As of 2015, stroke is the fifth leading cause of death in US and remains to be a major cause of long-term disability with limited treatment options. Angiotensin II (Ang II) is a peptide hormone that mediates its actions through two major types of receptors, angiotensin type 1 (AT1R) and type 2 (AT2R) receptors. While, AT1R causes vasoconstriction, inflammation and ischemic damage after stroke, AT2R is considered beneficial. Experimentally, angiotensin II type 1 receptor blockers (ARBs) have consistently improved stroke outcome yet clinical data showed controversial results with a trend towards worsened stroke outcome due to BP lowering. AT2R stimulation has emerged as a new tactic to reap the beneficial actions of AT1R blockade without affecting the blood pressure. Our work aimed to examine the beneficial effects of acute AT2R stimulation after stroke using the novel, non-peptide and orally available AT2R agonist, compound 21 (C21). We showed for the first time neurovascular protection and sustained functional improvement up to 7 days with a single C21 treatment after stroke. Furthermore, we have shown a sustained proangiogenic response with C21 treatment that was mediated through endothelial upregulation of brain derived neurotrophic factor
Our findings include characterization of IL-10 expression after ischemic stroke and highlighting its involvement in C21 mediated neuroprotection in vivo. Yet, C21 was still able to provide direct neuroprotection in primary neuron cultures. Lastly, we assessed the AT2R expression in type 2 diabetic GK rats and found lower expression levels after stroke compared to Wistars which lead to impaired efficacy to AT1R blockade after stroke. Our work provides better understanding of the mechanisms of the beneficial actions of AT2R stimulation after stroke and paves the way for translating C21 and future non-peptide AT2R agonists to the clinical setting as acute stroke treatment.

INDEX WORDS: Stroke; Angiotensin type 1 receptor (AT1R); Angiotensin type 2 receptor (AT2R); Compound 21 (C21); Angiotensin II Receptor Blockers (ARBs); Angiogenesis; Interleukin 10 (IL-10); Anti-inflammation; Neuroprotection; Vascular Protection.
MECHANISMS MEDIATING NEUROPROTECTION AND RECOVERY WITH AT2 RECEPTOR STIMULATION AFTER STROKE

by

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B.Pharm, Cairo University, Egypt, 2007

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2015
MECHANISMS MEDIATING NEUROPROTECTION AND RECOVERY WITH AT2 RECEPTOR STIMULATION AFTER STROKE

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May 2015
DEDICATION

I dedicate this thesis to my wife (Esraa) and my daughter (Alia) who spent years of their lives away from their home country and family in order for me to achieve this dream. I also dedicate this thesis to my family back home in Egypt; my parents (Yousry and Khadija) and my siblings (Mohamed, Rehab, and Lamia) whose encouragement and support kept me going in times of difficulties throughout my life.
ACKNOWLEDGEMENTS

Coming from a high-power distance culture, I had the mindset of waiting for directions from my boss and never questioning their suggestions. While this taught me to be respectful to others, it never allowed me to explore my inner potential. Working with my mentor, Dr Susan Fagan, has been an extreme privilege for me. Under her guidance, I learned to be an independent thinker and believe in my ability to implement my own ideas and make the change I want to see. I am very grateful for the opportunities that Dr Fagan provided to me during my PhD training. I had a successful project, great learning experience and was exposed to major professors and cutting edge research in our field through attending four international conferences. I was extremely fortunate to work with Dr Fagan’s great stroke team. Every single member of the team has helped me in at least one aspect of this work. I would like to thank the previous lab members, Anna Kozak, Ahmed Alhusban, and Sahar Soliman, and the current lab members, Tauheed Ishrat and Bindu Pillai for their time and help. Working with all of them was a pleasure and I really learned how to work efficiently in a successful productive team because of them.

I would like to express my sincere gratitude to my advisory committee members, Dr Bradley Phillips, Dr Adviye Ergul, Dr Krishnan Dhandapani and Dr Somanath Shenoy for their support and guidance. Their time and effort is greatly appreciated and I have learned a lot from their insightful suggestions, technical support and the study material they provided for me. Especially, I would like to thank Dr Adviye Ergul for giving me the opportunity to work with her on two prestigious review articles, which honed my
writing skills. Dr Ergul’s team especially Mohammed Abdelsaid and Trevor Hardigan has provided me with technical help in my studies. I also would like to thank other Clinical and Experimental Therapeutics (CET) program professors, Dr Azza El-Remessy and Dr Lakshman Segar. They taught me many things through interacting with them on both the personal and professional levels. Other CET postdoctoral and students have been best coworkers and friends. I could have never wished for a better work environment.
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Stroke Statistics

As of 2015, stroke is the fifth leading cause of death in the United States (US), accounting for about 1 of every 19 deaths. Stroke is also a leading cause of serious long-term disability in US; being among the top 18 diseases contributing to years lived with disability in 2010 [1]. Ischemic strokes represent 87% of all strokes, and are characterized by a discontinuation of the blood supply to a region of the brain [1]. Tissue plasminogen activator remains the only FDA approved drug treatment for management of acute ischemic stroke since 1996. Its use is limited by its narrow therapeutic window (up to 3-4.5 hours) and risk of intracranial hemorrhage making it useful for ≈ 3% of stroke patients [2]. Therefore, finding new drug candidates is imperative for this largely untreated disease.

Responding to an urgent need to develop new stroke treatments, preclinical research has developed more than 1000 neuroprotectants over the past two decades. About 100 of them reached clinical trials but failed to show benefit to stroke patients [3]. Recently, a new approach has emerged to enhance the endogenous reparative mechanisms in the brain with a global focus involving all cells in the neurovascular unit. It is conceivable that modulating the endogenous inflammatory response as well as enhancing neurovascular restoration (neurogenesis and angiogenesis) could improve long-term stroke outcome even
after the narrow time window for neuroprotection and salvaging the penumbra region is gone [4, 5].

**Stroke and Brain Inflammation**

Inflammation is a central component in the pathophysiology of ischemic stroke. Initially, hypoxia due to blood flow interruption activates inflammation. Progressive cell death in the ischemic core release danger molecules that further triggers inflammatory response and immune cells recruitment. It is believed that post-stroke inflammatory response contributes to both ischemic lesion progression and tissue repair [6]. Because of this Janus effect of inflammation, anti-inflammatory approaches have not been successful so far in the treatment of ischemic stroke clinically [7, 8]. Deep understanding of the stroke inflammatory cascade is imperative for designing innovative treatment strategies that target only the deleterious effects of neuroinflammation.

**Interleukin 10 and stroke:** Interleukin (IL) 10 (also known as cytokine synthesis inhibitory factor) is an anti-inflammatory cytokine that has a direct neuroprotective action. Interleukin 10 mediates its actions through binding to interleukin 10 receptor complex. IL-10 receptor complex is a heterodimer of two chains, IL-10R1 and IL-10R2. While IL-10R1 is specific to IL-10 signaling pathway, IL-10R2 can form receptor complexes for other cytokines as well. Interleukin 10 activates the JAK1-STAT3 (Janus Kinase-Signal Transducer and Activator of Transcription 3) signaling pathway that leads to the IL-10 anti-inflammatory response (figure 1.1) [9].

Several experimental in vivo studies have proven the anticipated protective role of IL-10 in ischemic stroke (table 1.1); Administration of exogenous IL-10 centrally and systemically decreases the infarct size in rats after permanent focal ischemia [10, 11],
while IL-10 knockout mice show larger infarct volume following middle cerebral artery occlusion [12]. Moreover, post-ischemic IL-10 gene transfer attenuated brain infarction in rats subjected to focal and global ischemia [13]. Interestingly, neuroprotection by systemic immune cells have also been shown to be mediated through interleukin 10 production. Boosting regulatory T cells function by histone deacetylase inhibition (HDACi) provides neuroprotection and limits neuroinflammation after stroke through IL-10 production [14, 15]. Similarly, exogenous administration of IL-10 producing regulatory B-cell subset limit CNS inflammation and reduce infarct volume following ischemic stroke in B-cell deficient mice [16, 17].

In in vitro models, IL-10 protects murine cortical and cerebellar neurons from excitotoxic damage and oxygen glucose deprivation (OGD) by activating phosphatidylinositide 3-kinases (PI-3K) and signal transducer and activator of transcription 3 (STAT-3) pathways (table 1.2) [12, 18, 19]. Despite the existing knowledge, the spatiotemporal expression and cellular sources of IL-10 following ischemic injury are still to be elucidated.

Clinically, subjects with lower IL-10 production are at increased risk of stroke [20]. Conversely, IL-10 is increased in serum and CSF of patients after ischemic stroke with conflicting reports on its correlation with improved versus worsened outcome [21-27]. I.V. administered IL-10 reduces inflammatory cytokines (IL-1β and TNF-α) production in blood of healthy volunteers.[28] As a drug, recombinant IL-10 has been tested in inflammatory conditions such as rheumatoid arthritis, Crohn’s disease and psoriasis but was not further pursued due to either lack of efficacy or systemic adverse effects [29]. While IL-10 use as a drug is hindered by its possible systemic immunosuppressive effect,
modulating endogenous IL-10 levels can provide neuroprotection and mitigate inflammation after stroke.

Table 1.1. Studies documenting IL-10 mediated neuroprotection in vivo.

<table>
<thead>
<tr>
<th>Author</th>
<th>Insult</th>
<th>Study design</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spera et al. 1998 [30]</td>
<td>pMCAO - SHRs</td>
<td>IV &amp; ICV recombinant human IL-10 (30 min &amp; 3 h after pMCAO) – sacrificed after 24 h</td>
<td>Central administration reduced infarct size by 20.7% while systemic administration reduced infarct by 30.7 to 40.3%</td>
</tr>
<tr>
<td>Grilli et al. 2000 [12]</td>
<td>pMCAO - IL-10 KO mice</td>
<td>Animals sacrificed after 24 h</td>
<td>Infarct was 30% larger in the IL-10 KO mice</td>
</tr>
<tr>
<td>Ooboshi et al. 2004 [13]</td>
<td>pMCAO &amp; global ischemia - SHRs</td>
<td>Adenoviral vectors encoding human IL-10 given ICV, 90 or 60 min after focal or global ischemia – sacrificed after 5 days</td>
<td>Reduced brain infarction in the treatment group with less infiltration of leukocytes and macrophages</td>
</tr>
<tr>
<td>Frenkel et al. 2005 [31]</td>
<td>2 h MCAO - mice</td>
<td>Induction of IL-10-producing CD4+ T cells – sacrificed after 24-72 h</td>
<td>Treatment reduced ischemic infarct size by 70% at 24 h and improved behavioral score</td>
</tr>
<tr>
<td>Liesz et al. 2009 [14]</td>
<td>Permanent MCAO - Mice</td>
<td>Regulatory T-cell depletion</td>
<td>Infarct expansion that was rescued by IL-10 substitution</td>
</tr>
<tr>
<td>De Bilbao et al. 2009 [32]</td>
<td>pMCAO - transgenic mice</td>
<td>Endogenous over expression (transgenic) – sacrificed after 4 days</td>
<td>Transgenic IL-10 mice showed a 40% reduction in infarct size</td>
</tr>
<tr>
<td>Perez-de Puig et al. 2013 [33]</td>
<td>pMCAO - IL-10 KO mice</td>
<td>Animals were kept in pathogen free environment and sacrificed after 7 days</td>
<td>IL-10 deficiency slightly increased infarct volume and neurologic deficits</td>
</tr>
<tr>
<td>Bodhankar et al. 2013 and 2014 [16, 17]</td>
<td>60 min MCAO - Wild type and B-cell deficient mice</td>
<td>Transfer of IL-10(+) B-cells – sacrificed at 96 h</td>
<td>Treatment reduced infarct volume and CNS inflammation</td>
</tr>
<tr>
<td>Liesz et al. 2013 [15]</td>
<td>Permanent cortical stroke - Mice</td>
<td>Histone deacetylase inhibition (HDACi) using trichostatin A</td>
<td>Treatment reduced infarct size and neuroinflammation</td>
</tr>
</tbody>
</table>

Table 1.2. Studies documenting IL-10 mediated neuroprotection in vitro.

<table>
<thead>
<tr>
<th>Author</th>
<th>Insult</th>
<th>Signaling pathways involved</th>
</tr>
</thead>
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<tr>
<td>Bachis et al. 2001[18]</td>
<td>Glutamate &amp; NMDA excitotoxicity</td>
<td>IL-10 prevents rat cerebellar granule cell death through blocking caspase-3 activation</td>
</tr>
<tr>
<td>Zhuo et al. 2009[34]</td>
<td>Glutamate induced toxicity</td>
<td>IL-10 provides direct trophic support to rat spinal cord neurons through the PI-3K/AKT &amp; STAT-3 pathways</td>
</tr>
<tr>
<td>Sharma et al. 2011[19]</td>
<td>OGD or glutamate toxicity</td>
<td>IL-10 directly protects rat primary cortical neurons by activating PI-3 kinase &amp; STAT-3 pathways</td>
</tr>
</tbody>
</table>

Table abbreviations: NMDA: N-methyl D-aspartic acid, OGD: oxygen glucose deprivation.

Renin-Angiotensin System in the Brain

One of the molecular pathways central to the pathophysiology of stroke and other neurovascular diseases is the brain renin-angiotensin system (RAS). There is a strong body of evidence that modulating the RAS is associated with endogenous neurovascular restoration and recovery in different brain disorders experimentally. However, clinical data is still lagging with no RAS modulating drug is currently approved for use as an acute treatment for ischemic stroke so far. Blood pressure lowering is considered to be the main reason behind the missed opportunity of harnessing the pleiotropic effects of RAS for the benefit of stroke patients.

Angiotensin (Ang) II is a peptide hormone that is generated from Ang I by the angiotensin-converting enzyme (ACE). Ang I itself results from cleavage of angiotensinogen by the enzyme renin. Circulating Ang II does not cross the blood brain barrier (BBB), but a local brain renin-angiotensin system exists that have been implicated in the pathophysiology of different CNS disorders [35, 36]. Different renin angiotensin system components are depicted in figure 1.2. Ang II activates two major types of receptors in the brain, angiotensin type one (AT1R) and type two (AT2R) receptors. AT1R is widely expressed in adults and mediates most Ang II effects including
inflammatory and vasoconstrictor actions. AT1R is further sub-classified into two types in the rodent brain, AT1a and AT1b. AT2R, which is less expressed in adults and becomes upregulated with diseases, has opposite effects, thus, promotes vasodilation and mitigates inflammation (Fig 1.3) [37, 38].

AT2R counteracts AT1R effects by: 1-mediating opposite actions, 2-mediating opposite signaling via activating phosphatase whereas AT1R activates kinase, 3-inhibition through direct binding (heterodimerization) and 4-downregulation of AT1R expression [39, 40]. Physiologically, AT2R actions are usually masked by the more abundant AT1R. However, AT2R is upregulated in pathological conditions and do not sensitize or internalize upon stimulation (unlike AT1R) [39]. Angiotensin-converting enzyme inhibitors (ACEI) and angiotensin type 1 receptor blockers (ARBs) have long been in clinical use as first line treatment for management of essential hypertension and associated cardiovascular disorders [41]. In general, while stimulation of AT1R by Ang II promotes harmful effects in different brain disorders, Ang II stimulation of AT2R is considered protective (Fig 1.3). The development of the non-peptide AT2R agonist, compound 21 (C21), offered a therapeutic opportunity to curb AT1R deleterious effects and allowed for the recent burst in literature studying the beneficial role of AT2R stimulation under different pathological conditions. Interestingly, AT2R blockade is also under clinical investigation as a possible treatment for neuropathic pain including post-herpetic neuralgia, further adding to the complexity of the function of this intriguing receptor [42].

In addition to the classical ACE/Ang II/ATR axis, also exists an alternative axis ACE2/Ang-(1-7)/Mas; Ang II is cleaved by angiotensin-converting enzyme 2 (ACE2) to angiotensin-(1-7). Ang-(1-7) binds to its receptor, Mas. Together with the AT2R,
ACE2/Ang-(1-7)/Mas axis constitutes the protective arms of the renin angiotensin system as opposed to ACE/AngII/AT1R arm [43-45]. On the other hand, Ang II can also be cleaved by aminopeptidase A to Ang III which is further cleaved to Ang IV by aminopeptidase N. Ang IV binds to angiotensin type 4 receptor (AT4R) which is also thought to be identical with the enzyme insulin regulated aminopeptidase (IRAP) (Fig 1.2) [46]. Ang IV/AT4R has been linked to cognition and memory in the brain [47]. The most recently discovered components of the RAS system are alamandine and its receptor, the Mas-related G-coupled receptor type D (MrgD) [48]. It is considered to have protective actions similar to Ang-(1-7), however, its function in brain disorders has not yet been examined.

**RENIN ANGIOTENSIN SYSTEM IN ISCHEMIC STROKE**

A. Role of different RAS components in ischemic stroke.

**Angiotensin II and stroke:** Angiotensin II plays a central role in ischemic stroke injury. Transgenic mice overexpressing angiotensin II show smaller penumbra area at 1 h and larger infarct size at 24 h after pMCAO, which is associated with lower regional cerebral blood flow [49]. Similarly, human renin and angiotensinogen double transgenic mice show reduced CBF, larger infarct volume and exaggerated ischemic brain damage [50, 51]. Reduction of angiotensin II levels in these mice by treatment with Aliskiren, a selective renin inhibitor, improves 7-day stroke neurological outcome [52]. In contrast, both angiotensinogen and AT1aR KO mice show smaller lesion volume and larger penumbra size at 1 but not 24 h [49, 53]. Taken together, higher Ang II levels appear to increase brain damage and worsen stroke outcome through AT1R activation. However, It is also important to keep in mind that Angiotensin II can also provide neuroprotection
through AT2R stimulation however this effect is usually masked by the predominant AT1R.

**ACE and stroke:** Chronic pretreatment with captopril for 28 days have been shown to successfully reduce infarct area by 25% after pMCAO [54]. Others have shown infarct reduction and neurological improvement upon 5-10 days pretreatment with candesartan but not ramipril or enalapril [55, 56]. Similar results were achieved when compared ramipril with telmisartan, and the combination of both did not provide additional benefit over telmisartan alone [57]. Another group showed infarct volume reduction with ACEI pretreatment in normotensive Wistar–Kyoto WKY but not spontaneously hypertensive rats (SHRs) despite greater blood pressure (BP) reduction [58]. We have previously shown neuroprotection with post-stroke treatment with the ACEI, enalapril. The reduction in the infarct size was more pronounced with a hypotensive dose of 10 mg/kg vs. a sub-hypotensive dose of 5 mg/kg enalapril [59]. However, enalapril treatment was not able to achieve the robust functional improvement we previously saw with the AT1R blocker, candesartan, under same experimental conditions [60]. Collectively, it appears that ACEIs as a class are less beneficial than AT1R blockers in ischemic stroke possibly because of the decrease in Ang II levels and subsequent less activation of AT2R in the brain.

**AT1R and stroke:** Angiotensin type 1-receptor blockers (ARBs) have long been studied as a potential treatment for acute ischemic stroke. Unlike the case with ACEI, preclinical studies with ARBs have unequivocally reported the neurovascular protective, anti-inflammatory and anti-oxidant effects of this class when given as pre- or post-stroke treatment. Chronic pretreatment with ARBs protects from cerebral ischemia and enhances recovery after stroke in both normotensive and hypertensive rats [61, 62]. Similar
neuroprotection and behavioral recovery was achieved following post-stroke treatment with ARBs [60, 63, 64]. In our hands post-stroke treatment with the ARB, candesartan, achieved neuroprotection and functional recovery after both permanent and transient MCAO using a single hypotensive dose of 1 mg/kg [65, 66]. In the clinical setting, while chronic ARBs administration has been shown to reduce the risk of ischemic stroke [67], acute post-treatment has not shown consistent protection for stroke patients; In the Acute Candesartan Cilexetil Therapy in Stroke Survivors (ACCESS) trial that was conducted on 342 patients, candesartan reduced the cumulative 12-month mortality and number of vascular events with no effect on BP [68]. This was followed by a larger multicenter trial that recruited 2029 patients from nine north European countries, the angiotensin-receptor blocker candesartan for treatment of acute stroke (SCAST) [69]. Candesartan failed to show benefit in SCAST, which was partially attributed to the associated BP lowering, despite the small difference in BP between the treatment and placebo groups (147/82 mm Hg vs 152/84 mm Hg). This could be the reason for the more interest given in recent years to the modulation of the protective arms of the angiotensin system after stroke to avoid any blood pressure change (Fig 1.4). Interestingly, we and others have shown beneficial stroke outcome with sub-hypotensive doses of ARBs and when co-administered with tPA [70].

In vitro, cell damage after 2 h OGD was higher in primary neurons isolated from WT mice compared to AT1aR KO mice as measured by LDH release assay. Pretreatment with the AT1R blocker losartan could reduce the damage level in WT neurons to that of AT1a KO neurons [49]. Interestingly, the protective effects of ARBs pretreatment against
OGD/R or glutamate excitotoxicity were mediated through AT1R blockade and not indirect AT2R stimulation [71, 72].

**AT2R and stroke:** Direct stimulation of the angiotensin II type 2 receptor (AT2R) has emerged as a tactic to reap the benefits of ARBs without the BP lowering effects. AT2R has been linked to neuroprotection in stroke (table); AT2R stimulation reduces infarct volume while AT2R knockout mice show larger infarct [73, 74]. Interestingly, studies from our lab and other groups have shown that ARBs mediate their beneficial effects in stroke through the unopposed AT2R (and possibly AT4R) stimulation [73, 75-78]. Targeting AT2R with a clinically translatable drug represents a promising treatment for ischemic stroke provided that the molecular mechanisms involved are fully understood. Until recently, the peptide, CGP42112, remained the only available AT2R agonist with its use being limited by its poor bioavailability and off-target effects. Development of compound 21 (C21) by Vicore Pharma (Gothenburg, Sweden) paved the way for studying the function of AT2R in different disease conditions [79].

**ACE2/Ang-(1-7)/Mas axis in stroke:** Components of the ACE2/Ang-(1-7)/Mas axis are upregulated acutely after experimental ischemic stroke [80]. ICV infusion of either Ang 1-7 or the ACE2 activator, diminazine aceturate (DIZE), attenuates cerebral infarct size and stroke neurological impairment via an anti-inflammatory effect [81-83]. Similarly, neuronal overexpression of ACE2 protects mice from MCAO-induced brain infarction and neurological deficit with a more pronounced protection in older animals [84, 85]. This protection was mediated via increasing cerebral blood flow, angiogenic factors, and Ang 1-7/Ang II ratio in the brain, and was independent of blood pressure change.
Recently, Ang-(1-7) has been shown to promote angiogenesis in the brain [86]. ICV infusion of Ang-(1-7) for 4 weeks increased rat brain endothelial proliferation and capillary density. This 4-week pretreatment enhanced brain ischemic tolerance by reducing cerebral infarct and behavioral deficit after MCAO. Interestingly, the use of eNOS inhibitor (L-NIO) or the Mas receptor antagonist, A-779, blocked the angiogenic and neuroprotective effects of Ang 1-7. These findings demonstrate that Ang 1-7 promotes angiogenesis and neuroprotection via Mas receptor stimulation and subsequent eNOS activation.

As discussed above, ACE2/Ang-(1-7)/Mas axis provides similar neuroprotection and neurovascular restoration to that of AT1R blockade and AT2R stimulation, and efforts to target this axis with a clinically feasible treatment are also ongoing (discussed in review by Regenhardt et al.) [87].

**Ang IV/AT4R axis in stroke:** A less defined component of the brain angiotensin system is the Ang IV/IRAP axis. Ang II is cleaved by aminopeptidase A to Ang III, which is further cleaved by aminopeptidase N to Ang IV. Ang IV has been shown to be cerebroprotective after ischemic stroke. When infused through the internal carotid artery after embolic stroke in rats, Ang IV achieved a dose dependent reduction in infarct size, neurological deficit and mortality at 24 h through NO-dependent redistribution of blood to the ischemic areas [88]. Ang IV is a potent inhibitor of insulin-regulated aminopeptidase (IRAP). As anticipated, IRAP KO mice show reduced 24 h infarct volume and increased compensatory blood flow after transient 2 h MCAO [89]. Ang IV/AT4R axis is studied more in relation to learning and memory as discussed later.
**Compound 21 (C21) as a potential stroke treatment:** C21 is the first nonpeptide AT2R agonist [90]. It is highly selective to AT2R versus AT1R (4000-fold) with a Ki value of 0.4 nM for the AT2R receptor and a Ki >10 µM for the AT1R receptor [90, 91]. Being non-peptide, hydrophilic and having a molecular mass of 497.6 g/mol, C21 is soluble in water and most solvents. While C21 is orally available (20–30%) with a half-life of 4 hours in rats [90], it could have poor CNS penetration through the intact blood brain barrier [92]. Yet, it is perceived to be able to adequately reach the brain after blood brain barrier (BBB) disruption. In preclinical models, C21 treatment has shown promising results in cardiovascular diseases including myocardial ischemia and pulmonary hypertension among other conditions [93-95]. While still in the preclinical phase, the above-mentioned criteria make C21 an excellent drug candidate for the treatment of ischemic stroke.
Figure 1.1. Schematic diagram representing IL-10/IL-10 receptor signaling.

Interleukin 10 mediates its actions through binding to interleukin 10 receptor complex. IL-10 receptor complex is a heterodimer of two chains, IL-10R1 and IL-10R2. While IL-10R1 is specific to IL-10 signaling pathway, IL-10R2 can form receptor complexes for other cytokines as well. Interleukin 10 activates the JAK1-STAT3 (Janus Kinase-Signal Transducer and Activator of Transcription 3) signaling pathway that leads to the IL-10 anti-inflammatory response. IL-10 has also been shown to increase AKT phosphorylation. IL-10 is thought to mediate its direct neuroprotective actions through increasing STAT3 and AKT phosphorylation.
Figure 1.1

[Diagram showing IL-10 signaling with IL-10R1 and IL-10R2 leading to p-AKT and p-STAT3, which result in Neuroprotection]
**Figure 1.2. Schematic diagram representing the renin angiotensin system components.** The ten amino acid peptide, angiotensin I (Ang I), is generated from angiotensinogen (AGN) via renin. Angiotensin converting enzyme (ACE) converts Ang I to Ang II. The octapeptide, Ang II, can be further cleaved to the heptapeptides; Ang-(1-7) through ACE2 or Ang III through aminopeptidase (AMP) A which is further cleaved to the hexa-peptide, Ang IV, by aminopeptidase (AMP) N. Ang II can stimulate both angiotensin receptors type 1 (AT1R) and type 2 (AT2R). Ang-(1-7) stimulates Mas receptor while Ang IV binds to and inhibits AT4R/IRAP. RAS components in green colored shapes have been shown to be beneficial for stroke outcome in preclinical studies while red colored ones are detrimental. Pharmacological modulators of RAS components are presented in boxes; the ones written in green have been shown to improve stroke outcome while the red ones worsen it. Of these pharmacological modulators, aliskiren, angiotensin converting enzyme inhibitors (ACEI), angiotensin receptors blockers (ARBs), diminazine aceturate (DIZE), and compound 21 (C21) are non peptides. Aliskiren, ACEI and ARBs are already in clinical use as antihypertensive drugs, DIZE is an antiprotozoal drug, while C21 is still in the translational phase.
Figure 1.2
Figure 1.3. Schematic diagram representing angiotensin type 1 and type 2 receptors actions in the brain after stroke. Angiotensin type 1 receptor (AT1R) is widely expressed in adults and mediates most Ang II effects including inflammatory and vasoconstrictor actions. Angiotensin type II receptor (AT2R), which is less expressed in adults and becomes upregulated with diseases, has opposite effects, thus, promotes vasodilation and mitigates inflammation. AT2R counteracts AT1R effects by: 1-mediating opposite actions, 2-mediating opposite signaling via activating phosphatase whereas AT1R activates kinase, 3-inhibition through direct binding (heterodimerization) and 4-downregulation of AT1R expression. The balance can be shifted toward the beneficial AT2R actions by either AT1R blockers (ARBs) or AT2R stimulation with compound 21 (C21).
Figure 1.3

ARBs → AT1R

AT1R

Inflammation
Vasoconstriction
Pro-oxidation
Cell death

Ang II

AT2R

Anti-inflammation
Vasodilation
Anti-oxidation
Anti-apoptosis
Angiogenesis
Neurogenesis

AT2R antagonizes AT1R by
1. Mediating opposite actions
2. Interfering with AT1R signaling
3. Decreasing AT1R expression

Tissue damage ↔ Tissue protection
Figure 1.4. Number of publications/year on angiotensin system modulation after stroke targeting the harmful axis Ang II/ACE/AT1R system vs. those targeting the protective axes, AT2R, Ang-(1-7)/ACE2/Mas, and Ang IV/AT4. There is an increasing trend in recent years towards exploring the protective arms of renin angiotensin system after stroke to harness the beneficial actions of AT1R blockade without blood pressure lowering effect.
Figure 1.4

![Graph showing the number of research articles per year from 1999 to 2014 for different angiotensin pathways. The graph compares Ang II/ACE/AT1R to AT2R, Ang-(1-7)/ACE2/Mas & Ang IV/AT4.]
PROBLEM STATEMENT AND SPECIFIC AIMS

Angiotensin type 1-receptor blockers (ARBs) have long been studied as a potential treatment for acute ischemic stroke. Several preclinical studies have reported the neurovascular protective, anti-inflammatory and anti-oxidant effects of ARBs when given as pre- or post-stroke treatment. In the clinical setting, while chronic ARBs administration has been shown to reduce the risk of ischemic stroke [67], acute post-treatment has not shown consistent protection for stroke patients; In the Acute Candesartan Cilexetil Therapy in Stroke Survivors (ACCESS) trial that was conducted on 342 patients, candesartan reduced the cumulative 12-month mortality and number of vascular events with no effect on blood pressure [68]. This was followed by a larger multicenter trial that recruited 2029 patients from nine north European countries, the angiotensin-receptor blocker candesartan for treatment of acute stroke (SCAST) [69]. Candesartan failed to show benefit in SCAST, which was partially attributed to the associated blood pressure lowering, despite the small difference between the treatment and placebo groups (147/82 mm Hg vs 152/84 mm Hg). Currently, blood pressure management in the acute phase of stroke remains controversial with the latest guidelines recommending blood pressure reduction only if >220/120 mmHg (or 185/110 mmHg for patients eligible for tPA) [1, 96].

Direct stimulation of the angiotensin II type 2 receptor (AT2R) has emerged as a tactic to reap the benefits of ARBs without the BP lowering effects. AT2R has been linked to neuroprotection yet the mechanisms involved are not fully elucidated [73, 74]. Interestingly, studies from our lab and other groups have shown that ARBs mediate their beneficial effects in stroke through the unopposed AT2R stimulation [73, 75-77]. Taken
together, these studies show that targeting AT2R with a clinically translatable drug represents a promising treatment for ischemic stroke provided that the molecular mechanisms involved are fully understood. Until recently, the peptide, CGP42112, remained the only available AT2R agonist with its use being limited by its poor bioavailability and off-target effects. Development of compound 21 (C21) by Vicore Pharma (Gothenburg, Sweden) paved the way for studying the function of AT2R in different disease conditions [79].

Recent reports have pointed out the involvement of IL-10 in C21-mediated beneficial effects in renal inflammation, and myocardial ischemia models through AT2R stimulation [97-99]. C21 has been shown to provide renoprotection by directly increasing the levels of anti-inflammatory cytokine IL-10 in the kidney via nitric oxide (NO) signaling [97]. Similarly, C21 is anti-inflammatory in lipopolysaccharide-activated THP-1 macrophages via increased interleukin-10 production [100]. Furthermore, C21 protects against myocardial ischemia through increasing IL-10 production by CD8(+)AT2R(+) T cells [98].

We hypothesized that modulating the angiotensin system through AT2R stimulation could provide neuroprotection early on in stroke and enhance recovery through stimulating angiogenesis and neurogenesis. We investigated the possible underlying mechanisms of C21-mediated neuroprotection after stroke through IL-10 upregulation.
Specific Aims:

**Aim 1:** Define the temporal and spatial expression of IL-10 and IL-10 receptor in the ischemic and contralesional hemispheres after ischemic stroke.

In this aim, we conducted immunofluorescent co-localization studies and Western blotting to examine the temporospatial expression and cellular localization of IL-10 and its receptor in both hemispheres of sham and stroked rats at 24 and 72 hours post-ischemia.

**Aim 2:** Investigate the neurovascular protective effect of the novel AT2 agonist, Compound 21, and determine the role of IL-10 in its neuroprotection after stroke.

This aim is further subdivided into 2 sub-aims:

**Aim 2A:** Investigate the neurovascular protective and proangiogenic effects of the novel AT2 agonist, Compound 21 (C21).

We used in vivo and in vitro experiments to examine the effect of C21 on neurovascular injury, behavioral outcome and angiogenesis after stroke ischemia reperfusion injury.

**Aim 2B:** Determine the role of IL-10 in C21-mediated neuroprotection in vivo and in vitro.

We conducted in vivo and in vitro IL-10 neutralization experiments to test the possible involvement of IL-10 in C21 mediated neuroprotection.


CHAPTER 2

ANTI-INFLAMMATORY IL-10 IS UPREGULATED IN BOTH HEMISPHERES
AFTER EXPERIMENTAL ISCHEMIC STROKE: HYPERTENSION BLUNTS THE
RESPONSE¹


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Abstract

**Background:** Exogenous administration of the anti-inflammatory cytokine, interleukin 10 (IL-10), is known to promote neuroprotection and mitigate neuroinflammation after ischemia. However, endogenous expression and localization of IL-10 and its receptor (IL-10R) in the post-ischemic brain are still to be elucidated. In this investigation we aimed at determining the temporospatial expression of IL-10 in the rat brain relative to its systemic levels after ischemic stroke.

**METHODS:** Wistar rats were subjected to either permanent (pMCAO) or 3-h temporary (tMCAO) middle cerebral artery occlusion and euthanized at either 24 or 72 h. IL-10/IL-10R levels were quantified in ischemic and contralesional hemispheres and compared to shams using multiplex bead array and Western blotting, respectively. Localization of IL-10/IL-10R with markers for neurons, microglia, astrocytes & endothelial cells were examined using double labeling immunofluorescence. IL-10 was also quantified in the brain tissue of spontaneously hypertensive rats (SHRs) at 24 h after tMCAO.

**RESULTS:** After both pMCAO and tMCAO in Wistars, IL-10 was significantly upregulated in both hemispheres by $\approx 50\%$ at 24 h while IL-10R expression significantly decreased only at 72 h in the ischemic hemisphere. IL-10 and IL-10R expression highly co-localized with viable neurons in the ischemic penumbra and contralesional hemisphere. In hypertensive rats, IL-10 showed no significant contralesional upregulation and declined significantly in the ischemic side at 24 h post-ischemia.

**CONCLUSION:** Our data highlights the involvement of the ischemic and contralesional neurons in the endogenous anti-inflammatory response after ischemic stroke through
increased production of IL-10. This increase in IL-10 is blunted in hypertensive animals and may contribute to worse outcomes.

**Introduction**

Innate inflammatory response is a major component of the pathophysiology of ischemic stroke [1, 2]. While preclinical studies have shown beneficial effects with adopting an anti-inflammatory approach in the treatment of ischemic stroke, results from clinical trials have been disappointing with no benefit or even worsened outcome in ischemic stroke patients after anti-inflammatory intervention [3, 4]. This can be partly explained by the lack of full understanding of the endogenous anti-inflammatory response in the brain following ischemia.

Animal studies have confirmed the anticipated neuroprotective role of the anti-inflammatory cytokine, interleukin 10 (IL-10), in ischemic stroke. Administration of exogenous IL-10 centrally and systemically decreases the infarct size in rats after permanent focal ischemia [5], while IL-10 knockout mice showed larger infarct volume following middle cerebral artery occlusion [6]. Moreover, post-ischemic IL-10 gene transfer attenuated brain infarction in rats subjected to focal and global ischemia [7]. In *in-vitro* models, IL-10 protects murine cortical and cerebellar neurons from excitotoxic damage and oxygen glucose deprivation by activating survival pathways [6, 8]. Clinically, lower IL-10 plasma levels have been associated with increased risk of stroke [9]. However, IL-10 is increased in the serum and CSF of patients after ischemic stroke with conflicting reports on its correlation with improved versus worsened outcome [10-12]. It is likely that the ratio of inflammatory versus anti-inflammatory cytokines may give a better picture of overall status in the acute stroke period.
Despite existing knowledge, the temporospatial expression and cellular sources of endogenous IL-10 and its receptor following ischemic stroke are still not known. In the current study, we aimed to elucidate the expression and cellular sources of IL-10 and its receptor in brain tissue of Wistar rats at 24 and 72 h post ischemia. In addition, we examined the change in IL-10 levels after stroke in hypertensive rats.
METHODS

**Middle cerebral artery occlusion (MCAO):** All experimental procedures were approved by the institutional Animal Care and Use Committee of the Charlie Norwood Veterans Affairs Medical Center. 36 Adult male Wistars and 16 spontaneously hypertensive (SHR) rats (270-320 g) underwent either permanent (pMCAO) (n=9), or 3 h-temporary (tMCAO) (n=25) middle cerebral artery occlusion using the suture model, or sham operation (n=18) as described [13]. Animals were sacrificed at 24 or 72 h and brain tissues were collected for molecular analysis and immunofluorescence.

**Multiplex array system:** Cytokine levels in brain homogenates and sera from Wistar rats were simultaneously analyzed using a multiplex array system (Bio-Plex 200; Bio-Rad) according to the manufacturer instructions. After calibrating the instrument, 25 µg for brain and 50 µL of sera were run in triplicates using 5-plex assay kits for tissue and sera respectively and compared to serial dilutions of the standards provided with the kits. Concentrations were determined using the Bio-Plex Manager software program (Bio-Rad version 4.1.1). Cytokine levels below detection limit were assigned a zero value.

**Enzyme linked immunosorbent assay (ELISA):** Brain homogenates from spontaneously hypertensive rats (SHRs) were analyzed for IL-10 using Rat IL-10 sandwich ELISA (RayBiotech) according to the manufacturer’s instructions.

**Western blotting:** Brain homogenate aliquots containing 50 µg protein were separated on SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-IL-10 Ra rabbit anti-rat antibody (1:500, Santa Cruz) [13]. Protein bands were quantified and normalized to β-actin using ImageJ software.
**Double-labelling Immunofluorescence:** 4 µm paraffin embedded brain sections were processed simultaneously. After rehydration, the sections were boiled in sodium citrate buffer (pH 6.0) for antigen retrieval, permeabilized and blocked in 10% horse serum with 1% BSA in TBS for 2 h at 25°C. For double labeling, two primary antibodies were incubated simultaneously overnight at 4°C at the following dilutions: rabbit anti-IL-10 (1/100; invitrogen), rabbit anti-IL-10Ra (1/100; Santa Cruz), mouse anti-neuronal nuclei (NeuN) (1/100; Millipore) a marker for neurons, mouse anti-glial fibrillary acidic protein (GFAP) (1/300; Sigma-Aldrich) a marker for astrocytes, mouse anti-CD68 (1/100; AbD Serotec) a marker for reactive microglia/macrophages, and goat anti-Von Willebrand factor (VWF) (1/100; Santa Cruz) a marker for endothelial cells. After washing, slides were incubated with fluorescent secondary antibodies, cover slipped with Vectashield mounting medium (Vector Laboratories) and viewed using Zeis Axio Observer.Z1 fluorescent microscope. Images were taken using 40x objective lens. Negative controls were prepared by omitting the primary antibodies.

**Statistical Analysis:** The results are expressed as the means ± SEM, and statistical analyses were performed with Student’s t-test using NCSS 2007 software. *P* values less than 0.05 were considered significant.
**Results**

**IL-10 is upregulated in both hemispheres at 24 h after MCAO in Wistars.**

We used the Multiplex array system (Bio-plex 200) to study the cytokine temporal profile in brains and sera of Wistar rats at subacute (24 h) and delayed (72 h) time points in response to MCAO. IL-10 significantly increased by about 50% in both hemispheres at 24 h after both permanent and temporary MCAO. On the other hand, inflammatory cytokines (IL-6, IL-1α & TNF-α) fold increase was much higher in the lesional hemisphere at 24 h with more upregulation after tMCAO then subsided at 72 h (Fig 2.1A). All cytokine serum levels were significantly upregulated at 24 h and further increased by 2-3 folds at 72 h (Fig 2.1B).

**Neurons are the main source of IL-10 after MCAO.**

Using double labeling immunofluorescence, we explored the spatial expression of IL-10 in sham Wistar rat brains and at 24/72 h after tMCAO using markers for neurons, astrocytes, activated microglia and endothelial cells. IL-10 showed strong co-localization with the neuronal marker, NeuN, in the ischemic penumbra and contralesional cortical, striatal and hippocampal neurons with less expression in the ischemic core neurons or activated microglia (CD68). IL-10 showed no co-localization with markers of astrocytes (GFAP) or endothelial cells (VWF) at the time points studied (Fig 2.2).

**Temporal and spatial interleukin 10 receptor (IL-10R) protein expression after tMCAO in Wistars.**

IL-10 executes its pleiotropic actions through binding to IL-10 receptor complex that is composed of two chains, IL-10Rα and IL-10Rβ. Of the two receptor subunits, IL-10Rα is
specific for the IL-10 signalling pathway while IL-10Rβ is part of other cytokine receptor complexes as well [14].

To test whether stroke modulates IL-10Rα expression, we performed Western blotting and immunostaining on Wistar brains subjected to tMCAO. IL-10Rα protein expression did not change at 24 h after tMCAO relative to baseline. However, it significantly declined in the stroked hemisphere at 72 h (Fig 2.3). Immunoflourescent co-localization showed strong IL-10R expression in the neurons (NeuN) and endothelial cells (VWF) of sham operated animals and this expression was extended to activated microglia (CD68) after tMCAO (Fig 2.4).

**IL-10 upregulation is lost in hypertensive animals after MCAO.**

Hypertension is associated with a pro-inflammatory milieu. For this reason, we examined the IL-10 levels in SHRs brains after sham operation and at 24 h after 3 h-tMCAO. Baseline IL-10 levels were less in hypertensive rats compared to Wistars. After MCAO, IL-10 was significantly decreased in the stroked hemisphere together with a slight increase in the contralesional hemisphere that was not significant (Fig 2.5).

**DISCUSSION**

This study investigated the endogenous expression of IL-10 and its receptor in the rat brain after experimental stroke. We show, for the first time, that IL-10 is upregulated in both hemispheres at 24 h after both p- and t-MCAO, in contrast to the inflammatory cytokines that were upregulated on the ischemic side. Moreover, immunostaining studies showed neurons to be the major source of IL-10 and its receptor. IL-10 receptor was also expressed in microglia & endothelial cells in accordance with the literature [8, 15]. Together with previous reports of localization of IL-6 and its receptor with ischemic brain
neurons [16], our data highlights the involvement of neurons in the ischemic inflammatory response being both the source and target of cytokine actions. The discrepancies in the cytokines levels between brain and serum at 72 h point to the strong involvement of the systemic immune response in cytokine production at this later time point and that serum cytokine levels do not necessarily reflect the local inflammation ongoing in the brain.

Hypertension is a major risk factor for ischemic stroke. The low baseline levels and loss of early upregulation of IL-10 in spontaneously hypertensive rats (SHRs) 24 h after MCAO shows an impaired anti-inflammatory response after ischemic stroke that might be associated with the impaired recovery and worsened outcome in these animals [17].

In summary, our study highlights the contribution of the ischemic and contralesional neurons to the anti-inflammatory response after ischemic stroke through upregulation of IL-10. This outcome is lost under the inflammatory milieu of hypertension. Taken together, these findings suggest that endogenous IL-10 could be a therapeutic target to reduce ischemic damage especially under hypertension. Despite this, the study is limited to the time points examined and doesn’t rule out a possible late role of IL-10 after 3 days post-ischemia involving other brain cells. While a recent study has elucidate the functional role of IL-10 in ischemic stroke [18], further investigations are needed to examine the mechanisms involved in IL-10 upregulation post-ischemia.
Competing interests

The authors declare no competing interests.

Acknowledgements

This work was funded by Veterans Affairs Merit Review (BX000891) & RO1-NS063965 to SCF.

Authors’ contributions

AF – Participation in the study design, conduction of experiments, data analysis, and drafting of the manuscript. AK – participation in the study design, conduction of experiments and data analysis. AA – participation in the conduction of experiments and data analysis. JAS – critically revising the manuscript and final edits to the manuscript. SCF – Study design, critically revising the manuscript and final edits to the manuscript.
FIGURE LEGENDS

Figure 2.1. Cytokines temporal profile in Wistar rat brains and sera after MCAO. (a) Brain cytokine analysis using Bio-plex 200 showed significant IL-10 upregulation by about 50% in both hemispheres at 24 h compared to baseline. Inflammatory cytokines were strongly increased in the ischemic hemisphere at 24 h then subsided at 72 h (b) Serum cytokine levels showed an ongoing increase at 24 and 72 h. Values are presented as mean ± SEM. i = ischemic hemisphere (red), c = contralesional hemisphere (blue). Solid line represents pMCAO, dashed line represents tMCAO. *, # Significantly different from the corresponding baseline values (p<0.05), ψ significantly different from the corresponding 24 h values (p<0.05). Baseline values correspond to sham manipulated animals.
Figure 2.2. Immunofluorescent localization of IL-10 in Wistar rat brains after tMCAO. IL-10 strongly co-localized with NeuN in the ischemic penumbra and contralesional cortical, striatal and hippocampal neurons with less expression in the ischemic core neurons or activated microglia (CD68). IL-10 showed no co-localization with markers of astrocytes (GFAP) or endothelial cells (VWF). Images were taken from 24 h brain sections. 72 h brain sections showed similar pattern. N=4 per group.
Figure 2.3. Temporal IL-10R expression in Wistar rat brains after tMCAO. Western blotting analysis showed no change in IL-10Rα protein expression in Wistars brains at 24 after tMCAO but it declined significantly at 72 h relative to baseline. # Significantly different from the sham right hemisphere (p<0.05), $ significantly different from the 72 h time point contralesional hemisphere (p<0.05), N=4-7 per group.
Temporal IL-10R protein expression in rat brain following tMCAO

Sham  tMCAO_24h  tMCAO_72h

contralesional side
ischemic side

IL-10Rα/β-actin (arbitrary units)

#,$

IL-10Rα (100 kDa)

β-actin
Figure 2.4. Immunofluorescent localization of IL-10R in Wistar rat brains after tMCAO. IL-10Rα strongly expressed in neurons (NeuN) and endothelial cells (VWF) of sham and stroked animals in addition to activated microglia/macrophages (CD68) after tMCAO but not astrocytes (GFAP). Images were taken from 24 h brain sections. 72 h brain sections showed similar results. N=4 per group.
Figure 2.5. IL-10 expression in SHR brains at 24 h after tMCAO. IL-10 levels were quantified in brains of sham and stroked SHR using ELISA. IL-10 significantly decreased in the ischemic hemisphere of SHRs with a slight non-significant increase contralesionally at 24 h after tMCAO relative to sham values. N=8 per group. * Significantly different from the sham right hemisphere (p<0.05), # significantly different from the stroked hemisphere (p<0.05), N=4-7 per group.
ELISA: IL-10 in SHR brain samples (8/31/12)

SHRs_Sham  
SHRs_tMCAO

0 100 200 300 400

left / non-stroked side  
right / stroked side

*, #
n=8 n=8

IL-10 conc. (pg/mg)

24 hours

* significantly different as compared to the stroked side.
# significantly different as compared to the shams.
BIBLIOGRAPHY


CHAPTER 3

C21 IS PROANGIOGENIC IN THE BRAIN AND RESULTS IN SUSTAINED
RECOVERY AFTER ISCHEMIC STROKE²


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Running head title: C21 enhances recovery after stroke

Conflicts of Interest and Source of Funding:

This work was funded by Veterans Affairs Merit Review (BX000891), RO1-NS063965 and the Jowdy Professorship to SCF. The authors declare no conflict of interest.

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• Word count: 5772
• Number of tables: 0
• Number of figures: 8
• Number of supplementary digital content files: 0
ABSTRACT

Introduction: Angiotensin II type 2 receptor (AT2R) stimulation has shown neuroprotection after stroke. However, the therapeutic utility of AT2R stimulation has been hampered by the lack of a specific agonist with favourable bioavailability. Compound 21 (C21), the first nonpeptide AT2R agonist, offers a potential option to enhance stroke recovery. This study aimed to investigate the effect of C21 administration on early and late stroke outcome, and the possible molecular mediators involved.

Methods: Rats were subjected to 3 h or 90 min middle cerebral artery occlusion (MCAO) and randomized to i.p. C21 (0.03 mg/kg) or saline at reperfusion. Animals were sacrificed at 24 h or 7 days and brains collected for molecular analysis and immunostaining, respectively. Functional outcome at days 1, 4 and 7 was assessed blindly. C21 angiogenic potential was assessed in vitro.

Results: After 3 h MCAO, C21 treatment reduced infarct size, and improved behavioural outcome at 24 h without affecting blood pressure. Co-administration of the AT2R antagonist (PD 123319) blocked these effects. On the molecular level, C21 decreased brain haemoglobin content, downregulated apoptotic and oxidative markers, and increased pro-survival molecules in the brain. After 90 min MCAO, C21 treatment showed sustained functional improvement at 7 days together with higher vascular density in the ischemic penumbra. In vitro, C21 showed a proangiogenic effect that was blocked with BDNF neutralization.

Conclusion: These findings demonstrate that a single dose of C21 is neurovascular protective and improves stroke outcome possibly through increasing neurotrophin activity, mitigating brain inflammation, and promoting antioxidant and proangiogenic effects.
CONDENSED ABSTRACT:

We investigated the effect of a single dose of compound 21 (C21), the first nonpeptide Angiotensin II type 2 receptor agonist, on early and late stroke outcome in rats. C21 treatment reduced infarct size, and improved behavioural outcome at 24 h without affecting blood pressure. On the molecular level, C21 decreased brain haemoglobin content, downregulated apoptotic and oxidative markers, and increased pro-survival molecules in the brain. Moreover, C21 treatment showed sustained functional improvement at 7 days together with higher vascular density in the ischemic penumbra. In vitro, C21 showed a proangiogenic effect that was blocked with BDNF neutralization.

Keywords: Compound 21; Stroke; Angiotensin II type 2 receptor; Blood pressure.
INTRODUCTION

Stroke remains the fourth leading cause of death in US and a leading cause of serious long-term disability. Tissue plasminogen activator (tPA) remains the only FDA approved treatment for ischemic stroke but benefits only 3-8.5% of stroke patients due to its narrow time window (3 - 4.5 h) and adverse effects. It is imperative to find new therapeutic strategies for stroke treatment [1].

Angiotensin II is a neuropeptide that binds to two major G-protein coupled receptors, type 1 (AT1R) and type 2 (AT2R). AT1R is highly expressed in adults and mediates most of the angiotensin II effects, including vasoconstriction and inflammation. AT2R, on the other hand, is less expressed in adults but is upregulated in pathological conditions. AT2R stimulation opposes AT1R actions by promoting anti-inflammatory, anti-proliferative and vasodilatory effects [2, 3].

Accumulating evidence from our lab and other groups has proven the neuroprotective and restorative capacities of AT1R blockers (ARBs) in experimental stroke models [4-7]. Indeed, we have shown that a single dose of the ARB, candesartan, after stroke can provide neuroprotection and sustained recovery after temporary middle cerebral artery occlusion [8]. However, results from the multicenter randomized stroke clinical trial, SCAST, showed lack of benefit with candesartan treatment, possibly due to the associated blood pressure lowering effect [9].

The beneficial effects of AT2R activation in ischemic stroke have been documented in the literature. Mice lacking AT2R exhibit larger infarct size [10]. In fact, we and others have shown that AT1R blockers mediate their neurovascular protective effects through the unopposed AT2R stimulation [10-13]. In two elegant studies, McCarthy et al. showed that
pretreatment [14] or delayed [15] intracerebroventricular administration of the AT2R agonist, CGP, improve stroke outcome. Despite this, the clinical utility of CGP is limited by its route of administration and bioavailability, together with its nonspecific effects [16].

Compound 21 (C21), the first selective nonpeptide AT2R agonist, has shown beneficial effects in different cardiovascular diseases including myocardial infarction [17] and pulmonary hypertension [18]. Still in the preclinical phase, this compound has a strong potential to be developed as stroke treatment because of its oral availability and anticipated minimal effect on blood pressure. Two very recent reports show that C21 is neuroprotective when infused centrally pre- and post-stroke induction and when given in multiple doses systemically as well [19, 20]. In our study, we aimed to investigate the effect of a single post-stroke dose of C21 on blood pressure, acute neurovascular injury and 7-days functional outcome in an ischemia reperfusion injury model, and elucidate the possible molecular mechanisms involved.

**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 Charlie Norwood Veterans Affairs Medical Center, Augusta, GA.

**Middle cerebral artery occlusion and drug treatment:**

Temporary middle cerebral artery occlusion (MCAO) was performed on adult male Wistar rats (280-320 g) using 4–0 silicon-coated nylon suture (Doccol 4037) as previously described [21]. Briefly, animals were anesthetized using 2-5 % isoflurane inhalation. The right common carotid artery bifurcation is dissected and the suture is advanced from the external carotid artery into the internal carotid artery to block the base of the middle
cerebral artery for the indicated time period of occlusion. Animals were allowed to recover after occlusion and re-anaesthetized at reperfusion. Animals were randomized to receive either intraperitoneal (i.p.) C21 (0.03 mg/kg) or saline at reperfusion. In some experiments, the AT2R blocker, PD123319, was given i.p. (3 mg/kg) in a separate syringe together with C21. The 0.03 mg/kg C21 dose has been shown previously to specifically stimulate the AT2R in the heart, an effect that was blocked with 3 mg/kg dose of PD 123319 [17]. Systemic PD 123319 administration has been shown to block AT2R in the brain [22]. For acute neurovascular endpoints and molecular analysis, animals were subjected to 3 h MCAO and sacrificed at 24 h. For long-term behavioral outcome and immunostaining, animals were subjected to 90 min MCAO and sacrificed at 7 days (Fig 1.A). The 3 h MCAO model has been established in our lab to induce reproducible robust ischemia reperfusion injury with >40% infarction size, significant hemorrhagic transformation, and exaggerated inflammatory response at 24 h [23, 24]. The 90 min MCAO was adopted for behavioral testing to achieve a substantial motor deficit with no mortality at 7 days. Sham animals were subjected to same surgical manipulation without actual MCAO occlusion.

**Blood pressure (BP) telemetry:**

Animals were implanted with telemetry transmitters (Data Sciences International, St. Paul, Minnesota, USA) according to the manufacturer’s instructions under 3-5% isoflurane inhalation anesthesia as reported previously [7]. Animals were allowed to recover for 10 days before induction of MCAO.

**Infarct size analysis and hemorrhage:**

At 24 h, rats were anesthetized with ketamine/xylazine intramuscular, transcardially perfused with 240 mL of ice cold PBS and the brains were removed. The brain tissue was
sliced into seven 2 mm-thick coronal sections (A to G) and stained with 2% 2,3,5-
triphenyltetrazolium chloride (TTC) (Sigma Chemical Co., Missouri, USA). Areas of the
infarct, stroked and non-stroked hemispheres were measured blindly using ImageJ software
(NIH) and infarct size was calculated with edema correction using the following formula:

$$100 \times \frac{\text{non-stroked} - (\text{stroked} - \text{infarct})}{\text{non-stroked}}.$$ 

Hemorrhage was quantified using a colorimetric hemoglobin detection assay kit
(Quantecrome, BioAssay Systems, Haywood, CA) as described previously [25].

**Molecular analysis:**

B, C, D, and E sections of the brain tissue were homogenized on ice in RIPA lysis buffer
using a glass pestle then briefly sonicated, centrifuged and supernatant was collected.

**Western blotting:** 50 µg protein aliquots were run on SDS-PAGE as described
previously,[21] transferred to nitrocellulose membranes and probed with the antibodies
against: p-Akt, Akt, p-eNOS, eNOS, iNOS, nNOS, cleaved capsase 3 (Cell Signaling),
AT1R, AT2R (Abcam), and BDNF (Santa Cruz). Bands optical densities were quantified
using ImageJ software (NIH), divided by the loading control (β-actin or GAPDH), and
normalized to the saline non-stroke side.

**Slot blot:** Nitrotyrosine immunoreactivity was measured as a biomarker for protein
nitration by peroxynitrite [26]. 40 µg of brain homogenate were immobilized onto a
nitrocellulose membrane using a slot-blot microfiltration unit. After blocking, the
membrane was reacted with anti-nitrotyrosine antibody (Millipore). Bands were quantified
using ImageJ software (NIH).

**Enzyme linked immunosorbent assay (ELISA):** Brain IL-10 levels were measured using
Rat IL-10 sandwich ELISA (RayBiotech) according to the manufacturer’s instructions.
**Behavioral testing:** All the tests were done by a blinded investigator.

**Modified Bederson score:** Animals were assigned a score from 0-3. The animal is given a point for each of the following: forelimb flexion when suspended by tail; decreased resistance to lateral push; and contralateral circling [27].

**Beam walk:** Animals were placed on a beam (60 cm long and 4.5 cm wide) for 1 minute and given 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 [28].

**Paw grasp:** Animal were suspended by tail and allowed to grasp a vertical pole. The Animal is given one of three scores: one for grasping by both forelimbs; two for grasping with only one forelimb and three for failure to grasp.

**Rotarod test:** Animals were pretrained for three days before MCAO. On day one, animals were habituated to stay on the rotating rod at a minimum speed of 4 rpm. On day two, animals were allowed to run at an accelerating speed of 4 to 15 rpm in 5 minutes. On day three, animals were allowed to run for an accelerating speed of 4 to 40 rpm in 5 minutes. Animals were tested before induction of stroke and at days 1, 4 & 7 after MCAO. The longest time the animal stayed on the rotating rod (4-40 rpm in 5 minutes) out of three trials, 5 minutes apart, was recorded and data was presented as percentage from baseline [29].

**Grip strength:** forelimb grip was tested using a Grip Strength Meter (Columbus instruments). The tested was repeated three times and the maximum grip force (in Newtons) was recorded.
**Immunofluorescence staining:**

At day 7, rats were anaesthetized with ketamine/xylazine and transcardially perfused with 180 mL of PBS followed by 120 mL of 10% formalin (Fischer Scientific). Brains were collected and kept in 10% Formalin for 24 h then paraffin embedded and cut into 4 µm thick sections. Sections from different animals were processed simultaneously as described previously [24]. Primary antibodies were incubated overnight at 4°C at the following dilutions: rabbit anti-laminin (1/50; Novus Biologics), rabbit anti-IL-10 (1/100; invitrogen), mouse anti-glial fibrillary acidic protein (GFAP) (1/300; Sigma-Aldrich) a marker for astrocytes, and mouse anti-CD68 (1/100; AbD Serotec) a marker for reactive microglia/macrophages. After washing, slides were incubated with fluorescent secondary antibodies, cover slipped with Vectashield mounting medium (Vector Laboratories) and viewed using Zeis Axio Observer.Z1 fluorescent microscope. Negative controls were prepared by omitting the primary antibodies.

Laminin-stained vascular profiles were counted using ImageJ software (NIH) in 5 different fields per section digitized from the ischemic border zone using a 20X objective lens. Similarly, IL-10, GFAP, and CD68 were quantified in 4 different fields from the ischemic border zone using ImageJ software (NIH).

**Cell culture:**

Human cerebromicrovascular endothelial cells (hCMECs) were provided as a gift from Dr. J. Zastre (University of Georgia). hCMECs were cultured in MCDB-131 complete medium (VEC technologies, NY). Cells were serum starved overnight in serum-free EMEM (ATCC) before experiments. C21 treatment was applied in three concentrations, 1, 10 and 100 nM. PD 123319 (0.1 µM) was applied 30 min before C21 treatment to assess
the involvement of AT2R. BDNF neutralization was achieved using anti-BDNF neutralizing antibody (NAb) (2 µg/ml) (Santa Cruz). Serum free EMEM served as control and solvent for treatments.

Western blotting on cell supernatant was conducted after concentrating 500 µl of media using Amicon Ultra 10K centrifugal filter devices (Millipore) to ~ 20 µl and boiling with 4X Laemmli buffer (BIO-RAD). Cell migration (wound healing), and tube formation assays were conducted as previously described [13].

**Statistical analyses**

Statistical tests were carried out using NCSS97 software. Differences between C21 and saline treatments were determined by Student’s un-paired t-test. A 2 way ANOVA was conducted for the PD123319 blocking experiments followed by Tukey’s post-hoc test for multiple comparisons. Data is presented as mean ± SEM.
RESULTS
C21 has no effect on blood pressure after 3 h MCAO.

A major limitation that led to ARBs’ failure in clinical trials of ischemic stroke was the associated acute blood pressure lowering effect [9]. We tested the effect of C21 on blood pressure at the selected treatment dose of 0.03 mg/kg given i.p. at reperfusion after 3 h MCAO, using continuous blood pressure telemetry. Stroke increased the blood pressure by 30 mmHg from baseline as we have previously shown and blood pressure remained elevated for 24 h [7]. C21 treatment had no effect on blood pressure compared to saline (Fig 3.1.B).

C21 is neurovascular protective at 24 h after 3 h MCAO.

Adopting the same study design of 3 h MCAO and treatment with C21 or saline at reperfusion, we tested the acute neurobehavioral and vascular outcome at 24 h on a different set of rats.

C21 treatment achieved a 40% reduction in infarct size (Fig 3.2.A), and improved functional outcome (Fig 3.2.B, C). Both effects were blocked with co-administration of the AT2R antagonist, PD123319, confirming that C21, at the given dose, mediates its neuroprotection through AT2R stimulation. Moreover, C21 treatment was associated with a decrease in hemoglobin excess into the ischemic side of the brain (Fig 3.2.D).

C21 treatment downregulates AT1R and upregulates AT2R expression after 3 h MCAO.

Since C21 mediates its actions through AT2R agonism, we examined the angiotensin II receptors expression using Western blotting in response to ischemic insult and after C21 treatment. AT1R shows as 2 bands at 45 and 60 kDa due to differential glycosylation while
AT2R shows as a single band at 40 kDa. AT1R and AT2R were upregulated in the stroked and non-stroked hemispheres, respectively, at 24 h after MCAO as compared to shams, which is consistent with the literature [30]. C21 treatment blunted the ischemia mediated AT1R receptor upregulation ipsilaterally (Fig 3.3.A), while further augmented the AT2R expression in the non-stroked hemisphere (Fig 3.3.B).

**C21 treatment increases pro-survival, neurotrophic and anti-inflammatory molecules.**

To elucidate the possible molecular mechanisms involved in C21 mediated neuroprotection, we examined the expression of different pro-survival and pro-apoptotic molecular markers using Western blotting and ELISA. C21 treatment enhanced the prosurvival Akt/eNOS/NO pathway as evident by increased Akt and eNOS phosphorylation in both hemispheres (Fig 3.4.A, B). This was associated with a reduction in the pro-apoptotic cleaved caspase 3 in the stroked hemisphere (Fig 4.C). Endothelial nitric oxide synthase enzyme (eNOS) activation has been associated with improved stroke outcome through increased nitric oxide (NO) production [31]. Furthermore, eNOS/NO activation has been reported to increase the neuroprotective cytokine, interleukin 10 (IL-10) [32], and the neurotrophin, BDNF [33, 34]. In accordance with these reports, C21 mediated eNOS phosphorylation was associated with IL-10 and BDNF upregulation in the stroked and non-stroked hemispheres, respectively (Fig 3.4.D, E).

**C21 ameliorates ischemia induced nitrative stress.**

Under the pro-oxidant milieu of ischemic stroke, NO can combine with superoxide anion to form the strong oxidant and pro-apoptotic, peroxynitrite. Furthermore, NO overproduction by the other two NOS isoforms worsens stroke outcome [35]. Therefore, we
further extended our molecular analysis to examine protein nitration (nitrotyrosine) as a marker of peroxynitrite formation, together with iNOS and nNOS protein levels. C21 treatment reduced iNOS and nNOS levels in the stroke and non-stroked hemispheres, respectively (Fig 3.5.A, B). The decrease in the iNOS levels in the ischemic side was associated with decreased nitrative stress as measured by protein nitration levels on slot blot (Fig 3.5.C).

**C21 improves long-term functional outcome at day 7 after 90 min MCAO.**

To examine the effect of C21 on long-term behavioral outcome, Wistar rats subjected to 90 min MCAO and treated with a single dose of C21 (0.03 mg/kg, i.p) or saline at reperfusion were tested blindly using a battery of behavioral tests at days 1, 4 and 7. C21 treated rats showed a sustained functional improvement on modified Bederson, beam walk and paw grasp scores (Fig 3.6.A-C). Moreover, C21 treatment was associated with full recovery to baseline values at day 7 on the rotarod and grip strength devices compared to only 70% recovery in the saline treated group (Fig 3.6.D, E). Sham animals showed no significant deficit on any of the tests (data not shown).

**C21 treated rats show higher vascular density and IL-10 expression at day 7 after 90 min MCAO.**

Immunoflourescent staining on brain sections collected at 7 days after 90 min MCAO showed increased vascular density in the ischemic border zone with C21 treatment as measured by laminin staining and comparing to shams (Fig 3.7.A). Moreover, C21 treated rats showed higher IL-10 expression in the ischemic border zone neurons (co-localization not shown), with no significant difference in the astrogliosis marker, GFAP, or activated migroglia/macrophage marker, CD68, between C21 and saline treatment (Fig 3.7.B, C, D).
C21 induces a BDNF dependent proangiogenic effect on hCMEC.

To examine the proangiogenic effect of C21 on brain endothelial cells, hCMEC, were treated with increasing concentrations of C21 (1-100 nM). Treatment increased hCMEC migration in a wound-healing assay and increased the BDNF and its pro-form levels in the supernatant in a dose dependent manner (Fig 3.8.A, B). The higher dose of 100 nM increased both cell migration and tube formation. Co-treatment with the AT2R blocker, PD123319, or blocking BDNF with neutralizing antibody (NAb) abrogated the pro-angiogenic effect (Fig 3.8.C, D).
DISCUSSION

This study shows the neurovascular protective effects and sustained functional improvement with a single dose of C21 after experimental ischemic stroke with no effect on blood pressure. 0.03 mg/kg C21 given i.p. after stroke reduced infarct size, decreased bleeding into the brain and achieved functional recovery, restoring motor abilities to pre-stroke values. Consistent with previous literature, we show the beneficial effects of AT2R agonism in a well-established model of ischemic stroke with clinically relevant drug and route of administration.

The balance between the AT1R and AT2R expression determine the Angiotensin II system function in the brain [36]. Previous studies have shown that AT2R expression is increased after ischemic stroke mainly in neurons with reports of no change or downregulation of AT1R [11, 30, 37]. Here we found an increase in expression of both receptor subtypes after ischemic insult with down regulation of AT1R and further upregulation of AT2R after C21 treatment. Our results demonstrate that AT2R, when stimulated, not only promotes its beneficial actions through its own upregulation but it can also outweigh the detrimental effects of AT1R through its downregulation.

AT2R stimulation is associated with neurotrophic, pro-survival and anti-inflammatory signals. The contralateral AT2R upregulation with C21 treatment coincided with an increase in the neurotrophin BDNF in the same hemisphere. AT2R mediated BDNF upregulation has been shown to enhance axonal plasticity after spinal cord injury [38]. BDNF itself is known to promote motor function after ischemic stroke when given exogenously [39-41]. Interestingly, one study showed prolonged neuroprotection up to 7 days after a single dose of BDNF [42]. In our hands, acute C21 treatment increased BDNF
at 24 h and achieved sustained motor recovery up to 7 days, a similar response to what we have reported previously with AT1R blockade [8]. Taken together, these findings demonstrate that early neurotrophic modulation could achieve long lasting functional recovery. We have also previously shown that AT1R antagonism promotes angiogenesis through AT2R dependent increase in BDNF expression in vitro [13]. C21 treatment achieved the same response. The proangiogenic effect of C21 in vitro correlated with higher vascular density in vivo in the ischemic borderzone at 7 days. Neovascularization in the ischemic penumbra has been correlated, clinically and experimentally, with longer survival and recovery after stroke [43, 44]. The proangiogenic effect of C21 could be one of the mechanisms by which it promotes recovery through BDNF.

C21 has been shown to lower renal and post-infarct cardiac inflammation via the anti-inflammatory cytokine, IL-10 [32, 45]. We have recently demonstrated that IL-10, which is also neuroprotective [46, 47], is increased in viable neurons after ischemic insult [24]. The increase in IL-10 with C21 could be a direct effect of neuronal AT2R stimulation promoting an autocrine protective effect against the ischemic insult. IL-10 upregulation was sustained up to 7 days after treatment. However, there was no difference in astrogliosis or activated microglia/macrophage infiltration with treatment. This shows that C21 attenuates cerebrovascular inflammation and could explain, at least in part, the dramatic reduction in infarct size after 3 h of occlusion.

C21 treatment was associated with an increase in the prosurvival p-eNOS, p-AKT and decreased cleaved caspase 3. Of the three nitric oxide synthase (NOS) isoforms, eNOS mediated nitric oxide (NO) production is protective in cerebral ischemia mainly through increased regional blood flow [48]. Recent reports have also shown that p-eNOS mediates
BDNF [33, 34] and IL-10 [32] upregulation. With C21, we found an increase in eNOS activation as measured by phosphorylation in both hemispheres, which can possibly be the underlying mechanism of BDNF and IL-10 upregulation through AT2R. Further mechanistic studies are needed in this regard.

The other two NOS isoforms, iNOS and nNOS, have been associated with worsened stroke outcome [49-52]. NO overproduction by these isoforms combines with superoxide anion to form the potent oxidant, peroxynitrite, which is upregulated after ischemia/reperfusion injury and triggers protein nitration and cell death [53-57]. Treatment with C21 reduced iNOS and nNOS expression together with a decrease in protein nitration in the stroked hemisphere, therefore, increasing cell survival through alleviation of nitrative stress.

Recently, Verdonk et al. demonstrated that C21, at high concentration, can activate both AT1R and AT1R in addition to other off-target effects [58]. This should be taken in consideration when translating C21 to the clinic since these off-target effects would likely affect the patients’ blood pressure and mask the protective effects of AT2R stimulation.

Our results add to recent reports of the neuroprotective effects of C21 when given systemically [19, 20], and further show that only a single dose is sufficient to achieve neurovascular protection after ischemia reperfusion injury. In addition we have shown sustained recovery with C21 possibly due to its proangiogenic action, an effect that is mediated by the neurotrophin BDNF.

In conclusion, this study demonstrates that acute AT2R stimulation with C21 provides neurovascular protection and enhances long-term recovery via multiple mechanisms.
Therefore, developing AT2R agonist drugs represent an attractive intervention to harness the beneficial effects of ARBs without affecting the BP.
ACKNOWLEDGEMENTS

The authors thank Vicore Pharma (Göteborg, Sweden) for the supply of C21 and Dr. Muscha Steckelings for the dosing information.

The authors thank Mr. Hiram Ocasio for his outstanding technical support with BP telemetry.
FIGURES LEGENDS:

Figure 3.1. C21 has no effect on blood pressure after 3 h MCAO. A schematic diagram depicts the in vivo study design (A). BP, as measured by telemetry, showed an increased from baseline of 95 mmHg to 125 mmHg after MCAO, decreased to 115 mmHg after reperfusion and remained elevated for 24 h. C21 administration at reperfusion didn’t affect the BP (n=5-6) (B).
A) Adult male Wistar rats

MCAO 3 h → Reperfusion + C21 i.p. (0.03 mg/kg)

21 h → BP, neurovascular/functional outcome, and molecular analysis

→ Functional outcome, and immunostaining

90 min MCAO → Behavioral testing

Day 1 → Day 4 → Day 7

B) Blood Pressure (mmHg)

- Saline
- C21

MCAO & treatment

Hour of day

80 90 100 110 120 130 140

6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 0 1 2 3 4 5 6 7
Figure 3.2. C21 is neurovascular protective at 24 h after 3 h MCAO. C21 administration (0.03 mg/kg, i.p.) at reperfusion reduced infarct size by 40 % as measured by TTC staining (A), and improved the functional outcome tests, modified Bederson (B) and beam walk (C) scores, at 24. The AT2R antagonist, PD123319 (3 mg/kg, i.p.), blocked these effects (n=4-6) (*p<0.05 vs the other three groups). C21 treated rats had >60% reduction in hemorrhagic transformation as measured by hemoglobin excess in the stroked vs non-stroked hemisphere (D).
Figure 3.3. C21 treatment downregulates AT1R and upregulates AT2R expression after 3 h MCAO. Compared to sham operated rats, stroke insult upregulated both AT1R (A) and AT2R (B) protein expression in the stroked and non-stroked hemispheres, respectively. C21 treatment modulated the AT receptors expression, downregulating AT1R and further augmenting the ischemia induced AT2R upregulation (n=4-6). Representative Western blot bands of the AT receptors expression are shown in panel (C).
Figure 3.4. C21 treatment increases pro-survival, neurotrophic and anti-inflammatory molecules. Western blotting analysis showed a C21 mediated increase in Akt and eNOS phosphorylation in both hemispheres (A, B) (n=4-6). Moreover, C21 reduced cleaved caspase 3 in the stroked hemisphere and increased the neurotrophin BDNF in the non-stroked hemisphere (C, E) (n=6). C21 treated rats showed an increase in the neuroprotective cytokine, IL-10, in the stroked hemisphere as measured by ELISA (D) (n=3-4).
**Figure 3.5. C21 ameliorates ischemia induced nitrative stress.** C21 administration reduced nNOS and iNOS protein expression in the non-stroked and stroked hemispheres, respectively, as measured by Western blotting (A, B) (n=6). Slot blot analysis showed a reduction in nitrative stress with C21 treatment as measured by nitrotyrosine formation (C) (n=6).
Figure 3.6. C21 improves long-term functional outcome at day 7 after 90 min MCAO. C21 administration at reperfusion after 90 min MCAO provided a sustained improvement in functional outcome for 7 days as measured by modified Bederson (A), beam walk (B), paw grasp (C), rotarod (D), and grip strength (E) tests (n=5-6) (*p<0.05 vs the corresponding time point in the saline treated group).
Figure 3.7. C21 treated rats show higher vascular density and IL-10 expression at day 7 after 90 min MCAO. Immunoflourescent staining on brain sections showed higher vascular density in the ischemic border zone with C21 treatment as measured by laminin staining (A) (n=5-6). Staining for IL-10 in the ischemic border zone showed higher expression with treatment (B) (n=3-4). There was no significant difference in astrogliosis or activated microglia/macrophage infiltration as measured by staining for GFAP and CD68, respectively (C, D) (n=5-6). Representative images of Laminin, IL-10, GFAP, and CD68 staining from the ischemic borderzone of C21 and saline treated rats (E).
Figure 3.8. C21 induces a BDNF dependent proangiogenic effect on hCMEC. C21 treatment (1-100 nM) achieved a dose dependent increased migration in a wound healing assay using hCMEC (A) (n=3) (*p<0.05 vs control and 1 nM dose, #p<0.05 vs control, 1 nM and 10 nM doses). The 100 nM dose of C21 significantly increased BDNF and its pro-form in the cell supernatant (B) (n=3-4) (§p<0.05 vs control and 10 nM dose). The 100 nM dose of C21 increased cell migration and tube-formation, and co-treatment with PD123319 or BDNF NAb blocked these effects (C, D) (n=3) (*p<0.05 vs control PD+C21 and PD, #p<0.05 vs control and C21+BDNF NAb).
BIBLIOGRAPHY


CHAPTER 4
THE ANGIOTENSIN TYPE II RECEPTOR AGONIST, COMPOUND 21, PROVIDES NEUROPROTECTION THROUGH INTERLEUKIN 10 UPREGULATION\textsuperscript{3}

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To be submitted.
Abstract

Introduction: We and others have shown that the angiotensin type 2 (AT2) receptor agonist, compound 21 (C21), provides neuroprotection and enhances recovery in rodent stroke models yet the mechanism involved is not known. Moreover, C21 treatment is associated with an anti-inflammatory response. Here we tested the hypothesis that C21 mediates neuroprotection by upregulating the neuroprotective and anti-inflammatory cytokine interleukin (IL)-10. Methods: Wistar rats (n=7-8) were subjected to 3 h MCA suture occlusion and treated at reperfusion with C21 (0.03 mg/kg) ± IL-10 neutralizing antibody (0.1 µg/kg) both given i.p. Endpoints at 24 h included: Infarct size, behavioral outcome, and molecular analysis. Primary rat neurons were used to test the direct neuroprotective effect of C21 in vitro. Results: C21 treatment reduced infarct size and increased IL-10 downstream survival signals, STAT3 and AKT phosphorylation, in the stroked hemisphere compared to saline. Anti-IL-10 co-treatment blocked the C21 induced reduction in infarct size and inflammatory/apoptotic markers as well as survival signal activation, but not the improvement in behavioral outcome. In vitro, C21 treatment reduced ischemic neurons apoptosis after oxygen glucose deprivation/reperfusion (OGD/R) insult as measured by cleaved caspase 3. However, C21 did not reduce LDH release. Conclusion: C21 provides acute neuroprotection after ischemia reperfusion injury through IL-10 upregulation. Further understanding of the mechanism of action will pave the way for translating C21 and future AT2 agonists to the clinical stroke setting.
Introduction

Angiotensin II is a peptide hormone that binds to four major types of receptors in the brain; angiotensin type 1 (AT1R), angiotensin type 2 (AT1R) receptors, Mas receptor, and angiotensin type 4 (AT4R). AT1R is the most extensively studied, and blockade of this receptor has long been shown to provide neuroprotection in experimental ischemic stroke. However, clinical data showed no benefit with the AT1R blocker, candesartan, treatment in the acute phase after stroke possibly because of the associated blood pressure lowering effect. We and others have recently shown that AT2R stimulation with the non-peptide agonist, compound 21 (C21), provides neuroprotection and functional recovery after experimental ischemic stroke without affecting the blood pressure.[1-4] Nevertheless, the mechanism underlying this neuroprotection is still not known.

While in vivo neuroprotection with C21 have been confirmed in four different labs including ours, direct neuroprotection in vitro is not well characterized. While the peptide AT2R agonist, CGP-42112, has been shown to protect primary cortical neurons against glucose deprivation, the authors failed to show similar protection with C21 [5]. Wu et al. showed neuroprotection with ARBs but not CGP-42112 pretreatment against OGD/R injury [6]. Interestingly, the protective effects of ARBs pretreatment against OGD/R or glutamate excitotoxicity were mediated through AT1R blockade and not indirect AT2R stimulation [6, 7].

Interleukin (IL) 10 is an anti-inflammatory cytokine that mediates its actions through activation of the JAK1-STAT3 (Janus Kinase-Signal Transducer and Activator of Transcription 3) signaling pathway [8]. IL-10 has been shown to provide direct neuroprotection in vivo and in vitro. Administration of exogenous IL-10 centrally and
systemically decreases the infarct size in rats after permanent focal ischemia [9], while IL-10 knockout mice show larger infarct volume following middle cerebral artery occlusion [10]. Moreover, post-ischemic IL-10 gene transfer attenuated brain infarction in rats subjected to focal and global ischemia [11]. Interestingly, neuroprotection by systemic immune cells such as regulatory T and B cells have also been shown to be mediated through interleukin 10 production [12-15]. In vitro, IL-10 protects murine cortical and cerebellar neurons from excitotoxic damage and oxygen glucose deprivation (OGD) by activating phosphatidylinositol 3-kinases (PI-3K) and signal transducer and activator of transcription 3 (STAT-3) pathways [10, 16, 17].

In our hands, we have seen upregulation of IL-10 with C21 treatment at 24 h in the ischemic hemisphere after 3 h MCAO. Moreover, we have seen increase in the number of IL-10 positive cells at 7 days with a single dose of C21 after 90 min MCAO. In this study, we aimed to examine the role of interleukin 10 in the neuroprotective effect of C21 using in vivo and in vitro ischemia reperfusion injury models.
METHODS

Experiments were approved by the Care of Experimental Animal Committee of Georgia Regents University/Institutional Animal Care and Use Committee (IACUC) of the Charlie Norwood Veterans Affairs Medical Center, Augusta, GA.

Middle cerebral artery occlusion and treatment:

Adult male Wistar rats (280-340 g) were subjected to 3 h middle cerebral artery occlusion (MCAO) followed by reperfusion for 21 h to achieve ischemia reperfusion injury in vivo as previously described [1]. Animals were treated according to a 2x2 study design using two separate syringes given intraperitoneal (i.p.) on each side of the body (Fig 4.1). C21 was given at a dose of 0.03 mg/kg, which has previously shown AT2R mediated neuroprotection in our hands using the same stroke model [1]. Anti-IL-10 neutralizing antibody was given at a dose of (0.1 mg/kg) based on the previously published literature. Animals were sacrificed at 24 h for molecular analysis. Behavioral outcome was assessed just before sacrifice. Sham animals were subjected to the same surgical procedure without actual MCAO occlusion.

Infarct size analysis:

At 24 h, rats were sacrificed via transcardial perfusion with ice cold PBS following ketamine/xylazine anesthesia. Brains were removed and sliced into seven 2 mm-thick coronal sections (A to G) and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma Chemical Co., Missouri, USA). Areas of the infarct, stroked and non-stroked hemispheres were measured blindly using ImageJ software (NIH) and infarct size was calculated with edema correction using the following formula: 100 x (non-stroked – (stroked – infarct))/non-stroked.
**Molecular analysis:**

B, C, D, and E sections of the brain tissue were homogenized on ice in RIPA lysis buffer using a glass pestle then briefly sonicated, centrifuged and supernatant was collected.

**Western blotting:** 50 µg protein aliquots were run on SDS-PAGE as described previously [18], transferred to nitrocellulose membranes and probed with the antibodies against: p-Akt, p-STAT3, STAT3, p-JNK, and cleaved capsase 3 (Cell Signaling). Bands optical densities were quantified using ImageJ software (NIH), divided by the loading control (GAPDH), and normalized to the saline non-stroke side.

**Enzyme linked immunosorbent assay (ELISA):** Brain IL-10 levels were measured using Rat IL-10 sandwich ELISA (RayBiotech) according to the manufacturer’s instructions.

**Behavioral testing:**

Behavioral assessment was conducted in a blinded fashion.

**Modified Bederson score:** Animals were assigned a score from 0-3. The animal is given a point for each of the following: forelimb flexion when suspended by tail; decreased resistance to lateral push; and contralateral circling [19].

**Beam walk:** Animals were placed on a beam (60 cm long and 4.5 cm wide) for 1 minute and given 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 [20].

**Paw grasp:** Animal were suspended by tail and allowed to grasp a vertical pole. The Animal is given one of three scores: one for grasping by both forelimbs; two for grasping with only one forelimb and three for failure to grasp.
**Primary neurons isolation and culture:**

Primary rat cortical neurons were isolated from embryonic day 17 (E17) pregnant Wistar rat embryos as described [21]. Brain cortices will be dissected and plated at 1 X 10^6 cells/mL on poly-D-lysine coated 24-well plates. Neurons will be cultured in neurobasal medium (GIBCO) containing 2% B27 (GIBCO) and 0.5 mM L-glutamine, to select for neurons. Staining for Microtubule-associated protein 2 (MAP-2, a neuronal marker, Abcam 1/300) and anti-glial fibrillary acidic protein (GFAP, a marker for astrocytes, Sigma-Aldrich, 1/300) showed that more than 90% of cells in our culture were neurons with less than 10% glia. Neurons were maintained in growth medium at 37 °C in a 95% air, 5% CO2 humidified atmosphere. Experiments were conducted between days in vitro (DIV) 10–12, when neurons express mature neuronal markers as previously characterized [21].

**OGD/R protocol:**

To mimic in vivo ischemia-reperfusion conditions, cells were subjected to oxygen glucose deprivation (OGD) for 3, 6 and 9 h followed by 6 h of reperfusion (R). Durations of OGD and reperfusion were based on our preliminary experiments. For OGD, cells were switched to glucose free DMEM (GIBCO) and incubated in a hypoxia chamber (ProOx C21, Biospherix, NY) (95% N2, 5% CO2, 37 °C). Reperfusion (R) was achieved by replacing the DMEM with the growth medium and further incubation under 95% air, 5% CO2 at 37 °C for 21 h. Cells were treated at reperfusion with C21 ± PD123319 (AT2R blocker, 1 µM) or anti-IL-10 neutralizing antibody (1 µg/mL).

**Statistical analyses**
Statistical tests were carried out using NCSS97 software. A 2 way ANOVA was conducted for the IL-10 blocking experiments in vivo followed by Tukey’s post-hoc test for multiple comparisons. Data is presented as mean ± SEM.

RESULTS:

**Interleukin 10 is involved in compound 21 mediated neuroprotection in vivo:**

Wistar rats were subjected to 3 h middle cerebral artery occlusion (MCAO) using the suture model followed by 21 h of reperfusion. Animals were divided into four groups according to a 2X2 study design and treated at reperfusion with 0.03 mg/kg C21 or saline i.p. with or without anti-IL-10 neutralizing antibody or IgG control as outlined in (Fig 4.1). As we have previously reported, C21 treated rats showed a reduction in infarct size compared to saline treatment. Moreover, C21 treatment reduced hemispheric swelling. Interleukin 10 (IL-10) was involved in the neuroprotective effect of C21 as manifested by abrogation of C21 mediated neuroprotection and hemispheric swelling reduction with IL-10 neutralization (Fig 4.2).

**Interleukin 10 is not involved in the C21 mediated behavioral recovery.**

C21 treatment improved bederson and beam walk scores at 24 h. IL-10 neutralization showed a trend towards blocking the improved recovery but did not reach significance suggesting that other mediators might be also involved and have stronger influence (Fig 4.3).

**Interleukin 10 neutralization blocks the C21 mediated increase in prosurvival signals and decrease in proinflammatory and proapoptotic signals.**

C21 treatment increased the prosurvival signals p-AKT and p-STAT3 and decreased the proinflammatory and proapoptotic signals p-JNK and cleaved caspase 3 in the ischemic
hemisphere. IL-10 neutralization blocked the increase in p-AKT and p-STAT3 and decrease in p-JNK and cleaved caspase 3 levels (Fig 4.4).

**C21 directly protects primary cortical neurons against ischemia reperfusion injury.**

Next, we wanted to test if C21 has a direct neuroprotective effect in vitro. For this, we used embryonic day 17 (E17) primary cortical neurons. We subjected the neuron culture to 3, 6 and 9 h of oxygen glucose deprivation (OGD) followed by 6 h of reperfusion to mimic the in vivo ischemia reperfusion injury model. We confirmed that the OGD model is working by measuring lactate dehydrogenase (LDH) release in the supernatant, which showed a time dependent increase after 3, 6 and 9 h of OGD (Fig 4.5). Similarly, OGD for 3, 6, and 9 h of OGD followed by 6 h of reperfusion resulted in more than 2-fold increase in LDH release. Interestingly, C21 treatment was not able to reduce the LDH release but significantly reduced cleaved caspase 3 at medium and high doses (100 and 1000 nM) suggesting an anti-apoptotic effect (Fig 4.6). The antiapoptotic and prosurvival effect of C21 was blocked with the AT2R antagonist PD123319 but not anti-IL-10 neutralizing antibody (Fig 4.7).

**DISCUSSION:**

These results show for the first time the involvement of IL-10 in the neuroprotective and anti-inflammatory effects of C21. Recent reports have pointed out the involvement of IL-10 in C21-mediated beneficial effects in renal inflammation, and myocardial ischemia models through AT2R stimulation [22-24]. C21 has been shown to provide renoprotection by directly increasing the levels of anti-inflammatory cytokine IL-10 in the kidney via nitric oxide (NO) signaling [22]. Similarly, C21 is anti-inflammatory in lipopolysaccharide-activated THP-1 macrophages via increased interleukin-10 production [25]. Furthermore,
C21 protects against myocardial ischemia through increasing IL-10 production by CD8(+)AT2R(+) T cells [23]. In our hands, IL-10 neutralization blocked the neuroprotective effect of C21 after ischemia reperfusion injury in vivo. However, this neuroprotection did not translate to better functional outcome. One of the potential mediators that could be involved in C21 mediated functional improvement is brain derived neurotrophic factor. We have previously published that BDNF is involved in the proangiogenic effect of C21. In addition, knocking down of BDNF abrogated the behavioral recovery with candesartan treatment (unpublished data).

We have previously shown that IL-10 colocalizes with neurons after ischemic stroke [26], others have also shown that IL-10 is involved in the systemic immune cells protective actions [12-15]. Anti-IL-10 neutralizing antibody was given in this study via the i.p. route and thus it can neutralize the interleukin 10 produced by resident brain cells as well as systemic immune cells.

While Lee et al. showed no direct neuroprotection with C21 treatment in vitro [5], we have seen a neuroprotective effect with C21 treatment manifested by increased prosurvival signals and reduction in apoptosis. The discrepancy between our results and the previous report could be due to the different in vitro stroke model used. While Lee et al. used only glucose deprivation, we used a more clinically relevant model combining both oxygen and glucose deprivation in addition to the reperfusion insult.

In conclusion, our results show that IL-10 is involved in C21 neuroprotective action in vivo. C21 elicited direct neuroprotection on primary neurons in vitro.
ACKNOWLEDGEMENTS

This study was supported by Veterans Affairs Merit Review (BX000891) & RO1-NS063965 to SCF.

The authors thank Vicore Pharma (Göteborg, Sweden) for the supply of C21 and AstraZeneca for the supply of candesartan.

CONFLICT OF INTEREST

The authors declare no conflict of interest.
Figure 4.1. Schematic diagram representing the study outline and treatment groups.

Wistar rats were subjected to 3 h MCAO using the suture model followed by reperfusion for 21 h. Treatments were administered i.p. at reperfusion in separate syringes. Animals were assigned to one of four treatment groups in a 2X2 study design as outlined in the table.
Figure 4.1

Wistar rats 280-340 g

MCAO

3 h

Reperfusion

21 h

Reperfusion

& treatment i.p.:

C21 (0.03 mg/kg) or saline

+ Anti-IL-10 (0.1 mg/kg) or IgG control

-Behavioral assessment

-Infarct size

-Molecular analyses

<table>
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<th>2X2 design</th>
<th>Anti-IL-10 neutralizing antibody</th>
<th>IgG antibody (Isotype control)</th>
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<td>C21 + IgG control</td>
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<tr>
<td>Saline</td>
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Figure 4.2. Interleukin 10 is involved in C21 mediated neuroprotection in vivo. A) Representative TTC stained brain sections from the four treatment groups showing infarct size and hemispheric swelling. B) C21 treatment reduced infarct size at 24 h after 3 h MCAO. Co-treatment with anti-IL-10 neutralizing antibody abrogated the neuroprotective effect of C21 treatment. C) C21 treatment reduced hemispheric swelling at 24 h, an effect that was blocked with anti-IL-10 co-administration. N=7-9 per group. *p<0.05 vs other groups, #p<0.05
Figure 4.2

A) 

<table>
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<tr>
<th></th>
<th>Sal + IgG</th>
<th>C21 + IgG</th>
<th>C21 + IL-10 Ab</th>
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B) 

% Infarct Size

C) 

% Hemispheric swelling

* p < 0.05

# p < 0.01
Figure 4.3. Interleukin 10 neutralization does not prevent the C21 mediated functional outcome improvement. A, B) C21 treatment improved bederson and beam walk scores while IL-10 neutralization did not prevent the functional improvement. N=7-9 per group. *p<0.05 vs Sal+IgG and Sal+IL-10
Figure 4.3

A) Bederson score

B) Beam walk score

[Bar graphs showing the comparison of Bederson score and Beam walk score across different groups marked as S1 + HgO, C21 + HgO, C21 + IL-10 Ab, and S1 + IL-10 Ab]
Figure 4.4. Interleukin 10 neutralization blocks the C21 mediated increase in prosurvival signals and decrease in proinflammatory and proapoptotic signals. C21 treatment increased the prosurvival signals p-AKT and p-STAT3 and decreased the proinflammatory and proapoptotic signals p-JNK and cleaved caspase 3 in the ischemic hemisphere. Animals treated with C21 and anti-IL-10 showed low p-AKT and p-STAT3 and high p-JNK and cleaved caspase 3 levels similar to that of saline treated animals.
Figure 4.4

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Figure 4.5. Schematic diagram of the in vitro study design. A) Primary rat cortical neurons isolated from embryonic day 17 (E17) Wistar rat embryos were subjected to 3, 6 or 9 h of oxygen and glucose deprivation (OGD) followed by 6 h of reperfusion. B) Immunostaining of primary culture for the neuronal marker MAP2 at day 10 in vitro (DIV 10). C) Time dependent increase in cell death after 3, 6, and 9 h of OGD as manifested by increased lactate dehydrogenase release (LDH) release into the supernatant.
Figure 4.5

A) Primary rat cortical neurons isolated from E17 Wistar rat embryos

3, 6, or 9 h Oxygen-glucose deprivation (OGD)

6 h Reperfusion (R) & treatment

-LDH release assay on supernatant

-Molecular analyses on cell lysate

C21 (10, 100 & 1000 nM)

B) Experiments were conducted at 9-10 days in vitro (DIV)

MAP2 staining

C) Primary rat cortical neurons

Oxygen-glucose deprivation (OGD) 3, 6 or 9 h

-LDH release assay on supernatant

![Graph showing LDH release assay](image)
Figure 4.6. C21 does not reduce LDH release but reduces cleaved caspase 3 after OGD/R. Neurons were subjected to 3, 6 and 9 h of OGD followed by 6 h of reperfusion in glucose containing medium under normoxic conditions. C21 did not affect LDH release into the supernatant (A) but decreased cleaved caspase 3 in cell lysate (B) in 100 and 100 nM concentrations.
A) Primary rat cortical neurons \\
Oxygen-glucose deprivation (OGD) & Reperfusion (R) & treatment C21 (10, 100 & 1000 nM) \\
LDH release assay on supernatant \\

B) Primary rat cortical neurons \\
Oxygen-glucose deprivation (OGD) & Reperfusion (R) & treatment C21 (10, 100 & 1000 nM) \\
Cleaved caspase 3 on cell lysate \\

C.Caspase 3 \\
β-actin
Figure 4.7. C21 anti-apoptotic effect is mediated through AT2R stimulation. Neurons were subjected to 6 h of OGD followed by 6 h of reperfusion. C21 in 100 and 100 nM concentrations decreased cleaved caspase 3 and increased the prosurvival p-AKT, which was blocked with AT2R antagonism using PD123319 but not IL-10 neutralization.
Figure 4.7

Primary rat cortical neurons → Oxygen-glucose deprivation (OGD) → Reperfusion (R) & treatment with C21 (100 & 1000 nM) ± PD123319 (1μM) or anti-IL-10 (1 μg/mL) → Cleaved caspase 3 on cell lysate

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<td>+ PD</td>
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<td>+ anti-IL-10</td>
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C.Caspase 3
p-AKT (Ser473)
β-actin


CHAPTER 5
IMPAIRED RESPONSE TO POST-STROKE CANDESARTAN TREATMENT IN A MODEL OF TYPE 2 DIABETES: RELATIONSHIP TO ANGIOTENSIN RECEPTORS EXPRESSION

Abdelrahman Y. Fouda, Trevor Hardigan, Sahar Soliman, Anna Kozak, Bindu Pillai, Adviye Ergul, and Susan C. Fagan

To be submitted
ABSTRACT

Introduction: Angiotensin receptor blockers (ARBs) have long been studied in preclinical stroke models and have shown promising results. However, most of the studies have been conducted in young normotensive/normoglycemic animals. The current study tests the ability of candesartan to improve stroke outcome in the presence of pre-existing type 2 diabetes, a major risk factor for ischemic stroke.

Methods: Type 2 diabetic Goto-Kakizaki (GK) rats, were subjected to 3 h middle cerebral artery occlusion (MCAO), randomized to receive either intravenous saline or candesartan (1 mg/kg) at reperfusion. Blood pressure (BP), behavioral outcome, vascular integrity as well as oxidative and nitrative stress markers were assessed at 24 h. Angiotensin receptors type 1 and type 2 (AT1R and AT2R) expression was examined and compared to normoglycemic Wistars. Moreover, endothelium mediated relaxation to AT2R stimulation was assessed in basilar arteries from GK rats.

Results: In GK rats, candesartan reduced the stroke-induced elevation in blood pressure only modestly. It failed, however, to improve neurobehavioral outcome, preserve vascular integrity or reduce oxidative/nitrative stress or apoptotic markers at 24 h. Stroke reduced the expression of AT2R in GK rats, a known mediator of recovery. Such an effect was not ameliorated by candesartan treatment. Conversely, in Wistars, stroke upregulated AT2R expression, an effect that was augmented by candesartan treatment. AT2R stimulation failed to improve the impaired endothelium mediated relaxation in GK rats’ basilar arteries.

Conclusion: GK rats have reduced sensitivity to post-stroke candesartan treatment. AT2R downregulation after stroke and the failure of candesartan to rescue its expression could be the reason behind this blunted response.
INTRODUCTION

Diabetes is a major risk factor for ischemic stroke and accounts, either alone or in combination with hypertension, for ~ 26% of ischemic strokes cases [1]. Moreover, stroke outcome is usually worse in diabetic/hyperglycemic patients [2]. Experimental studies attributed this worsened outcome to increased oxidative stress, as well as changes in cerebrovascular structure and myogenic tone [3].

The utility of angiotensin II type-1 receptor blockers (ARBs) has been well documented in preclinical models of ischemic stroke in normotensive/normoglycemic animals [4-6] as well as in hypertensive animals [7, 8]. Reports from our lab and other groups attributed the beneficial outcomes to the indirect stimulation of angiotensin II type-2 receptor (AT2R), through increased availability of its endogenous ligand, angiotensin II [9-11]. In the diabetic milieu, pretreatment with ARBs for 14 days improved stroke outcome in STZ-induced type 1 diabetes in rats [12] and type 2 diabetic mice [13]. Post-stroke treatment with ARBs, however, has not been tested in experimental models of diabetes.

In spite of the compelling experimental evidence on the usefulness of ARBs in acute stroke, results from clinical trials showed conflicting results; in a pilot clinical trial – ACCESS – candesartan treatment achieved favorable outcome after stroke. However, a larger multicenter trial – the SCAST – showed no benefit with post-stroke candesartan treatment [14]. One plausible explanation for the discrepancy between preclinical and clinical studies is the heterogeneity of the patient population in a clinical setting versus the use of otherwise healthy young animals in an experimental setting. It is possible that different co-morbidities affect the subject response to the same drug and dosage regimen.
Diabetes and hyperglycemia have been shown to activate local tissue renin angiotensin systems. Diabetes activates renal renin angiotensin system, an effect that has been implicated in the development of diabetic nephropathy [15]. Similarly, high glucose levels are known to activate the renin angiotensin system in different cell types, including vascular smooth muscle cells [16] and cardiomyocytes [17]. Therefore, acute angiotensin system modulation after stroke may yield different outcomes in diabetic versus non-diabetic animal models. In this study, we aimed to assess the effect of acute candesartan treatment on stroke outcome in a rat model of type 2 diabetes. The results of this study might help explain the discrepancy between preclinical and clinical outcomes of candesartan treatment after stroke.
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

Adult male Wistar, and Goto-Kakizaki (GK) rats (Taconic), weighing between 280–300 grams, were either sham-operated or subjected to 3 h middle cerebral artery occlusion (MCAO) using intraluminal suture model as described previously [18]. Animals were randomized to receive 1 mg/kg of candesartan (a gift from AstraZeneca) or saline at reperfusion via tail vein injection (Fig 5.1.A). At 24 h, animals were deeply anaesthetized with ketamine/xylazine intramuscular, transcardially perfused with 240 mL of ice cold PBS. Brains were harvested and the two hemispheres were snap-frozen separately as described previously [18]. The duration of ischemia/reperfusion and dose of 1 mg/kg of candesartan were selected to compare the response of GK rats to our previous studies in the control Wistars using the same experimental conditions with the same surgeon performing the strokes. Sham animals were subjected to the same surgical procedure without actual MCAO occlusion.

Neurobehavioral Assessment

Neurobehavioral outcome was evaluated at 24 h following stroke using Bederson score (0-3). Animals were blindly assessed for forelimb flexion, decreased resistance to lateral push and circling movement and given one point for each.

Hemoglobin Content (Vascular integrity) Measurement
Hemorrhage was quantified by measuring tissue hemoglobin in the ischemic side of the brain after perfusing the blood. Brain tissues were homogenized in 10% glycerol-Tris buffered saline solution containing Tween-20. Hemoglobin content was quantified using colorimetric hemoglobin detection assay (QuantiChrom Hemoglobin Assay Kit, BioAssay Systems) according to the manufacturer’s protocol.

**Slot Blot**

Detection of 4-hydorxynonenal (4-HNE), a marker of lipid peroxidation, and nitrotyrosine (NY), a marker of nitrosative stress, was done using slot blot technique. Briefly, proteins in tissue homogenate were immobilized on nitrocellulose membrane using Whatman Minifold slot blot system. Non-specific binding was blocked using 5% milk in TBST for 60 minutes. Membranes were then incubated with primary anti-nitrotyrosine antibody (Millipore) or anti-4 hydroxynonenenal (Alpha Diagnostic), followed by peroxidase-labeled goat anti-mouse IgG. Densitometric measurements were done using Image-J software.

**Western Blot**

Protein expression was measured by western blotting as described previously [18]. 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 cleaved caspase-3 (Cell Signaling) and Angiotensin II Receptors Type-1 (Abcam - ab9391) and type-2 (Abcam - ab9391). GAPDH (Cell Signaling) and β-actin (Sigma Aldrich) were used as loading controls. Densitometric measurements of bands were done using Image-J software.

**Blood Pressure Telemetry**
Blood pressure telemetry transmitters (Data Sciences) were implanted according to the manufacturer’s protocol and as described previously [4]. Blood pressure was measured continuously and recorded for 2 days before and 1 day after MCAO.

**Determination of Basilar Artery Reactivity**

Isometric tension exerted by the vessels was recorded via a force transducer using the wire-myograph technique (Danish Myo Technologies, Denmark). The myograph chambers were filled with Krebs buffer (NaCl 118.3, NaHCO$_3$ 25, KCl 4.7, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, CaCl$_2$ 1.5 and Dextrose 11.1 mM), gassed with 95% O$_2$ and 5% CO$_2$ and maintained at 37°C. Basilar arteries were isolated from GK and Wistar rats and vessel segments were mounted in the chamber using 40 µm-thin wires and adjusted to a baseline tension of 0.4g. Viability was tested by response to 70 mM KCl. Vessels were then preconstricted to 60% of the baseline tension with 100 nM serotonin (5-HT) and cumulative dose response curves to the specific AT2R agonist C21 (0.1 nM - 1 µM) were generated. Relaxation response was expressed as % change of 5HT response. In additional experiments, vessels were equilibrated and then incubated with 100 nM C21 ± 1 µM PD123319 (AT2R antagonist) for 30 min. Vessels were then preconstricted with 5HT and endothelium-dependent relaxation to acetylcholine (Ach, 1 nM - 1 µM) was assessed. Sensitivity (EC$_{50}$) and area under the curve (AUC) values were calculated from the respective dose-response equations. C21 was provided as a kind gift from Vicore Pharma (Göteborg, Sweden) and PD123319 was purchased from Sigma- Aldrich.

**Statistical Analysis**
All statistics were carried out using NCSS 8 software. Results were expressed as mean ± standard error of the mean (±SEM). Data was statistically analyzed using two-sample unpaired Student’s t-test. Results were considered statistically significant at $p < 0.05$. 


RESULTS

GK rats show reduced sensitivity to blood pressure lowering with candesartan after stroke.

In order to confirm the presence of hyperglycemia in the GK animals under study, we measured blood glucose daily for 8-12 days before MCAO. Animals had an average blood glucose level of 162.6 ± 15.9 mg/dL confirming the presence of persistent hyperglycemia. GK rats recorded a mean baseline arterial pressure of 109 ± 2 mm Hg, about 10 mm Hg higher than Wistars, consistent with the previously published data by other groups [19]. Middle cerebral artery occlusion resulted in ~30 mm Hg increase in blood pressure. Treatment with candesartan (1 mg/kg) at reperfusion reduced blood pressure but failed to bring it down to pre-stroke levels suggesting an impaired sensitivity to angiotensin blockade after stroke (Fig 5.1.B). It is worth mentioning that in our previous studies, the same candesartan dose administered at reperfusion successfully reduced the elevation in blood pressure in normotensive normoglycemic Wistars [4] and in hypertensive rats [7].

Post-stroke candesartan treatment fails to improve neurobehavioral outcome or preserve vascular integrity after stroke in GK rats.

Administration of candesartan at reperfusion did not result in a significant improvement in neurobehavioral outcome of GK rats at 24 h (Fig 5.2.A). Bederson score decreased only modestly with the treatment (3 versus 2.7 for saline and candesartan-treated groups, respectively). Similarly, candesartan treatment did not significantly preserve vascular integrity in GK rats as assessed by tissue hemoglobin content (Fig 5.2.B), although there was a trend towards vascular preservation (21% decrease in tissue hemoglobin with the treatment).
Post-stroke candesartan treatment fails to reduce oxidative/apoptotic markers after stroke in GK rats.

We examined the effect of candesartan treatment on lipid peroxidation, protein nitration and apoptosis. Levels of the oxidative stress markers, 4-hydroxynonenal (4-HNE), and nitrotyrosine (NY) were comparable in saline- and candesartan-treated animals (Fig 5.3.A, 3.B). Although there was a trend of decreasing cleaved caspase-3 level, a marker of apoptotic cell death, the effect of treatment was not significant (Fig 5.3.C). Taken together, these results show an impaired response to post-stroke candesartan treatment in GK rats.

GK rats have reduced AT2R expression after stroke.

To investigate the possible underlying causes of this differential response to candesartan treatment in GKS versus Wistars, we examined the angiotensin receptors (AT1R and AT2R) expression at 24 h in both rats in response to MCAO with and without candesartan treatment. Sham operated Wistar and GK rats showed no significant difference in angiotensin receptors expression (Fig 5.4.A, B). AT2R expression was upregulated after MCAO in Wistars in the contralateral hemisphere as compared to shams. Candesartan treatment further augmented the MCAO-induced AT2R upregulation. GK animals (Fig 5.4.C), on the other hand, showed reduced AT2R expression after stroke and candesartan treatment failed to enhance receptor expression (Fig 5.4.D).

AT1R showed as two bands at 45 and 60 kDa, due to differential protein glycosylation and were quantified separately. Both bands showed similar trend, with stronger upregulation of the 45 kDa band in the stroked hemisphere after MCAO in Wistars (Fig 5.4.E). Candesartan treatment decreased the AT1R expression in Wistars but failed to decrease it in GK rats (Fig 5.4.F).
AT2R stimulation fails to improve basilar artery endothelial mediated relaxation in GK rats.

To further examine the differential response to AT2R stimulation in GK and Wistar rats, basilar arteries reactivity in response to increasing doses of the AT2R agonist, C21 (0.1 nM - 1 µM), was examined using wire-myograph. Basilar arteries of Wistar but not GK rats showed a small (7-10%) relaxation to C21 starting at 10$^{-7}$ M (data not shown). Since the response was small, in the next set of experiments, the ability of C21 to enhance endothelium-dependent relaxation was measured. Preincubation with C21 (100 nM) enhanced relaxation (AUC) to Ach in control animals (vehicle 146.7 ± 3.9% vs C21 pretreatment 229.6 ± 11.6%), which was abolished by the blockade of AT2R (176.9 ± 25.5%, $p=0.007$) (Fig 5.5.A). Similarly, C21 improved sensitivity (EC$_{50}$) in control animals as indicated by a leftward shift in the dose-response curve (vehicle 110.5 ± 32 nM vs C21 pretreatment 11.9 ± 2 nM) which was abolished in the presence of PD123319 (93.8 ± 31 nM, $p=0.04$) (Fig 5.5.A). Basilar artery relaxation (AUC) was impaired in diabetic GK rats (93.9±1.8%) as we have shown previously [20] and C21 had no effect (98 ± 8.9%) indicating a disease and treatment interaction ($p<0.001$) (Fig 5.5.B).
DISCUSSION

Collectively, our results show a lack of benefit of post-stroke candesartan treatment in type 2 diabetic GK rats, as opposed to our previous findings in Wistars. This was correlated with: 1. Post stroke downregulation of AT2R in GKS versus its strong upregulation in Wistar controls, 2. The ability of candesartan to further upregulate AT2R and downregulate AT1R in Wistars but not GKS, and 3. Enhanced cerebrovascular relaxation to AT2R stimulation in Wistar but not GK rats.

In our previous studies, candesartan improved neurovascular stroke outcome in Wistar rats after 3 h MCAO when given I.V. at reperfusion at a dose of 1 mg/kg [4, 18]. This effect was associated with amelioration of oxidative stress and BP reduction to baseline values. Subjecting diabetic GK rats to same experimental conditions showed no improvement with candesartan treatment. While this is the first report to our knowledge to examine post-stroke treatment with ARBs in a diabetic model, other groups have demonstrated that chronic pretreatment with ARBs successfully reduce stroke severity in diabetic rodents [12, 13]. This beneficial role of chronic pretreatment with ARBs on stroke outcome was attributed to amelioration of oxidative and inflammatory milieu of diabetes. Our results show that such effect cannot be achieved acutely with a single dose post-stroke. Clinically, chronic candesartan treatment is associated with reduction in stroke incidence [21]. Moreover, preadmission use of ARBs is associated with reduced 30 day mortality after stroke [22]. Interestingly, in a meta-analysis of 4 clinical trials, ARBs provided less protection against stroke incidence in diabetic as compared to non-diabetic patients, demonstrating a relative reduced efficacy of ARB pretreatment as well in the diabetic setting [23].
While AT1R is plays a detrimental role after stroke, AT2R activation improves stroke outcome [24]. Furthermore, previous studies suggest a cross talk between the two receptors. AT1R activation inhibits the expression of AT2R mRNA while its blockade increases the AT2R expression [25]. Likewise, overexpression of AT2R downregulates AT1R expression [26] and its stimulation promotes a positive self-feedback [27]. In accord with a previous study by Kagiyama et al. [28] who used quantitative autoradiography to probe for AT2R in Sprague-Dawley rats after transient MCAO, we found a contralesional AT2R upregulation after stroke in Wistar rats. As expected, candesartan further augmented AT2R expression in Wistar rats after stroke. On the other hand, GK rats showed AT2R downregulation after stroke that was not rescued by candesartan. This downregulation of the receptor could be attributed to the enhanced proteolytic activity in the diabetic brain after stroke. In coherence with our findings, a recent study has reported the degradation of the neurotrophic tyrosine kinase receptor, TrkB, in the diabetic brain resulting in reduced neuroprotection against hypoxic injury [29].

In a recent study, C21 has been reported to elicit vasorelaxation in basilar arteries from spontaneously hypertensive rats (SHRs) [30]. Using the same C21 concentration range, we did not see significant vasorelaxation relaxation in basilar arteries from Wistars or GK rats. However, preincubation with C21 enhanced the endothelium mediated relaxation in Wistars but not in GK rats. These findings suggest an impaired AT2R sensitivity in GK animals at baseline compared to Wistars. This reduced sensitivity and further AT2R downregulation after stroke could be the reasons behind reduced candesartan efficacy in these animals compared to Wistar controls. Further studies are warranted, however, to
prove the causative relationship between diabetes and the dysregulation of AT2 receptor expression and sensitivity.

**Conclusion:** This study demonstrates the effect of underlying disease condition on the response to ARBs treatment after stroke. Under same experimental conditions, candesartan treatment failed to improve stroke outcome in GK rats as compared to Wistars. Differential expression and activity of the angiotensin system receptors might account for the decreased drug sensitivity under different co-morbid conditions. This study highlights the importance of studying stroke therapeutics in experimental models with different underlying diseases to better mimic the stroke clinical setting.
ACKNOWLEDGEMENTS

This study was supported by Veterans Affairs Merit Review (BX000891) & RO1-NS063965 to SCF.

The authors thank Vicore Pharma (Göteborg, Sweden) for the supply of C21 and AstraZeneca for the supply of candesartan.

CONFLICT OF INTEREST

The authors declare no conflict of interest.
FIGURE LEGENDS

Figure 5.1. GK rats show reduced sensitivity to blood pressure lowering with candesartan after stroke.

A) Schematic diagram of the study design and endpoints. B) Continuous blood pressure monitoring by telemetry showed an average basal blood pressure of 109 ± 2 mm Hg. MCAO increased blood pressure abruptly to 147 ± 4 mm Hg. Treatment with a single dose of candesartan reduced blood pressure to an average of 120 ± 3 mm Hg during the first 24 h, versus 135 ± 3 mm Hg for saline-treated animals. X-axis represents the time of day in hours. Animals were stroked at the same time (9:00) everyday to control for any diurnal variation in blood pressure. Light is turned off in animal facility from 18:00 to 6:00 as represented by black line on X-axis. MAP: mean arterial pressure.
A) Type 2 diabetic GK rats

- 3 h MCAO
- 21 h Reperfusion

Neurovascular outcome and molecular analysis

- Reperfusion & IV injection of saline or Candesartan (1 mg/kg)

B) MAP (mmHg) vs Hour of day

- Graph showing MAP changes over a 24-hour period with MCAO, reperfusion, and saline or Candesartan 1mg/kg injections.

- Key: - Saline, - Candesartan 1mg/kg
Figure 5.2. Post-stroke candesartan treatment does not ameliorate neurovascular deficits after stroke in GK rats.

A) Neurobehavioral testing was conducted using the three-point Bederson score. Animals scored 3 and 2.7 on average in the saline and candesartan-treated groups, respectively. B) Assessment of hemoglobin content in brain tissue homogenate was conducted using a specific ELISA kit. Hemoglobin content decreased from 80.9 ± 25 to 64 ± 19 µg/ mg protein with treatment. The change, however, did not reach significance.
A) Bederson Score

B) Hemoglobin Content (μg/mg protein)
Figure 5.3. Post-stroke candesartan treatment does not reduce oxidative/apoptotic markers after stroke in GK rats.

D, E) Analysis of 4-HNE and NY levels in both saline and candesartan-treated groups was conducted using slot blot. No change was observed in the oxidative or nitrative stress markers with treatment at 24 h. F) Quantification of cleaved caspase-3 at the same time point showed a trend towards decreasing the cell death marker (18% decrease) with the treatment, that did not reach significance (n= 6 and 8 in saline and candesartan-treated groups, respectively).
Figure 5.4. MCAO upregulates AT2R expression in Wistars but downregulates the receptor in GKs.

A, B) Western blotting showed no difference in angiotensin receptors expression between sham Wistars and GK rats. Since there was no difference in expression between sham Wistars and GKs, receptors’ expression after stroke was normalized to the respective sham that was given an arbitrary value of 1. C) Quantification of AT2R expression showed a significant upregulation in contralateral hemisphere of Wistars. Candesartan treatment further increased the receptor expression. D) GK rats showed a significant decrease in AT2R expression that was not rescued by candesartan treatment at 24 h. E, F) Quantification of AT1R expression showed a significant upregulation in the ischemic hemispheres of Wistars and GKs at 24 h. Candesartan decreased the receptor expression in Wistars with no change with treatment in GKs. (n= 3-4, 6, 6 for sham, saline and candesartan groups, respectively, * = p <0.05).
**Figure 5.5.** AT2R stimulation enhances basilar artery endothelium mediated relaxation in Wistar but not GK rats.

*A*) Preincubation with C21 enhanced relaxation to Ach in control animals (vehicle 146.7 + 3.9 vs. C21 pretreatment 229.6 +11.6) which was abolished by the blockade of AT2R (176.9 + 25.5, \( p = 0.007 \)). Similarly, C21 improved sensitivity in control animals (vehicle 110.5+32 vs. C21 pretreatment 11.9+2), which was abolished in the presence of PD129319 (93.8+31, \( p = 0.04 \)).

*B*) Basilar artery relaxation (AUC) was impaired in diabetic GK rats (93.9+1.8%) as compared to controls (146.7+3.9%) and C21 had no effect (98 + 8.9%) indicating a disease and treatment interaction (\( p < 0.001 \)).
Bibliography


CHAPTER 6
INTEGRATED DISCUSSION

The aim of this dissertation was to investigate the potential role of AT2R stimulation with the novel non-peptide agonist, compound 21, on stroke outcome and the molecular mechanisms involved. Firstly, we have shown neurovascular protective effects and sustained functional improvement with C21 treatment after experimental ischemic stroke with no effect on blood pressure. This was manifested by infarct size reduction, decreased bleeding into the brain, and improved functional outcome at 7 days with only one single treatment post-stroke. On the molecular level, C21 increased the beneficial mediators, brain derived neurotrophic factor (BDNF) and interleukin 10 (IL-10). As we have previously published with the ARB candesartan, C21 induced a robust proangiogenic response in vivo and in vitro that was shown to be mediated through BDNF. Secondly, we examined the expression of IL-10 in the brain after stroke and its involvement in the neuroprotective effect of C21 in vivo and in vitro. We showed that IL-10 is upregulated in both hemispheres at 24 h after both permanent and temporary MCAO, in contrast to the inflammatory cytokines that were upregulated on the ischemic side. Moreover, immunostaining studies showed neurons to be the major source of IL-10 and its receptor. IL-10 receptor was also expressed in microglia & endothelial cells in accordance with the literature [1, 2]. Together with previous reports of localization of IL-6 and its receptor with ischemic brain neurons [3], our data highlights the involvement of neurons in the ischemic inflammatory response being both the source and target of cytokine actions.
Using IL-10 neutralization experiments, we have shown that C21 mediates its neuroprotection in vivo, at least partly, through IL-10 upregulation. At the same time, C21 provided direct neuroprotection in vitro that was mediated through AT2R stimulation.

Lastly, we have shown a lack of benefit of post-stroke candesartan treatment in type 2 diabetic GK rats, as opposed to our previous findings in Wistars. This was correlated with: 1. Post stroke downregulation of AT2R in GKs versus its strong upregulation in Wistar controls, 2. The ability of candesartan to further upregulate AT2R and downregulate AT1R in Wistars but not GKs, and 3. Enhanced cerebrovascular relaxation to AT2R stimulation in Wistar but not GK rats.

Consistent with previous literature, we have shown the beneficial effects of AT2R agonism in a well-established model of ischemic stroke with clinically relevant drug and route of administration. Convincingly enough, four reports from four different groups (including ours) emerged in 2014 and independently verified the neuroprotective effect of C21 treatment after stroke [4-7]. The first report by Joseph et al. tested C21 in endothelin-1 induced MCAO in rats. The authors showed that pre- or post-stroke treatment with central C21 infusion or multiple systemic doses (0.03 or 0.1 mg/kg/day, i.p.) reduce the infarct size, improves behavioral outcome and ameliorates brain inflammation at 24 and 72 h after stroke. Min et al. adopted a permanent MCAO model using electrocoagulation in mice. The authors again showed neuroprotection, behavioral recovery and decreased inflammation with pre- or post-stroke multiple systemic doses (0.01 mg/kg/day, i.p.) for up to 7 days after stroke. This study was unique in showing reduction in BBB disruption and cerebral edema with C21. McCarthy et al., employed MCAO in conscious spontaneously hypertensive rats (SHRs) by administering endothelin-1 to the middle cerebral artery (MCA). Pre- and post-
stroke central administration of C21 reduced the infarct area and augmented microglial activation. The authors showed that delayed administration of C21 up to 6 h after stroke, though directly administered into the brain, could achieve neuroprotection. They also showed cerebral vasorelaxation with C21 ex-vivo corroborating the in-vivo increase in cerebral blood flow seen by Min et al.. In our report, we used temporary MCAO for 3 h or 90 min using suture model in rat. While our results confirmed the neuroprotection, reduced inflammation and behavioral recovery with post-stroke systemic C21 treatment (0.03 mg/kg i.p.), we unequivocally showed no change in blood pressure with treatment using continuous blood pressure telemetry. Furthermore, we showed sustained functional and motor improvement for up to 7 days with only one single dose administered after reperfusion. This single dose achieved sustained angiogenic response and increased vascularity at 7 days in the ischemic border zone, an effect that was attributed to BDNF upregulation with C21 treatment of brain endothelial cells in vitro. In these four reports, C21 mediated its neuroprotection through AT2R stimulation as confirmed by the use of AT2R KO mice in Min et al. report or the AT2R blocker, PD 123319, in the other three.

<table>
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<tr>
<th>Author</th>
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<tr>
<td>Iwai et al. 2004[8]</td>
<td>pMCAO in AT2R deficient mice - sacrificed at 24 h</td>
<td>No treatment</td>
<td>AT2R deficient mice showed larger infarct and neurological deficit with more decrease in cerebral blood flow</td>
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<tr>
<td>McCarthy et al. 2009[9]</td>
<td>ET-1 induced MCAO in conscious SHRs – sacrificed at 72 h</td>
<td>Pre-treatment with CGP42112 ICV (0.1 to 10 ng/kg/min)</td>
<td>CGP42112 dose-dependently reduced cortical infarct volume post-stroke and improved behavioral outcome</td>
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<td>McCarthy et al. 2012[10]</td>
<td>ET-1 induced MCAO in conscious SHRs - sacrificed at 72 h</td>
<td>Multiple post-stroke treatments with CGP42112 ICV (3 µg/kg)</td>
<td>CGP42112 reduced total infarct volume by about 80% and improved motor function</td>
</tr>
<tr>
<td>Authors</td>
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<td>Lee et al. 2012[11]</td>
<td>30 min tMCAO in mice – sacrificed at 24 h</td>
<td>Single post-stroke treatment with CGP42112 IP (1 mg/kg)</td>
<td>CGP42112 improved functional outcomes and reduced infarct volume</td>
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<td>Joseph et al. 2014[12]</td>
<td>ET-1 induced MCAO in rats – sacrificed at 72 h</td>
<td>Multiple pre &amp; post-stroke treatments with C21 ICV and IP (0.03 mg/kg/day)</td>
<td>Central and systemic treatments reduced infarct size and neurological deficits with no change in cerebral blood flow</td>
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<td>Min et al. 2014[5]</td>
<td>pMCAO – AT2R deficient mice – sacrificed after 5 d</td>
<td>Multiple treatment with C21 IP (0.01 mg/kg/day)</td>
<td>Treatment reduced the ischemic area, improved neurological deficit, and improved the decrease in cerebral blood flow after MCAO</td>
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<tr>
<td>McCarthy et al. 2014</td>
<td>ET-1 induced MCAO in conscious SHRs - sacrificed at 72 h</td>
<td>Multiple pre- &amp; post-stroke treatments with C21 ICV</td>
<td>C21 decreased infarct volume, improved motor deficit and enhanced microglia activation.</td>
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<td>Alhusban et al. 2014</td>
<td>3 h and 90 min MCAO in rats – sacrificed at 24 h and 7 d respectively</td>
<td>Single treatment with C21 IP (0.03 mg/kg) at reperfusion</td>
<td>C21 reduced infarct size and improved behavioral outcome at 24 h and 7 d, together with increasing vascular density in the ischemic penumbra.</td>
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Table abbreviations: MCAO: middle cerebral artery occlusion, pMCAO: permanent MCAO, tMCAO: transient MCAO, IP: intraperitoneal, ICV: intracerebroventricular, SHRs: spontaneously hypertensive rats, ET-1: endothelin-1, min: minutes, h: hours, d: days.

AT2R stimulation vs AT1R blockade in stroke: Our novel work on the AT2R stimulation is an extension to a long and comprehensive history of lab publications on AT1R blockade after stroke. We have replicated with C21 many of the parameters that we previously measured with AT1R blockade as discussed in the following: 1. Neurovascular protection and behavioral recovery: AT2R stimulation and AT1R blockade provide comparable protection from stroke. McCarthy et al. compared 24 h pretreatment with either C21 or Candesartan i.p. infusion at doses of 0.1 and 0.5 mg/kg/day respectively in the same experiment. Similar improvement in motor deficit at 24 h and infarct size reduction at 72 h were achieved with both treatments. In our hands, C21 treatment elicited similar
neurovascular protection to candesartan when administered under same experimental protocol. Treatment with either agent at reperfusion after 3 h MCAO reduced cerebral infarct size and hemorrhagic transformation as measured by brain hemoglobin content at 24 h. Likewise, single treatment with either agent alone successfully enhanced function recovery and increased brain vascular density after stroke. 2. Cerebral blood flow: Both treatments increased cerebral blood flow after ischemic stroke. 3. Inflammation and oxidative stress: Both treatments ameliorated brain inflammation and oxidative stress. 4. Angiogenesis: We have seen a strong proangiogenic response with candesartan and C21 in vivo and in vitro. In vitro, both C21 and candesartan induced a BDNF dependent pro-angiogenic response on human cerebromicrovascular endothelial cells (HCMECs). 5. Effect on blood pressure. While AT2R stimulation achieved neuroprotection after stroke without lowering the BP, ARBs treatment could provide neuroprotection both in hypotensive and sub-hypotensive doses. Provided the similarities in actions elicited by AT1R blockade and AT2R stimulation, it would be interesting to see the effect of combination treatment of AT2R agonism and AT1R antagonism on stroke outcome.

This dissertation provides a better understanding of the mechanisms of the beneficial actions of AT2R stimulation after stroke. it will also provide a strong foundation to translate C21 or future similar drugs to the clinical setting as a stroke treatment.
BIBLIOGRAPHY