevertheless, improvement plateaued early in the individual treatment groups. Only the sequential combination treatment was significantly improved as compared to saline treatment on days 7 and 14 after stroke.

*Sequential treatment preserves candesartan-induced VEGF upregulation and induction of a prolonged proangiogenic state in vivo.*

We have previously shown an enhanced VEGF expression and a proangiogenic response *in vivo* after candesartan treatment (Guan et al., 2011c; Kozak et al., 2009; Soliman et al., 2014). The proangiogenic response was accompanied by MMP activation, especially MMP-2 (Kozak et al., 2009). In this study, we tested whether the proangiogenic effect of
candesartan treatment was maintained in the low-dose treatment regimen and whether early minocycline treatment affected such a response. There was ~30% increase in MMP-2 activity in both the candesartan only and the sequential treatment groups that did not reach significance at the tested sample size (Fig 5.3A). Minocycline treatment alone or in combination with candesartan did not decrease MMP-2 or MMP-9 at 14 days (Fig 5.3A, B). VEGF expression in the sequential combination group measured at 14 days was comparable to that of the candesartan only group (Fig 5.3C). Both treatment regimens resulted in significantly higher VEGF levels as compared to the saline treatment. Further, we tested whether VEGF upregulation led to increased vascular density. Laminin staining showed ~2, 3 and 2.3-fold increase in the cortex, penumbra and contralateral hemisphere, respectively, with candesartan treatment as compared to saline-treated group (Fig 5.3D-F). Sequential combination treatment enhanced vascular density by 1.8, 3.8 and 2.8-fold relative to saline treatment in the respective brain regions. Of note, a single dose of minocycline enhanced vascular density at the cortex region by ~90%. To differentiate between neovascularization and preservation of the already existing vasculature prior to the insult, co-localization of Ki67, a marker of proliferating cells, with the endothelial cell marker, CD-31, was attempted. In agreement with VEGF data, candesartan only and the sequential combination groups showed significantly elevated neovascularization as compared to the saline or the minocycline only groups (Fig 5.3G, H).

**Simultaneous minocycline and candesartan treatment abolishes candesartan-induced VEGF upregulation and proangiogenic state in vitro.**

We have previously demonstrated VEGF upregulation and enhanced proangiogenic steps in human brain endothelial cells in response to candesartan treatment *in vitro* (Soliman et
Here, we optimize the combination timing to preserve VEGF upregulation and the subsequent proangiogenic state. We tested the effect of prior or simultaneous minocycline treatment on candesartan-induced VEGF upregulation. Simultaneous minocycline treatment prevented candesartan-induced VEGF upregulation in brain endothelial cells (Fig 5.4). Spacing of the two agents was not enough to restore candesartan’s ability to induce VEGF expression. A second dose of candesartan was necessary to restore candesartan-induced VEGF upregulation, lending further support to our animal treatment regimen. To assess the functional outcome of simultaneous treatment, we quantified different angiogenic steps in vitro in response to different candesartan concentrations with or without simultaneous minocycline treatment. Endothelial cell proliferation was reduced by 36% in response to minocycline treatment (Fig 5.5A). Candesartan treatment (0.1, 1 and 10 µg/ml) enhanced cell proliferation by 37, 59 and 47%, respectively. Simultaneous minocycline treatment resulted in a decreased proliferative potential, as compared to their respective candesartan only treatment groups. To assess whether the reduced BrdU incorporation observed with minocycline treatment was due to an anti-proliferative or a direct cytotoxic effect of the tested minocycline concentration, we measured LDH release in the cell culture media. LDH concentration did not increase with minocycline treatment either alone or in combination with different concentrations of candesartan (Fig 5.5B). Endothelial cell migration followed the same pattern as cell proliferation. Minocycline treatment reduced migration by 27 and 41% at 18 and 24 hours, respectively, as compared to the control group (Fig 5.5C- D). Candesartan treatment, on the other hand, exerted a pro-migratory effect. At 18 hours, candesartan, at different concentrations, enhanced endothelial cell migration by about
40%. At 24 hours, candesartan treatment displayed a dose-dependent pro-migratory response, resulting in 41, 59 and 74% increase in endothelial cell migration in response to treatment with 0.1, 1 and 10 µg/ml of candesartan, respectively. Simultaneous treatment with minocycline significantly reduced candesartan-induced pro-migratory effects as compared to their respective candesartan only treatment groups. Although there was a trend of decreasing endothelial cell alignment into tube-like structures with minocycline treatment, this effect did not reach significance at the tested sample size (Fig 5.5E). Candesartan treatment induced a profound dose-dependent increase in the number of tube-like structures at 24 hours. Simultaneous minocycline treatment, however, abolished endothelial cell response to candesartan treatment.

Discussion

The main findings of this study include an enhanced neurobehavioral recovery at 14 days with the sequential minocycline and candesartan treatment. This was accompanied by preservation of candesartan-induced VEGF upregulation and a subsequent proangiogenic state. In brain microvascular endothelial cells, 6-hour spacing of minocycline and candesartan treatment, together with a second dose of candesartan rescued VEGF upregulation. Simultaneous treatment, however, abolished candesartan-induced VEGF upregulation and proangiogenic effects in vitro.

Minocycline displays MMP inhibitory characteristics as well as antioxidant, antiapoptotic and anti-inflammatory effects, among other non-antibiotic properties [reviewed by (Garrido-Mesa et al., 2013)]. Minocycline treatment reduced infarct volume in suture and embolic MCAO models (Wang et al., 2003; Xu et al., 2004) and was effective at reducing brain edema and neurological deficit in an experimental intracerebral hemorrhage model.
Furthermore, minocycline has shown promising neuroprotective effects in several non-stroke brain injury models, including Huntington’s disease, Parkinson’s disease, amyotrophic lateral sclerosis and spinal cord injury (Garrido-Mesa et al., 2013). Due to its well-documented non-antibiotic properties, supported by evidence of neuroprotection in different brain injury models, we attempted early minocycline treatment after focal cerebral ischemia.

Chronic angiotensin II Type 1 (AT1) receptor blockade has been shown to increase cerebrovascular density in the presence or absence of an ischemic insult (Forder et al., 2005; Munzenmaier and Greene, 2006). Furthermore, in an animal model of focal cerebral ischemia, administration of a single candesartan dose at reperfusion induced a prolonged VEGF upregulation (Soliman et al., 2014) and increased vascular density at 7 days (Kozak et al., 2009). In human brain endothelial cells, candesartan treatment induced a proangiogenic response mediated by VEGF, among other factors (Alhusban et al., 2013; Soliman et al., 2014). Of note, we have previously reported enhanced MMP activity, especially MMP-2, 24 hours after treatment with 1 mg/ kg of candesartan, which could explain VEGF upregulation and the proangiogenic state since MMPs and VEGF are interrelated (Chetty et al., 2010; Ebrahem et al., 2010; Hollborn et al., 2007; Lee et al., 2005). The benefits of candesartan treatment, however, could be masked by its blood pressure lowering effect. Current guidelines advise against blood pressure reduction in the first 24 hours after stroke (Jauch et al., 2013). We, therefore, attempted a combination of minocycline at reperfusion followed by low-dose candesartan treatment starting 24 hours after MCAO.
While several studies have demonstrated the detrimental consequences of MMP activation early after stroke (Jin et al., 2010), caution against late MMP inhibition emerged when it was accompanied with reduced markers of neurovascular remodeling (Zhao et al., 2006). The dual “destructive-protective” nature of MMPs, depending on the timing, calls for fine-tuning of the enzymatic activity in order to improve long term neurovascular recovery after stroke. In our treatment regimen, early minocycline administration at reperfusion, either alone or in the combination treatment group, preserved MMP activity 14 days after MCAO, allowing for neurovascular recovery. Minocycline treatment preserved vascular structure in the ischemic hemisphere, as evident by enhanced laminin staining. Vascular density was ~ 90% and 40% higher in the ischemic cortex and the ischemic border zone, respectively in minocycline-treated animals as compared to their saline-counterparts. However, this effect was not accompanied by enhanced vascular density in the contralateral hemisphere or increased cell proliferation, suggesting a vascular protective rather than a neovascularization effect of minocycline treatment. Minocycline actions as an MMP-inhibitor, anti-inflammatory, anti-apoptotic and antioxidant drug could all explain the vascular protective effect observed 14 days after a single minocycline treatment. Nonetheless, a recent report demonstrated increased neovessel formation and expression of tight junction proteins in the ischemic border zone 4 weeks after MCAO in response to a single minocycline treatment. Neovascularization was attributed to reduced tissue loss with the treatment. This is not in discord with our results, since tissue and vascular preservation at 14 days, demonstrated in our current study, could possibly lead to enhanced angiogenesis at later time points. (Yang et al., 2014). Therefore, minocycline treatment at reperfusion preserved the brain’s inherent angiogenic response as well as
candesartan-induced neovascularization. Candesartan treatment induced a prolonged increase in VEGF expression in both candesartan only and the sequential combination groups. This was accompanied by enhanced neovascularization and increased vascular density in both hemispheres.

In order to further study the interaction potential, we treated brain endothelial cells with either minocycline or candesartan or the combination of both drugs. Candesartan treatment enhanced different angiogenic steps in vitro in a dose-dependent fashion, as shown previously (Soliman et al., 2014). Minocycline, on the other hand, blocked angiogenesis and reduced the candesartan-induced proangiogenic effect. Such inhibition of angiogenesis was accompanied by, and possibly resulted from, inhibition of candesartan-induced VEGF upregulation. We attempted to space the treatments in vitro to test whether this approach would rescue VEGF production. Only when an additional dose of candesartan was added, did VEGF upregulation start again, lending further support to our in vivo dosage regimen.

With the continuous failure of stroke therapeutics in the clinical setting, there is an urgent need to develop new strategies that augment the brain’s reparative capacity. In conclusion, this study suggests a novel, sequential therapy with two well-tolerated, FDA-approved drugs as a possible tactic to enhance recovery after cerebral ischemia.
Bibliography


    endothelial growth factor and matrix metalloproteinases in the induction of

10. Engelhorn, T., Goerike, S., Doerfler, A., Okorn, C., Forsting, M., Heusch, G.,
    Schulz, R., 2004. The angiotensin II type 1-receptor blocker candesartan increases
    cerebral blood flow, reduces infarct size, and improves neurologic outcome after


    from focal ischemia with angiotensin II type 1 receptor blockade in the rat. Am J


    tetracycline analogs minocycline and doxycycline inhibit angiogenesis in vitro by
    a non-metalloproteinase-dependent mechanism. Cancer Chemother Pharmacol 36,
    418-424.

15. Go, A. S., Mozaffarian, D., Roger, V. L., Benjamin, E. J., Berry, J. D., Blaha, M.
    J., Dai, S., Ford, E. S., Fox, C. S., Franco, S., Fullerton, H. J., Gillespie, C.,
    Hailpern, S. M., Heit, J. A., Howard, V. J., Huffman, M. D., Judd, S. E., Kissela,


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Table 5.1: Treatment regimens administered to different animal groups at reperfusion for 7 days after stroke.
**Figure 5.1: Combining minocycline and candesartan sequentially decreases infarct volume more than each agent alone.** A: Representative images of cresyl violet-stained coronal sections 14 days after MCAO, collected from animals treated with saline, minocycline, candesartan or a sequential combination of minocycline and candesartan. B: Quantification of infarct volume 14 days after MCAO (n= 4, * significantly different from saline group, ψ significantly different from minocycline group, P< 0.05).
Figure 5.1: Combining minocycline and candesartan sequentially decreases infarct volume more than each agent alone.
**Figure 5.2: Sequential combination of minocycline and candesartan improves neurobehavioral outcome more than each agent alone.** A-D: Assessment of neurobehavioral and motor function 1, 3, 7 and 14 days after MCAO using modified Bederson score (A), beam walk (B), rotarod performance (C) and grip strength tests (D) in animals treated with saline, minocycline, candesartan or a sequential combination of minocycline and candesartan (n= 6-10, * significantly different from saline group, ψ significantly different from minocycline only group, ς significantly different from candesartan only group, P< 0.05).
Figure 5.2: Sequential combination of minocycline and candesartan improves neurobehavioral outcome more than each agent alone.
Figure 5.2: Sequential combination of minocycline and candesartan improves neurobehavioral outcome more than each agent alone.
Figure 5.3: Sequential combination of minocycline and candesartan preserves candesartan-induced proangiogenic response in vivo. Early minocycline treatment does not inhibit MMP-2 or MMP-9 activity at 14 days. A-C: Quantification of MMP-2 (A), MMP-9 (B) and VEGF (C) in the ipsilateral hemispheres of brains collected 14 days after MCAO from different treatment groups (n= 3-4). D: Diagrammatic illustration of the regions assessed for laminin staining. E: Representative micrographs of laminin-stained vessels in the ischemic border zone 14 days after MCAO. F: Quantification of laminin-stained vessels in the cortex, ischemic border zone and contralateral hemisphere 14 days after MCAO. G: Representative micrographs of neovascularization in the ischemic border zone 14 days after MCAO as demonstrated by ki76 (marker of proliferating cells), co-localized with CD-31 (endothelial cell marker). H: Quantification of ki67 and CD-31 co-localization in the ischemic border zone 14 days after MCAO (n= 3-4, * significantly different from saline group, ψ significantly different from minocycline only group, P<0.05).
Figure 5.3: Sequential combination of minocycline and candesartan preserves candesartan-induced proangiogenic response in vivo. Early minocycline treatment does not inhibit MMP-2 or MMP-9 activity at 14 days.
Figure 5.3: Sequential combination of minocycline and candesartan preserves candesartan-induced proangiogenic response in vivo. Early minocycline treatment does not inhibit MMP-2 or MMP-9 activity at 14 days.
Figure 5.3: Sequential combination of minocycline and candesartan preserves candesartan-induced proangiogenic response in vivo. Early minocycline treatment does not inhibit MMP-2 or MMP-9 activity at 14 days.
Figure 5.4: Simultaneous combination of minocycline and candesartan abolishes candesartan-induced VEGF upregulation in vitro. Quantification of VEGF expression in human brain endothelial cell lysate after 2-hour OGD/ reoxygenation (n=3-8, * significantly different from untreated control (Ctrl), P< 0.05, Cand I and II: Candesartan single and two treatments, respectively. Sequential/ Cand I and Sequential/ Cand II: Sequential combination of minocycline with one or two candesartan treatments, respectively).
Figure 5.4: Simultaneous combination of minocycline and candesartan abolishes candesartan-induced VEGF upregulation in vitro.
**Figure 5.5: Simultaneous combination of minocycline and candesartan decreases candesartan-induced proangiogenic effect in vitro.**

A, E: Quantification of endothelial cell proliferation (A) and tube formation (E) after 2-hour OGD and 24-hour reoxygenation. B: Determination of LDH concentration in cell culture media collected from brain endothelial cells exposed to 2-hour OGD and 24-hour reoxygenation (n=3, * significantly different from untreated control, # significantly different from the corresponding candesartan concentration without minocycline co-treatment. *, #: P< 0.05; ***, ###: P< 0.005). C: Representative micrographs of endothelial cell migration at 18 hours in individual treatments or simultaneous combination and compared to the untreated control. D: Quantification of endothelial cell migration after 2-hour OGD and 18- or 24-hour reoxygenation (n=3, * significantly different from untreated control at the same time point, # significantly different from the corresponding candesartan concentration without minocycline co-treatment at the same time point, *,#: P< 0.05; **,**#: P< 0.01; ***,###: P< 0.005).
Figure 5.5: Simultaneous combination of minocycline and candesartan decreases candesartan-induced proangiogenic effect in vitro.
Figure 5.5: Simultaneous combination of minocycline and candesartan decreases candesartan-induced proangiogenic effect in vitro.
CHAPTER 6
INTEGRATED DISCUSSION

The aim of this dissertation is to investigate the mechanisms of angiogenesis and neurovascular protection by ARB treatment after experimental focal cerebral ischemia. Several groups have reported improved neurobehavioral outcome and reduced infarct lesion in response to ARBs administered after experimental stroke (Brdon et al., 2007; Engelhorn et al., 2004; Kozak et al., 2009). We have shown a concomitant preservation of vascular barrier function 24 hours after candesartan treatment. This was, paradoxically, accompanied by increased cerebrovascular density at 7 days and enhanced proangiogenic potential of cerebrospinal fluid (CSF) (Kozak et al., 2009). In a follow-up study by Guan et al., it was reported that candesartan treatment at reperfusion upregulates VEGF-A and B expression in the contralateral and ipsilateral hemispheres, respectively (Guan et al., 2011). We, therefore, studied the proangiogenic time course after a single candesartan treatment in an experimental model of acute ischemic stroke. Moreover, we demonstrated a causal relationship between VEGF-A and B upregulation and the promotion of a proangiogenic state following candesartan treatment.

Angiogenesis is a key for recovery (Beck and Plate, 2009; Ergul et al., 2012; Quaegebeur et al., 2011; Xiong et al., 2010). In fact, interventions enhancing angiogenesis have been investigated in preclinical stroke trials, leading to improved functional recovery and reduced lesion volume (Guo et al., 2009; Simao et al., 2012; Wang et al., 2004). Endothelial cells are a rich source of growth factors and molecular cues to stimulate
neurorestorative processes (Chopp et al., 2007). Indeed, angiogenesis and neurogenesis are two tightly related processes in health and disease (Li et al., 2006; Madri, 2009; Ohab et al., 2006; Shen et al., 2004; Xiong et al., 2010). Angiogenesis, therefore, represents a viable target to improve recovery after an acute ischemic insult (Beck and Plate, 2009). Of importance, continuous failure of direct neuroprotectants in clinical trials has led leaders of the field to evaluate experimental and clinical trial design and to delineate hurdles to translation. A major flaw in experimental study design was the focus on a single cell type as a target for treatment, overlooking the potential interactions with the surrounding environment (Bushnell et al., 2006). The angiogenic approach to neuroprotection, on the other hand, takes into consideration the fundamental interactions between vasculature and neurons. Moreover, creating an angiogenic milieu is a druggable target and several safe and FDA-approved medications have been shown to switch on angiogenesis (Guo et al., 2009; Simao et al., 2012; Wang et al., 2004).

In chapter 3, we investigated the proangiogenic time course after a single candesartan administration. We reported the induction of a prolonged proangiogenic state, in both brain tissue and CSF, up to 72 hours after a single treatment (Figures 3.1 and 3.2). Of note, the increase in VEGF-A and B expression was more prolonged in the contralateral as compared to the ipsilateral hemisphere. The upregulation pattern of VEGF-A and B suggests the involvement of the contralateral hemisphere in ameliorating acute injury as well as in mediating recovery and promoting a proangiogenic state (Figure 3.2 A-D). The prolonged enhancement of the proangiogenic potential of CSF for up to 72 hours (Figure 3.1 A-C) suggests the release of growth factors within the vicinity of the
brain and further supporting the notion of the communication between the two hemispheres.

To further understand the molecular pathways involved in candesartan’s actions, we measured HIF-1α expression, an upstream signaling molecule of VEGF-A, 8 and 24 hours after MCAO (Figure 3.3 A). We observed an early stabilization of HIF-1α with candesartan treatment at 8 hours, an effect that continued till 24 hours post-MCAO. In saline-treated animals, however, HIF-1α levels were comparable in both hemispheres at 24 hours. Our data suggest the involvement of HIF-1α stabilization in mediating candesartan-induced VEGF-A upregulation and the subsequent proangiogenic state.

Further, our in vivo as well as in vitro studies show antioxidant and anti-nitrative effects of candesartan treatment (Figure 3.3 B and S1). It has been shown previously that tyrosine nitration hampers VEGF-A signaling, via nitration of PI3K/ Akt pathway molecules (Abdelsaid et al., 2010). It is, therefore, possible that the proangiogenic effect of candesartan treatment is mediated via enhanced VEGF-A expression as well its signaling pathway.

In addition to being a potent angiogenic factor, VEGF-A has shown neurogenic and neuroprotective effects (Jin et al., 2002; Jin et al., 2000). However, the permeabilizing effect of VEGF-A has always been a concern. Systemic administration of VEGF-A 1 hour after MCAO was associated with increased bleeding and ischemic damage (Zhang et al., 2000). In our study, we show that early VEGF-A upregulation was accompanied by a concomitant preservation of angiopoietin-1 (Figure 3.3 C). This finding might, at least partly, explain the angiogenic/ barrier protective paradox. However, one of the limitations of our findings is the correlative nature of the study. Further studies are warranted to
evaluate the exact role and contribution of angiopoietin-1 preservation in the barrier protective effect of candesartan treatment. Accordingly, we do not exclude the possibility of the involvement of other mechanisms and pathways in mediating such a response.

VEGF-B, a potent survival factor and an understudied molecule especially in a stroke setting, was also increased after candesartan treatment in both hemispheres. Of the measured time points, maximum upregulation of VEGF-B was reported 8 hours post-treatment in the ischemic hemisphere, consistent with its prosurvival role (Figure 3.2 C, D). The role of VEGF-B in mediating an angiogenic response is, however, debatable (Li et al., 2009; Li et al., 2008; Zhang et al., 2009). In our study, we have shown the contribution of VEGF-B to the in vitro angiogenic response elicited by candesartan treatment (Figures 3.4 C-F, 3.5 and 3.7). It is not clear, however, if this response is elicited by a direct angiogenic effect of VEGF-B or it is secondary to enhancing cell viability. The direct neuroprotective role of VEGF-B prompted us to investigate the role of candesartan-mediated VEGF-B upregulation in ameliorating acute injury in chapter 4 of this dissertation. Silencing VEGF-B expression using lentiviral shRNA particles against VEGF-B abrogated candesartan’s ability to decrease infarct volume and to improve neurobehavioral outcome (Figures 4.3 and 4.4). Taken together, VEGF-B might represent a safe and a druggable target to improve recovery after cerebral ischemic injury.

The effect of VEGF-B on vascular permeability is not yet clear. Overexpression or intravitreal delivery of VEGF-B resulted in a loss of retinal barrier function as demonstrated by two independent groups (Song et al., 2012; Zhong et al., 2011). Nevertheless, VEGF-B 186 isoform did not increase blood brain barrier leakiness in an animal model of motor neuron degeneration (Poesen et al., 2008). It has been recently
reported that VEGF-B might represent a more balanced approach towards cardiac
neovascularization (Kupatt and Hinkel, 2014). Whether this conclusion can be
extrapolated to the brain remains a question.

It has been documented by our lab that indirect unopposed AT2 receptor stimulation
in response to ARB treatment mediates its proangiogenic effect via BDNF upregulation.
Blocking AT2 receptors or the BDNF receptor, TrkB, abolished candesartan-induced
proangiogenic response (Alhusban et al., 2013). This data is in harmony with our findings
since BDNF and VEGF-A are interrelated (Chen et al., 2005; Chen et al., 2013; Li et al.,
2006). Candesartan’s benefit, however, could be mediated through mechanisms beyond
AT2 receptor stimulation such as the direct antioxidant and anti-nitrative effects. We,
therefore, conclude that candesartan, by its pleiotropic mechanisms of action, exerts
angiogenic and neurovascular protective effects.

Although ARB treatment has been promising in preclinical studies, employment in a
clinical setting was disappointing. The Scandinavian Candesartan Acute Stroke Trial
(SCAST) showed a lack of benefit when candesartan was administered acutely after stroke
(Sandset et al., 2011). In fact, blood pressure lowering and the subsequent reduction in
brain perfusion were always a concern with antihypertensive intervention in the acute
phase after ischemic stroke (Fischer and Rothwell, 2011). Another recent study (CATIS)
investigated the effect of acute blood pressure lowering after stroke using different classes
of antihypertensive medications or a combination of more than one class (He et al., 2014).
The study reported a lack of benefit by blood pressure reduction in the acute post-stroke
phase. American Heart Association 2013 guidelines recommended against blood pressure
lowering within the first 24 hours after ischemic cerebral injury (Jauch et al., 2013).
Accordingly, a subhypotensive dose of candesartan started at 24 hours was attempted to avoid the risk of blood pressure lowering in chapter 5 of this work.

Pathophysiology of ischemia/reperfusion injury is rather complicated. It involves massive production of free radicals that attack cellular structural and regulatory components. A group of matrix degrading enzymes, matrix metalloproteinases, is activated as a consequence of the ischemic event, resulting in loss of blood brain barrier integrity. The resulting infiltration of cellular components, including leukocytes, into the brain initiates an inflammatory reaction and creates more free radicals. Cellular death pathways via apoptosis and necrosis then ensue (Pradeep et al., 2012). It is believed that cellular death continues for days after an ischemic event. The full extent of damage is not achieved until 2-6 weeks after the onset of an ischemic insult (Valtysson et al., 1994). Due to the complexity of stroke pathophysiology, polytherapy represents an attractive and promising approach to treatment (Gladstone et al., 2002). However, combining agents with different mechanisms of actions requires optimization of treatment timing to avoid possible interactions.

The success of minocycline in a multitude of CNS injury models (Kim and Suh, 2009; Sanchez Mejia et al., 2001; Zhu et al., 2002) as well as the pleiotropic actions of this drug (Garrido-Mesa et al., 2013) make it a promising candidate for combination therapy in an ischemic stroke setting. Minocycline exerts antioxidant, antiapoptotic, anti-inflammatory and MMP-inhibitory characteristics, thereby, targeting multiple steps in ischemia/reperfusion pathophysiology (Garrido-Mesa et al., 2013). The anti-angiogenic and MMP-inhibitory effects of minocycline treatment, however, (Gilbertson-Beadling et al., 1995; Jung et al., 2014; Tamargo et al., 1991; Yao et al., 2004) call for optimization of
treatment timing when combined with candesartan. In fact, early activation of MMPs in the course of ischemia/reperfusion injury has been found to be detrimental to stroke outcome. MMPs degrade blood brain barrier, resulting in edema, hemorrhagic transformation and inflammation. On the other hand, MMP inhibition 7-14 days after an ischemic insult has been correlated with impaired functional recovery (Zhao et al., 2006). Therefore, we attempted early minocycline treatment at reperfusion, followed by daily low-dose candesartan treatment, starting 24 hours after injury and lasting for 7 days. Neuroprotection provided by this treatment regimen exceeded minocycline or candesartan individual treatment regimens (Figures 5.1 and 5.2). Whether candesartan-induced proangiogenic state was affected by earlier minocycline treatment was a question. To address this concern, angiogenic response was assessed 14 days after the ischemic insult. VEGF-A upregulation and increased neovascularization were observed in both candesartan only and the sequential combination groups (Figure 5.3). Our results show the efficacy of a multiple low-dose candesartan treatment regimen in inducing a proangiogenic response and improving neurobehavioral outcome. In addition, 24 hour spacing of minocycline and candesartan treatments allows for safe combination and avoids antagonistic interactions. Of support, our in vitro data show decreased angiogenic response with simultaneous minocycline and candesartan treatment (Figures 5.4 and 5.5).

In conclusion, our data provide evidence for the involvement of VEGF-A and B in the angiogenic and neurovascular protective effects of ARB treatment after focal cerebral ischemia. In addition, this study suggests the rational sequential combination therapy of early minocycline treatment followed by multiple low doses of candesartan after the first 24 hours, in order to harness the benefits of combination therapy and to avoid early
antihypertensive intervention. **Figure 6.1** depicts the mechanisms of protection by minocycline and candesartan and the rationale of the sequential combination treatment.

**Figure 6.1:** A diagram depicting the rationale of minocycline and candesartan combination treatment to optimize proangiogenic therapy. Mino: Minocycline; Cand: Candesartan.
Nevertheless, this study is not without limitations. Our studies were conducted in young, male and otherwise healthy animals. Further studies in aged, female and co-morbid condition animal models should be conducted before taking this treatment regimen to a clinical setting.

**Innovation and Therapeutic Implications**

In this dissertation, we investigate the use of candesartan in the acute treatment of ischemic stroke. Candesartan offers the advantages of being a small molecule, FDA-approved and having a known safety profile. In addition, we study acute treatment rather than preventive measures, which has more clinical applicability. Moreover, we take into account recent clinical trials and treatment guidelines that advise against early antihypertensive therapy. Combination of candesartan with another safe, tolerated and FDA-approved drug, minocycline, offers more promise by tackling different targets in the ischemia/ reperfusion cell death cascade. We are the first to study rational, sequential combination therapy for ischemic stroke in a preclinical setting. To our knowledge, no drug has been identified so far that enhances the expression of VEGF-B, a safe angiogenic and survival factor. Lastly, promoting angiogenesis to achieve neuroprotection takes into account the concept of the neurovascular unit and the possible interactions between different cell types in the complex brain tissue.
Bibliography


by angiotensin receptor antagonism involves differential VEGF expression in both hemispheres after experimental stroke. PLoS One 6, e24551.


37. Song, Z., Qian, X., Le, Y.-Z., 2012. REGULATION OF RPE BARRIER FUNCTION BY VEGF-B. Heart 98, E114.


SUMMARY

Our findings demonstrate the involvement of VEGF-A and B in mediating the benefits of ARBs treatment after ischemic stroke. Optimization of angiogenic therapy using rational sequential combination with minocycline increases the translational potential and clinical applicability of this study.
APPENDIX

CO-AUTHOR MANUSCRIPTS

List of manuscripts co-authored by Sahar A. Soliman in chronological order:


5. Fouda A, **Soliman S**, Kozak A, Pillai B, Ergul A, Fagan SC. *Impaired Response to Post-Stroke Candesartan Treatment in a Model of Type II Diabetes: A Correlation Study with AT Receptors Expression*. (Submitted to Neurochem Intl- Feb 2014)

6. Shanab A, El-Azab M, Sabbineni H, **Soliman S**, Matragoon S, Fagan SC, El-Remessy AB. *Candesartan preserves retinal vasculature in ischemic retinopathy: Role of inducible nitric oxide synthase (iNOS) and Hemeoxygenase-1 (HO-1)*. (Submitted to J Pharmacol Exp Ther- Feb 2014)

