Improving Stroke Outcome through Increasing the Activity of the BDNF/TrkB System

Ahmed Yusuf Ahmed Alhusban, Pharm.D.

(Under the Direction of Susan C Fagan Pharm.D.)

ABSTRACT

Despite significant improvement in our understanding of stroke pathophysiology and management, it is still a leading cause of death and disability worldwide. Interventions directed toward blood pressure reduction have proven beneficial in reducing stroke incidence and recurrence. Extrapolating these possible beneficial effects to the acute phase has been avoided, which limits exploitation of possible blood pressure independent effects of some antihypertensive agents. BDNF has been demonstrated to improve stroke outcome, by its dual ability to induce angiogenesis and neurogenesis. The aim of this work is to assess the interaction between blood pressure reduction with candesartan and BDNF/TrkB mediated improvement in functional outcome after stroke. To achieve this aim, the interaction between candesartan and BDNF was assessed in normotensive and hypertensive animals and in human cerebrovascular endothelial cells (hCMECs). Furthermore, the involvement of BDNF in candesartan induced long-term functional outcome improvement was assessed using shRNA mediated BDNF knockdown in normotensive animals. Additionally, the ability of candesartan to affect BDNF/TrkB activity after stroke was evaluated in hypertensive animals. To dissect the

involvement of blood pressure reduction in candesartan mediated effects, a sub-hypotensive dose of candesartan and intervention to override candesartan induced hypotensive effect was also used. Candesartan was found to positively interact with BDNF/TrkB both *in vitro* and *in vivo*. This interaction was detected in normotensive and hypertensive animals, without regard to whether they have been exposed to cerebral ischemia or not. Additionally, this positive interaction was demonstrated to be independent of its hypotensive effect. Interestingly, the angiogenic effect of candesartan was found to be mediated by BDNF/TrkB activity which was shown to be modulated by AT2 signaling. In conclusion, the positive interaction between candesartan and BDNF/TrkB is not dependent on its hypotensive effect.

INDEX WORDS: stroke, hypertension, BDNF, angiotensin receptor blockers, candesartan, angiogenesis, functional outcome, neurovascular protection, AT2.

IMPROVING STROKE OUTCOME THROUGH INCREASING THE ACTIVITY OF THE BDNF/TRKB SYSTEM

By

Ahmed Yusuf Ahmed Alhusban, Pharm. D.,
Jordan University of Science and Technology, Jordan, 2008

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

2013

IMPROVING STROKE OUTCOME THROUGH INCREASING THE ACTIVITY OF THE BDNF/TRKB SYSTEM

by

AHMED YUSUF AHMED ALHUSBAN

Major Professor: Susan C. Fagan
Committee: Adviye Ergul, MD, Ph.D.
William D. Hill, Ph.D.
Somanath PR Shenoy, Ph.D.
Azza B El-Remessy, Ph.D.,R.Ph., FAHA

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2013

DEDICATION

To my mother; the lady to whom all the gratitude after the almighty God for everything I have ever achieved and will achieve in my life. The lady who was the friend when I most needed a friend; the judge when my path goes astray; the motivator and supporter at times of stress; the beacon that guided my soul through all stages of my life……

To my father; the man who was giving everything and expecting nothing. The man whose simplicity, pure heart, and devotion taught me to face the most serious condition with faith, and determination; and to hold no grudge against anyone ……

ACKNOWLEDGMENTS

First, I would like to thank my mentor, Dr. Susan Fagan for all the support, guidance and great patience that she demonstrated through the last four years. Her unique approach in mentoring is unquestionably instrumental in everything I achieved during my graduate studies and in building my scientific character. Constantly offering challenges and opportunities, Dr. Fagan guided me to explore my potentials and helped me to develop skills to tackle any problem in the most efficient way. These attribute are essential in developing an independent scholar and a future investigator, which I hope I was and will be.

I was fortunate to work with Anna Kozak, a highly disciplined teacher and an extraordinary friend by all means. Her help and support were invaluable. The values and professional attitude that I got from her will live with me throughout my life.

The time, guidance and suggestions offered by my committee members were invaluable, exceptional and beyond all expectations. I would like to thank them all for all their effort and support.

A special thank and acknowledgment is to the exceptional friend that I was blessed to have, Maha Abdalla. Her encouraging, stimulating discussions and thoughtful insights are things that I will never forget. I would also like to acknowledge the enormous help I

got from Abdelrahman Fouda and Bindu Pillai during the adoption of new functional outcome assessment tools in the lab.

“The difference between the impossible and the possible is in a person’s determination”

Tommy Lasorda

TABLE OF CONTENTS

LIST OF FIGURES

SUPPLEMENTARY FIGURES

Problem statement and specific aims:

The objective of this study is to assess the interaction between blood pressure reduction and improving stroke outcome through BDNF/TrkB system activation. Data from our lab have demonstrated the neurovascular protective effect of blood pressure reduction after stroke. In addition our data demonstrated the ability of a single dose of candesartan to improve long-term functional outcome after stroke. This finding suggests that blood pressure reduction may not be essential to improve stroke outcome. Interestingly, we demonstrated that stroke outcome improvement was associated with the induction of a proangiogenic state in the brain. Analyzing angiogenic mediators induced by candesartan, we were able to demonstrate the partial involvement of vascular endothelial growth factor (VEGF) in the proangiogenic state. This finding highlights the involvement of other angiogenic mediators in the observed proangiogenic state.

Based on the previous discussion we **hypothesized** that blood pressure reduction improves functional outcome and recovery after cerebral ischemia by increasing the expression of brain derived neurotrophic factor (BDNF) in the brain. This hypothesis was tested using the following specific aims:

Aim 1: To assess the interaction between candesartan and BDNF in vivo and in human cerebromicrovascular endothelial cells (hCMECs).

The working hypothesis in this aim is that candesartan increases the expression of BDNF which mediates the angiogenic effects of candesartan in hCMECs. To test this hypothesis three experiments were used; in the first one the effect of candesartan on the expression of BDNF in animals with preexisting hypertension was assessed. Spontaneously hypertensive rats (SHRs) were randomized to receive either 0.3mg/kg candesartan or saline and the expression of BDNF in the brain were assessed using immunoblotting. In the second and third experiments the *in vitro* interaction between candesartan, BDNF and hCMECs was assessed; in addition the functional consequences and the contribution of BDNF to such interaction were assessed through quantifying different aspects of hCMECs behavior in response to candesartan and angiotensin II treatment to mimic the pathophysiology of hypertension.

Aim 2: To quantify the expression of BDNF in animals treated with candesartan after experimental stroke.

The working hypothesis in this aim is that blood pressure reduction after experimental ischemia using candesartan will increase the expression of BDNF in the brain. To test this hypothesis two experiments were completed. In experiment#1, the expression of BDNF after stroke in animals with and without preexisting hypertension was compared. Both SHR and WKY will be subjected to 3 hours of middle cerebral artery occlusion (MCAO) and randomized to receive either 1mg/kg single dose candesartan or saline at the time of reperfusion. The expression of BDNF was assessed using immunoblotting. In the second experiment, the contribution of blood pressure reduction to candesartan-induced expression of BDNF was assessed by randomizing WKY rats to receive either

0.3mg/kg candesartan (a sub-hypotensive dose of candesartan) or saline three hours after MCAO. The expression of BDNF was quantified using immunoblotting.

Aim 3: To determine the contribution of BDNF to the recovery of animals treated with candesartan after experimental stroke.

The hypothesis of this aim was that BDNF mediates the neurorestorative effects of candesartan through promoting angiogenesis, neurogenesis and neuronal plasticity. Testing this hypothesis involves knocking down the expression of BDNF in wistar rats using shRNA mediated silencing and exposing SHRs to MCAO. After three hours of MCAO, animals were reperfused and randomized to receive either 1mg/kg candesartan or saline.

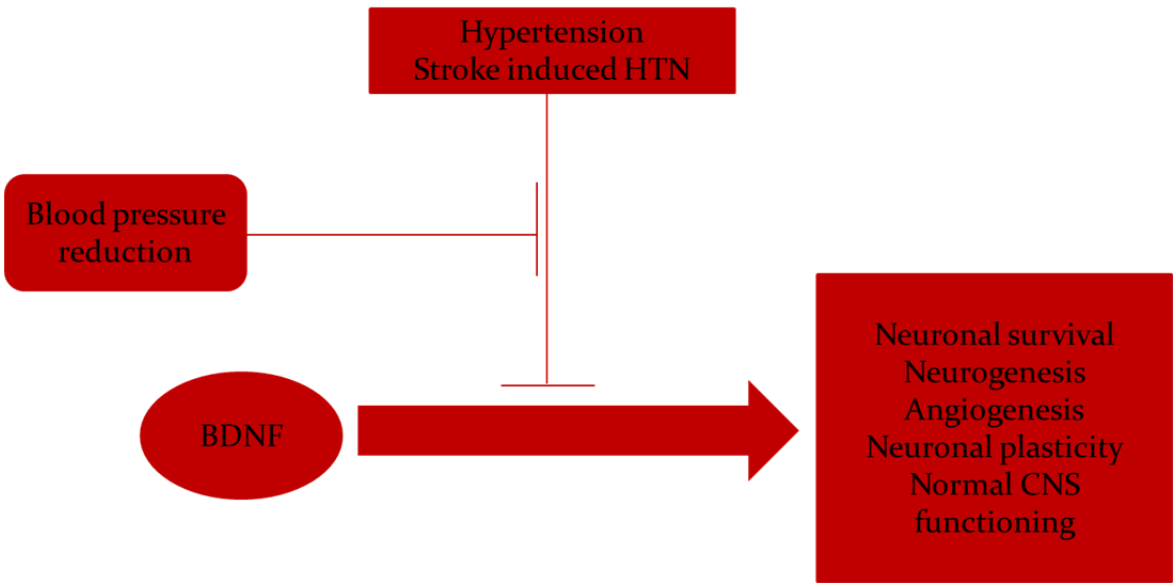


Figure 1-1: Schematic diagram of the proposed hypothesis.

Chapter 1

Review of the relevant literature and rationale

1.1 Cerebral ischemia overview:

Stroke is the fourth leading cause of death following coronary heart diseases (CHD), cancer and chronic lung restrictive diseases (CLRD); and accounted for 1 out of every 18 deaths in the United States in 2008 [1]. Annually around 795000 individuals experience a new or recurrent strokes, among those, 610000 have new strokes. In 2010, the direct and indirect costs of stroke in the US were 73.3 billion dollars [1] . Although 80-85% of patients survive their first stroke, the outcome is still suboptimal with stroke being the leading cause of disability in the United States [1] .

1.1.1 Risk factors of stroke

Stroke is a neurologic disease with multiple risk factors [2]. These risk factors can be categorized into modifiable and non-modifiable factors [2]. Modifiable risk factors include, but are not limited to, hypertension, diabetes, dyslipidemia, cigarette smoking and atrial fibrillation [2]. Non-modifiable risk factors include age, gender, race, and family history [2].

1.1.2 *Pathophysiology of stroke:*

Stroke can be categorized into either ischemic or hemorrhagic based on the pathophysiology involved, with nearly 88% of strokes being categorized as ischemic stroke [3]. The pathophysiology of ischemic stroke involves either an embolic event or a local thrombosis that will lead to blockade of a cerebral blood vessel [3]. The development of a block in cerebral circulation reduces blood flow and consequently , leads to parenchymal ischemia [4]. Due to the limited ability of the brain to store nutrients, reduced cerebral blood flow (CBF) jeopardizes brain functioning [4]. The extent of brain damage is determined by both the level of cerebral blood flow reduction and duration [5].

Experimental stroke research identified critical values of CBF below which prominent changes in neuronal function are detected [4, 6, 7]. The first of these values marks the level of CBF below which neurons develop reversible loss of function, this value is coined the functional threshold or electric threshold [6, 7]. Further reduction of CBF below the second critical point (ion pump failure) results in irreversible membrane changes and neural death. Brain tissue with CBF values between these two levels constitute the ischemic penumbra [5, 6]; whereas tissues with CBF level below the second value are termed ischemic core [6].

Theoretically; if the blood flow to the penumbral area is restored in a timely manner, complete restoration of neuronal function is expected [4]. The only FDA approved drug for the management of ischemic stroke-tissue plasminogen activator (tPA) - functions through this mechanism and is intended to lyse the clot and restore

CBF [8, 9]. Unfortunately, the use of tPA is limited by a short time window of administration and a fear of hemorrhage formation [10].

Despite the importance of restoring blood flow to the ischemic area, reperfusion may result in further injury to the ischemic tissue [4, 11]. Reperfusion induced injury is induced through a number of pathophysiologic mechanisms including: disruption of blood brain barrier (BBB) integrity, endoplasmic reticulum stress, oxidative stress, no reflow phenomenon and vascular dysfunction among other mechanisms [4, 11].

If CBF reduction is severe enough or perfusion is not restored, ischemic core extends into the penumbra and permanent irreversible changes ensue [4]. The most critical of these changes is excitotoxicity and the resulting apoptotic cell death [12]. During ischemia, adenosine triphosphate (ATP) production is reduced [6]. The plasma membrane contains a number of ATPase ionic pumps that are necessary to maintain homeostasis across the membrane [7]. Reduced ATP production renders these pumps dysfunctional which results in loss of membrane function and increased extracellular potassium (k+) levels [7]. Altered k+ homeostasis leads to depolarization and concomitant excessive release of the neurotransmitter glutamate [12-14]. Increased glutamate levels will lead to excessive stimulation of its receptors including ionotropic NMDA (N-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) [12-15]. This leads to increased levels of intracellular calcium and the consequent activation of multiple intracellular signaling pathways that culminate in cell death [12-14].

1.2 Stroke and hypertension

1.2.1 Epidemiology

Among the many risk factors of stroke, hypertension is the most prevalent [1, 3]. The life time risk of stroke doubles among individuals with hypertension as compared to individuals with blood pressure <120/80 mmHg [1] and a continuous positive correlation between blood pressure and the occurrence of stroke has also been found [16]. The effect of hypertension is not limited to increasing the risk of stroke as it is associated with worse outcome and a higher risk of hemorrhagic transformation among stroke victims [17-19].

1.2.2 Pathophysiology

Hypertension increases the risk of stroke through a number of different mechanisms including, but not limited to, remodeling of resistance arteries and endothelial dysfunction [3].

Vascular remodeling in resistance arteries is a well-recognized effect of hypertension [20, 21]. In addition, an association between vascular remodeling and increased prevalence of cardiovascular events has been suggested [22]. Based on structural studies, two forms of vascular remodeling have been characterized [23]. These structural vascular changes perturb cerebral blood flow auto regulation [24-26]. In addition, they render the cerebral vasculature more sensitive to the vasoconstrictive effect of angiotensin II [27].

Endothelial dysfunction has been identified as an early predictor of end-organ damage in patients with mild hypertension [23]. It has been also identified as a key factor in the pathogenesis of atherosclerosis [3]. Endothelial dysfunction is caused by

reduced bioavailability of nitric oxide due to increased oxidative stress [23]. The development of endothelial dysfunction is essential to the development of atherosclerosis and the consequent increased risk of cardiovascular events [21, 26, 28].

Reduced nitric oxide bioavailability might reduce the expression of growth factors, as has been noted in eNOS knockout animals which have been shown to have lower expression of growth factors like vascular endothelial growth factor (VEGF) and brain derived neurotrophic factor (BDNF) [29]. In addition, increased oxidative stress, the initiating factor in endothelial dysfunction, has been found to reduce brain expression of a number of growth factors that have been linked to improved stroke outcome such as BDNF [30, 31]. Following cerebral ischemia, hypertension was also found to reduce the expression of BDNF in the brain [32].

1.2.2 *Blood pressure reduction and stroke:*

Treatment with antihypertensive agents has been demonstrated to decrease the incidence and recurrence of stroke [16, 33-36]. In the primary prevention setting, data from Heart Outcome Prevention Evaluation study (HOPE) demonstrated the beneficial effect of antihypertensive agents in reducing the incidence of stroke in high risk patients[34]. In a subanlysis of HOPE results, treatment with ramipril reduced the incidence of stroke (relative risk=0.68 [95% CI, 0.56-0.84]) as compared to placebo [34]. Interestingly, the reduction in stroke incidence and other primary outcomes was detected despite a minimal difference in blood pressure between ramipril and placebo treated groups (136/76 mm Hg and 139/77 mm Hg, respectively) [34].

The effect of lowering blood pressure is more pronounced in the secondary prevention setting. Data from the United Kingdom Transient Ischaemic Attack Aspirin

Trial (UK-TIA) demonstrated that reducing diastolic blood pressure by 5mmHg reduces the risk of stroke recurrence by 34% (SD 7%) [16]. Similarly, a 10mmHg reduction in systolic blood pressure resulted in a 28% (SD 8%) reduction in stroke recurrence [16].

These findings are further supported by data from the Perindopril Protection Against Recurrent Stroke Study (PROGRESS) [35]. In this study the effect of perindopril only treatment was compared to perindopril/ indapamide combination and placebo treatments [35]. Patients treated with the combination had a higher reduction in blood pressure as compared to perindopril only treated patients (12.3/5 mm Hg (SE, 0.5/0.3), and 4.9/2.8 mm Hg (SE, 0.6/0.3), respectively) [35]. The difference in blood pressure reduction was translated into more robust reduction in stroke recurrence (43% (95% CI, 30–54) versus 5% (95% CI, –19to 23), respectively) [35]. Interestingly, PROGRESS results demonstrated that both normotensive and hypertensive subjects derived similar benefit from blood pressure reduction [35]. It also suggests that achieving low normal blood pressure levels (approximately 115/75 mm Hg) in patients who had a previous stroke effectively reduces stroke recurrence [35].

An important consideration in blood pressure management after stroke is the time of treatment induction [33]. Early initiation of antihypertensive agents has been avoided due to concerns about the presence of a j shape relationship between blood pressure and stroke outcome [33]. Accordingly, the induction of antihypertensive agents has been generally delayed until 3-7 days after stroke except when the blood pressure exceeds 180/105 in patients receiving tPA or 220/120 in patients not receiving tPA [10].

Data from UK-TIA refuted the presence of the often-mentioned j-shape relationship, which opened the arena for assessing the effectiveness of early induction

of antihypertensives in the management of stroke [16]. To assess the safety of acutely reduce blood pressure after stroke, Acute Candesartan Cilexetil Therapy in Stroke Survivors (ACCESS) trial randomized 342 patients to receive either candesartan or placebo within 36 hours of hospitalization after stroke [37]. Early initiation of candesartan reduced the 12-month mortality and the number of vascular events (OR = 0.475; 95% CI, 0.252–0.895) [37]. This effect was not associated with differences in the occurrence of undesirable effects [37]. Interestingly, the observed beneficial effect of early candesartan treatment was observed in the absence of appreciable differences in blood pressure levels between the two groups [37].

In contrast to results from ACCESS study, results from Scandinavian Candesartan Acute Stroke Trial (SCAST) suggested a lack of benefit from early administration of candesartan [38]. These results are further supported by a sub-analysis of Prevention Regimen for Effectively Avoiding Second Stroke (PRoFESS) which reported the lack of effect from early administration of telmisartan after stroke [39].

In light of these conflicting results, it is still unknown whether blood pressure reduction is a prerequisite for the beneficial effect of antihypertensive agents on stroke outcome.

1.3 Angiotensin Receptor Blockers (ARBs) and Stroke

Clinical and experimental data have highlighted the ability of antihypertensive agents that modulate the renin angiotensin aldosterone system (RAAS) to reduce the incidence and recurrence of ischemic stroke [36, 37, 40-43]. These agents have been

also demonstrated to effectively reverse hypertension induced vascular changes when compared to other antihypertensive agents [21, 23, 26].

To assess the potential of AT1 blockade to reduce hypertension induced end organ damage, Nishikawa assessed the effectiveness of candesartan in different models of hypertension [44]. In stroke-prone spontaneously hypertensive rats (SHRSP), candesartan reduced the incidence of stroke, and development of both albuminuria and left ventricular hypertrophy [44]. Interestingly, reduction in stroke incidence was observed even in doses that had minimal or no effect on blood pressure [44].

Further support to these findings was provided by the work of Ito et al. [45] they demonstrated the ability of chronic pretreatment with candesartan to confer neuroprotection in spontaneously hypertensive rats subjected to middle cerebral artery occlusion (MCAO) [45]. In their work, they used different doses of candesartan and compared it to both captopril and nicardipine as positive controls [45]. Similar to the Nishikawa findings [44], Ito et al. demonstrated that candesartan induced neuroprotection is not mediated by blood pressure reduction [45].

The work of Nishikawa [44]and Ito et al. [45] reported the beneficial effect of chronic pretreatment of candesartan on stroke outcome. In addition, Ito et al. reported the loss of neuroprotective effect of candesartan when administered for three days only before ischemia induction [45]. To verify whether the neuroprotective effect of candesartan is affected by the time of administration, Engelhorn et al. used different combinations of pretreatment, post treatment and their combination [46]. Both single dose and chronic post treatment with candesartan have neuroprotective effects [46]. Interestingly, the combination of pretreatment and post treatment had superior

neuroprotective effect when compared to other groups [46]. Similar results were reported by Lou et al. [47]

Following up on the possible lack of association between ARBs induced neuroprotection and blood pressure, Dai et al. used intracerebroventricular (ICV) infusion of irbesartan at a dose that is unable to affect vascular AT1 signaling [48]. ICV infusion of irbesartan improved stroke outcome and counteracted stroke induced expression of stress related proteins, mainly c-fos and c-jun [48].

The protective effect of ARBs was thought to be totally mediated through antagonizing the well-established harmful effects of angiotensin II binding to AT1 receptor. Zhou et al. demonstrated that candesartan induced neuroprotection was associated with an increase in AT2 expression in both WKY and SHRs [49]. Additionally, they demonstrated a higher expression of AT1 in SHR when compared to WKY whereas the expression of AT2 was lower in SHR animals [49].

In exciting work by Iwai et al., they reported the loss of ARB- induced neuroprotection in AT2 knockout animals [50]. In addition, they demonstrated an aggravated ischemic insult in AT2 knockout mice [50]. The proposed neuroprotective effect of AT2 stimulation was confirmed by the work of McCarthy et al. [51] Using ICV infusion of the peptide AT2 agonist CGP42112 alone or in combination with the AT2 antagonist PD123319 in SHRs [51], they demonstrated a robust neuroprotective effect of chronic pre stroke stimulation of central AT2 receptor [51].

Data from our lab has consistently demonstrated the neurovascular protective effect of candesartan [40-43, 52, 53]. This effect was exhibited as a robust reduction in hemorrhagic transformation in addition to the well reported neuroprotective effect of

candesartan [42, 43, 52]. Additionally, Kozak et al. reported that a single dose of candesartan administered at the time of reperfusion was able to improve long term functional outcome [41]. This finding supports previous reports that have demonstrated lack of association between candesartan induced neuroprotection and its ability to reduce blood pressure [44, 45]. Interestingly, candesartan induced neuroprotection was associated with the induction of a proangiogenic state in the brain [41]. This proangiogenic sate was found to be partially mediated by VEGF [41]. To further explore the effect of candesartan on angiogenesis after stroke, Guan et al. demonstrated the ability of candesartan to differentially regulate the expression of VEGF in the brain after stroke [52]. Additionally, they demonstrated the ability of candesartan to upregualte the expression of a number angiogenic mediators including BDNF [52].

1.4 Improving stroke outcome

Improving recovery after central nervous system injury requires both angiogenesis and neurogenesis induction [54] where neuronal stem cells (NSC) that are present in certain areas of the brain [54, 55] even in adults [55],"supported by their local vasculature, are thought to proliferate, migrate to, and differentiate at injury sites, affecting variable degrees of structural and functional recovery" [54]. This process suggests the presence of a complex mutual interaction between cerebral endothelial cells and neurons that is mediated by vascular endothelial growth factor (VEGF) and brain derived neurotrophin factor (BDNF) [54]. BDNF is a particularly promising target to improve stroke outcome due to its angiogenic [54, 56, 57] and neurogenic [54, 55] effects and wide spread expression in the brain [55].

1.4.1 BDNF and stroke outcome

BDNF is a member of the neurotrophin family that is widely expressed in a variety of cells types including neurons, endothelial cells and vascular smooth muscle cells[58]. This wide expression of BDNF correlates with the variety of effects that it produces as it has been found to promote angiogenesis [56, 58], neurogenesis [55, 59]and neuronal plasticity [55, 59]. Additionally it exerts neuroprotective roles and is involved in the development of the cardiovascular system[58] and normal functioning of the CNS [55].

BDNF effects are mediated through binding to two types of receptors: tropomycin related kinase-B (TrkB) and p75NTR, that are known to mediate opposing effects in tissues where TrkB mediates survival signaling while p75NTR generally induces apoptosis and axonal growth inhibition [55, 58].

Data from experimental cerebral ischemia studies highlights the significant role of BDNF in stroke recovery and outcome improvement. Ploughman et al. has reported on the detrimental effect of knocking down BDNF expression in the brain on stroke outcome where animals with reduced BDNF expression have worse motor recovery as compared to animals with normal expression of BDNF[60]. Similarly, experimental tactics aimed at increasing BDNF levels in the brain have resulted in improved recovery and reduced extent of neuronal damage following cerebral ischemia [61-65]. On the other hand, Qin et al. has found that animals expressing a BDNF variant (val66met) that is less active than the wild type BDNF had worse stroke outcome that was attributed to reduced angiogenesis after stroke [66]. Despite these promising experimental results, their translational impact is limited due to the invasiveness of the used techniques and

their associated risks. In addition, the systemic administration of BDNF is also limited because of the poor pharmacokinetic profile of BDNF [55, 67]. Therefore, attention should be directed toward agents having the ability to stimulate the BDNF/TrkB signaling [55].

1.4.2 BDNF and ARBs

In an interesting work by *Krikov* et al., they suggested a positive association between candesartan and BDNF/TrkB system. They demonstrated the ability of candesartan to increase the expression of TrkB but not BDNF at the mRNA level [68]. Recently Guan et al. demonstrated the ability of candesartan to significantly increase the expression of BDNF following experimental cerebral ischemia [52]. Additionally, Kishi et al. demonstrated an association between telmisartan induced neuroprotection in SPSHR and BDNF signaling [69]. Interestingly, the effect of ARBs on BDNF/TrkB activity has been recently demonstrated in other tissues. Ola et al. demonstrated the ability of telmisartan to increase BDNF expression in the retina of diabetic animals [70]. This increase was associated with amelioration of oxidative stress as measured by GSH levels [70].

ARBs induced neuroprotection in stroke models has been attributed to unopposed stimulation of AT2 receptor. Recently, Namsolleck et al. demonstrated a pro-recovery effect of AT2 stimulation in spinal cord injury [71]. Interestingly, they reported the ability of the AT2 receptor agonist C21 to increase BDNF expression in neurons [71].

In conclusion, ARBs offer an intriguing mechanism to upregualte BDNF expression in brain after stroke to improve stroke outcome and enhance recovery.

References

1. *Roger, V.L., et al., Heart disease and stroke statistics--2012 update: a report from the American Heart Association. Circulation, 2012. 125(1): p. e2-e220.*
2. *Go, A.S., et al., Heart disease and stroke statistics--2013 update: a report from the American Heart Association. Circulation, 2013. 127(1): p. e6-e245.*
3. *Sierra, C., A. Coca, and E.L. Schiffrin, Vascular mechanisms in the pathogenesis of stroke. Curr Hypertens Rep, 2011. 13(3): p. 200-7.*
4. *Pundik, S., K. Xu, and S. Sundararajan, Reperfusion brain injury: focus on cellular bioenergetics. Neurology, 2012. 79(13 Suppl 1): p. S44-51.*
5. *Heiss, W.D., The ischemic penumbra: how does tissue injury evolve? Ann N Y Acad Sci, 2012. 1268: p. 26-34.*
6. *Astrup, J., B.K. Siesjo, and L. Symon, Thresholds in cerebral ischemia - the ischemic penumbra. Stroke, 1981. 12(6): p. 723-5.*
7. *Astrup, J., et al., Cortical evoked potential and extracellular K+ and H+ at critical levels of brain ischemia. Stroke, 1977. 8(1): p. 51-7.*
8. *Hacke, W., et al., Randomised double-blind placebo-controlled trial of thrombolytic therapy with intravenous alteplase in acute ischaemic stroke (ECASS II). Second European-Australasian Acute Stroke Study Investigators. Lancet, 1998. 352(9136): p. 1245-51.*
9. *Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. N Engl J Med, 1995. 333(24): p. 1581-7.*
10. *Jauch, E.C., et al., Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. Stroke, 2013. 44(3): p. 870-947.*
11. *Eltzschig, H.K. and T. Eckle, Ischemia and reperfusion--from mechanism to translation. Nat Med, 2011. 17(11): p. 1391-401.*
12. *Lo, E.H., T. Dalkara, and M.A. Moskowitz, Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci, 2003. 4(5): p. 399-415.*
13. *Lipton, P., Ischemic cell death in brain neurons. Physiol Rev, 1999. 79(4): p. 1431-568.*
14. *Shimizu-Sasamata, M., et al., Attenuated neurotransmitter release and spreading depression-like depolarizations after focal ischemia in mutant mice with disrupted type I nitric oxide synthase gene. J Neurosci, 1998. 18(22): p. 9564-71.*
15. *Oguro, K., et al., Knockdown of AMPA receptor GluR2 expression causes delayed neurodegeneration and increases damage by sublethal ischemia in hippocampal CA1 and CA3 neurons. J Neurosci, 1999. 19(21): p. 9218-27.*
16. *Rodgers, A., et al., Blood pressure and risk of stroke in patients with cerebrovascular disease. The United Kingdom Transient Ischaemic Attack Collaborative Group. BMJ, 1996. 313(7050): p. 147.*

17. *Li, C., et al., Blood pressure control and risk of stroke: a population-based prospective cohort study. Stroke, 2005. 36(4): p. 725-30.*
18. *Bowes, M.P., et al., Acute hypertension, but not thrombolysis, increases the incidence and severity of hemorrhagic transformation following experimental stroke in rabbits. Exp Neurol, 1996. 141(1): p. 40-6.*
19. *Fagan, S.C. and J.H. Garcia, Hemorrhagic transformation in focal cerebral ischemia: influence of time to artery reopening and tissue plasminogen activator. Pharmacotherapy, 1999. 19(2): p. 139-42.*
20. *Park, J.B. and E.L. Schiffrin, Small artery remodeling is the most prevalent (earliest?) form of target organ damage in mild essential hypertension. J Hypertens, 2001. 19(5): p. 921-30.*
21. *Schiffrin, E.L. and R.M. Touyz, From bedside to bench to bedside: role of renin-angiotensin-aldosterone system in remodeling of resistance arteries in hypertension. Am J Physiol Heart Circ Physiol, 2004. 287(2): p. H435-46.*
22. *Rizzoni, D., et al., Prognostic significance of small-artery structure in hypertension. Circulation, 2003. 108(18): p. 2230-5.*
23. *Schiffrin, E.L., Remodeling of resistance arteries in essential hypertension and effects of antihypertensive treatment. Am J Hypertens, 2004. 17(12 Pt 1): p. 1192-200.*
24. *Baumbach, G.L. and D.D. Heistad, Remodeling of cerebral arterioles in chronic hypertension. Hypertension, 1989. 13(6 Pt 2): p. 968-72.*
25. *Harper, S.L. and H.G. Bohlen, Microvascular adaptation in the cerebral cortex of adult spontaneously hypertensive rats. Hypertension, 1984. 6(3): p. 408-19.*
26. *Rehman, A. and E.L. Schiffrin, Vascular effects of antihypertensive drug therapy. Curr Hypertens Rep, 2010. 12(4): p. 226-32.*
27. *Schiffrin, E.L., L.Y. Deng, and P. Larochelle, Morphology of resistance arteries and comparison of effects of vasoconstrictors in mild essential hypertensive patients. Clin Invest Med, 1993. 16(3): p. 177-86.*
28. *Touyz, R.M., Molecular and cellular mechanisms in vascular injury in hypertension: role of angiotensin II. Curr Opin Nephrol Hypertens, 2005. 14(2): p. 125-31.*
29. *Chen, J., et al., Endothelial nitric oxide synthase regulates brain-derived neurotrophic factor expression and neurogenesis after stroke in mice. J Neurosci, 2005. 25(9): p. 2366-75.*
30. *Salim, S., et al., Potential contribution of oxidative stress and inflammation to anxiety and hypertension. Brain Res, 2011. 1404: p. 63-71.*
31. *Hennigan, A., et al., Deficits in LTP and recognition memory in the genetically hypertensive rat are associated with decreased expression of neurotrophic factors and their receptors in the dentate gyrus. Behav Brain Res, 2009. 197(2): p. 371-7.*
32. *Lee, T.H., et al., Hypertension downregulates the expression of brain-derived neurotrophic factor in the ischemia-vulnerable hippocampal CA1 and cortical areas after carotid artery occlusion. Brain Res, 2006. 1116(1): p. 31-8.*

33. *Luders, S., Drug therapy for the secondary prevention of stroke in hypertensive patients: current issues and options. Drugs, 2007. 67(7): p. 955-63.*
34. *Yusuf, S., et al., Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. N Engl J Med, 2000. 342(3): p. 145-53.*
35. *Randomised trial of a perindopril-based blood-pressure-lowering regimen among 6,105 individuals with previous stroke or transient ischaemic attack. Lancet, 2001. 358(9287): p. 1033-41.*
36. *Schrader, J., et al., Morbidity and Mortality After Stroke, Eprosartan Compared with Nitrendipine for Secondary Prevention: principal results of a prospective randomized controlled study (MOSES). Stroke, 2005. 36(6): p. 1218-26.*
37. *Schrader, J., et al., The ACCESS Study: evaluation of Acute Candesartan Cilexetil Therapy in Stroke Survivors. Stroke, 2003. 34(7): p. 1699-703.*
38. *Sandset, E.C., et al., The angiotensin-receptor blocker candesartan for treatment of acute stroke (SCAST): a randomised, placebo-controlled, double-blind trial. Lancet, 2011. 377(9767): p. 741-50.*
39. *Yusuf, S., et al., Telmisartan to prevent recurrent stroke and cardiovascular events. N Engl J Med, 2008. 359(12): p. 1225-37.*
40. *Fagan, S.C., et al., Hypertension after experimental cerebral ischemia: candesartan provides neurovascular protection. J Hypertens, 2006. 24(3): p. 535-9.*
41. *Kozak, A., et al., Candesartan augments ischemia-induced proangiogenic state and results in sustained improvement after stroke. Stroke, 2009. 40(5): p. 1870-6.*
42. *Elewa, H.F., et al., Blood pressure lowering after experimental cerebral ischemia provides neurovascular protection. J Hypertens, 2007. 25(4): p. 855-9.*
43. *Kozak, W., et al., Vascular protection with candesartan after experimental acute stroke in hypertensive rats: a dose-response study. J Pharmacol Exp Ther, 2008. 326(3): p. 773-82.*
44. *Nishikawa, K., Angiotensin AT1 receptor antagonism and protection against cardiovascular end-organ damage. J Hum Hypertens, 1998. 12(5): p. 301-9.*
45. *Ito, T., et al., Protection against ischemia and improvement of cerebral blood flow in genetically hypertensive rats by chronic pretreatment with an angiotensin II AT1 antagonist. Stroke, 2002. 33(9): p. 2297-303.*
46. *Engelhorn, T., et al., The angiotensin II type 1-receptor blocker candesartan increases cerebral blood flow, reduces infarct size, and improves neurologic outcome after transient cerebral ischemia in rats. J Cereb Blood Flow Metab, 2004. 24(4): p. 467-74.*
47. *Lou, M., et al., Sustained blockade of brain AT1 receptors before and after focal cerebral ischemia alleviates neurologic deficits and reduces neuronal*

injury, apoptosis, and inflammatory responses in the rat. J Cereb Blood Flow Metab, 2004. 24(5): p. 536-47.

48. *Dai, W.J., et al., Blockade of central angiotensin AT(1) receptors improves neurological outcome and reduces expression of AP-1 transcription factors after focal brain ischemia in rats. Stroke, 1999. 30(11): p. 2391-8; discussion 2398-9.*
49. *Zhou, J., et al., AT1 receptor blockade regulates the local angiotensin II system in cerebral microvessels from spontaneously hypertensive rats. Stroke, 2006. 37(5): p. 1271-6.*
50. *Iwai, M., et al., Possible inhibition of focal cerebral ischemia by angiotensin II type 2 receptor stimulation. Circulation, 2004. 110(7): p. 843-8.*
51. *McCarthy, C.A., et al., Angiotensin AT2 receptor stimulation causes neuroprotection in a conscious rat model of stroke. Stroke, 2009. 40(4): p. 1482-9.*
52. *Guan, W., et al., Vascular protection by angiotensin receptor antagonism involves differential VEGF expression in both hemispheres after experimental stroke. PLoS One, 2011. 6(9): p. e24551.*
53. *Guan, W., et al., Acute Treatment with Candesartan Reduces Early Injury After Permanent Middle Cerebral Artery Occlusion. Transl Stroke Res, 2011. 2(2): p. 179-185.*
54. *Madri, J.A., Modeling the neurovascular niche: implications for recovery from CNS injury. J Physiol Pharmacol, 2009. 60 Suppl 4: p. 95-104.*
55. *Mocchetti, I. and M. Brown, Targeting neurotrophin receptors in the central nervous system. CNS Neurol Disord Drug Targets, 2008. 7(1): p. 71-82.*
56. *Kermani, P. and B. Hempstead, Brain-derived neurotrophic factor: a newly described mediator of angiogenesis. Trends Cardiovasc Med, 2007. 17(4): p. 140-3.*
57. *Sun, C., et al., The effect of brain-derived neurotrophic factor on angiogenesis. J Huazhong Univ Sci Technolog Med Sci, 2009. 29(2): p. 139-43.*
58. *Caporali, A. and C. Emanueli, Cardiovascular actions of neurotrophins. Physiol Rev, 2009. 89(1): p. 279-308.*
59. *Marini, A.M., et al., Role of brain-derived neurotrophic factor and NF-kappaB in neuronal plasticity and survival: From genes to phenotype. Restor Neurol Neurosci, 2004. 22(2): p. 121-30.*
60. *Ploughman, M., et al., Brain-derived neurotrophic factor contributes to recovery of skilled reaching after focal ischemia in rats. Stroke, 2009. 40(4): p. 1490-5.*
61. *Mahmood, A., D. Lu, and M. Chopp, Intravenous administration of marrow stromal cells (MSCs) increases the expression of growth factors in rat brain after traumatic brain injury. J Neurotrauma, 2004. 21(1): p. 33-9.*
62. *Schabitz, W.R., et al., Effect of brain-derived neurotrophic factor treatment and forced arm use on functional motor recovery after small cortical ischemia. Stroke, 2004. 35(4): p. 992-7.*

63. *Schabitz, W.R., et al., Intravenous brain-derived neurotrophic factor enhances poststroke sensorimotor recovery and stimulates neurogenesis. Stroke, 2007. 38(7): p. 2165-72.*
64. *Muller, H.D., et al., Brain-derived neurotrophic factor but not forced arm use improves long-term outcome after photothrombotic stroke and transiently upregulates binding densities of excitatory glutamate receptors in the rat brain. Stroke, 2008. 39(3): p. 1012-21.*
65. *Kurozumi, K., et al., BDNF gene-modified mesenchymal stem cells promote functional recovery and reduce infarct size in the rat middle cerebral artery occlusion model. Mol Ther, 2004. 9(2): p. 189-97.*
66. *Qin, L., et al., Genetic variant of BDNF (Val66Met) polymorphism attenuates stroke-induced angiogenic responses by enhancing anti-angiogenic mediator CD36 expression. J Neurosci, 2011. 31(2): p. 775-83.*
67. *Jang, S.W., et al., A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone. Proc Natl Acad Sci U S A, 2010. 107(6): p. 2687-92.*
68. *Krikov, M., et al., Candesartan but not ramipril pretreatment improves outcome after stroke and stimulates neurotrophin BNDF/TrkB system in rats. J Hypertens, 2008. 26(3): p. 544-52.*
69. *Kishi, T., Y. Hirooka, and K. Sunagawa, Telmisartan protects against cognitive decline via up-regulation of brain-derived neurotrophic factor/tropomyosin-related kinase B in hippocampus of hypertensive rats. J Cardiol, 2012. 60(6): p. 489-94.*
70. *Ola, M.S., et al., Telmisartan Ameliorates Neurotrophic Support and Oxidative Stress in the Retina of Streptozotocin-Induced Diabetic Rats. Neurochem Res, 2013.*
71. *Namsolleck, P., et al., AT2-receptor stimulation enhances axonal plasticity after spinal cord injury by upregulating BDNF expression. Neurobiol Dis, 2013. 51: p. 177-91.*

Chapter 2

AT1 RECEPTOR ANTAGONISM IS PROANGIOGENIC IN THE BRAIN: BDNF A NOVEL MEDIATOR

Alhusban A, Kozak A, Ergul A, Fagan SC. J Pharmacol Exp Ther. 2013 Feb; 344(2):348-59.

Abstract

Candesartan is an angiotensin II type 1 receptor blocker (ARB) that has been to shown to limit ischemic stroke and improve stroke outcome. In experimental stroke, candesartan induces a proangiogenic effect that is partly due to vascular endothelial growth factor (VEGF). Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family that has been reported to have angiogenic effects and play an important role in recovery after stroke. The purpose of this investigation was to determine the role of BDNF in the proangiogenic effect of candesartan in the brain under hypertensive conditions. Accordingly, spontaneously hypertensive rats were treated with candesartan and brain tissues were collected for quantification of BDNF expression. In addition, human cerebromicrovascular endothelial cells were treated with either low dose (1fM) or high dose (1μM) angiotensin II alone or in combination with candesartan (0.16μM) to assess the effect of candesartan treatment and BDNF involvement in the behavior of endothelial cells. Candesartan significantly increased the expression of BDNF in the SHR ($p<0.05$). In addition, candesartan reversed the antiangiogenic effect of the 1μM dose of AngII ($p=0.0001$). The observed effects of candesartan were ablated by neutralizing the effects of BDNF. Treatment with the AT2 antagonist PD-123319 significantly reduced tube-like formation in endothelial cells. AT2 stimulation induced the BDNF expression and migration ($p<0.05$). In conclusion candesartan exerts a proangiogenic effect on brain microvascular endothelial cells treated with angiotensin II. This response is due to increased BDNF expression and is mediated through stimulation of the AT2 receptor.

Introduction:

Angiotensin II type 1 receptor blockers (ARBs) have been shown to be vascular protective and seem to have a particularly robust effect in reducing the incidence of cerebrovascular events [1-3]. Acutely, ARBs have been shown to improve outcome in experimental stroke [4] and the long term functional benefit was accompanied by an augmented proangiogenic state [1]. This angiogenic effect was only partially attributed to an increase in VEGF expression [1].Interestingly, the angiogenic response of candesartan was maintained even in non-stroked rats [1].

Subsequently, it was demonstrated that candesartan increased the expression of a number of genes for proangiogenic growth factors, including brain derived neurotrophic factor (BDNF), following experimental cerebral ischemia [5]. BDNF is a member of the neurotrophin family that is expressed in a number of cell types and has been shown to have potent neurogenic, neuroprotective and angiogenic effects [6, 7]. The proangiogenic effects of ARBs are hotly debated, however, [1, 8, 9] and may be tissue and situation dependent [10]. In the brain, it is unclear whether the effects of ARBs are due to blood pressure lowering or a direct effect of candesartan on endothelial cells.

Glycogen synthase kinase- 3β (GSK-3β) is a serine threonine kinase that plays a key role in gene expression regulation [11, 12]. Recently, Li *et al.* demonstrated the involvement of GSK-3β in the recovery after CNS ischemic insults [13]. They demonstrated the involvement of GSK inhibition in regulating neural stem cells-

endothelial cells cross talk [13]. This cross talk was found to be mediated through soluble growth factors like BDNF [14].

The purpose of this investigation was to determine whether candesartan-mediated blood pressure lowering increased BDNF protein expression in the brain in vivo and whether BDNF is involved in the proangiogenic effect of candesartan in vitro. In addition the involvement of GSK-3β in candesartan mediated effects was assessed.

Materials and methods:

Animals: The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Charlie Norwood Veterans Affairs Medical Center (09-04-008). Male spontaneously hypertensive rats (SHRs) (280-300g, n=4-6 per group) were subjected to middle cerebral artery occlusion (MCAO) sham surgery and randomized to receive a single IV dose of candesartan 0.3mg/kg, hydralazine 1mg/kg, or saline. In addition, male wistar rats (280-300g, n=4 per group) were subjected to the same surgery and randomized to receive either a single IV dose of candesartan 1mg/kg or saline. Twenty four hours later the rats were euthanized and the brains were harvested and flash frozen in liquid nitrogen.

Western blotting: To assess BDNF expression, the right and left hemispheres were separated and processed as described previously [5] and the blots probed with antiBDNF (1:250, abcam) and β-Actin (1:10000, sigma). For the in vitro experiments; human cerebrovascular endothelial cells (hCMECs) were cultured to confluence and serum starved for 10 hours followed by incubations with either 1ƒM or 1μM angiotensin II (AngII). After 6 hours, candesartan 0.16μM was added to the cells and incubated for

16 hours and then homogenized and processed for immunoblotting. To assess the involvement of AT2 receptor in BDNF expression HCMECs were serum starved for 16 hours and pretreated with PD-123319(0.1μM) 30 minutes before being incubated with AngII (1fM or 1μM) for 6 hours. Candesartan or vehicle were introduced in the media for 10 hours. To further confirm AT2 involvement, cells were serum starved for 16 hours followed by treatment with either the AT2 agonist CGP-121141A (0.1μM) or vehicle for 16 hours. The expression of AT1, AT2 and the phosphorylation status of GSK-3β were assessed using the same above treatment paradigm. Blots were probed with mouse monoclonal AT1 antibody (Abcam; 1:1000); rabbit monoclonal AT2 antibody (Abcam; 1:1000); p-S9GSK-3β (cell signaling; 1:1000) and total GSK-3β (cell signaling; 1:1000) Protein expression was quantified as the relative optic density of the protein band normalized to actin using NIH-image J software.

Cell culture: hCMECs were provided as a generous gift from Dr. J. Zastre (UGA College of Pharmacy). hCMECs were cultured in minimum essential media (ATCC) supplemented with EGM-2 SingleQuot Kit Suppl. & Growth Factors (Lonza) and 10% FBS (Atlanta Biologicals) and p30-34 were used in the experiments.

Treatments: Candesartan was provided as a generous gift from Astra-Zeneca. Hydralazine was purchased from Sigma-Aldrich (St. Louis, MO) and was reconstituted with 0.9% normal saline. AngII was purchased from Sigma-Aldrich (St. Louis, MO) and was reconstituted and diluted to the desired concentration using serum free media. BDNF neutralization was achieved using 100 nM K252a (Trk receptor inhibitor) dissolved in 25% DMSO both purchased from Sigma-Aldrich (St. Louis, MO), 10ng/ml anti-BDNF neutralizing antibody (Abcam; Cambridge, MA) and 0.4ug/ml TrkB-Fc

(soluble BDNF receptor chimera. R&D systems; Minneapolis, MN). The involvement of AT2 receptor was assessed using the AT2 antagonist PD-123319 (0.1μM) and the AT2 receptor agonist CGP-42112A (0.1μM) both purchased from Sigma-Aldrich (Sigma-Aldrich; St. Louis, MO). All the inhibitors were added to the media 30 minutes before AngII treatment.

Dose and time study: hCMECs were cultured until confluence and serum starved for 10 hours . Cells were incubated with six different concentrations of AngII (0-1μM) for 2, 6and8 hours and the cells were homogenized and processed for immunoblotting. BDNF expression was quantified as the relative density of the BDNF band to the corresponding β-actin or GADPH bands. The calculated relative density was normalized to the relative density of the control band, and reported as fold change.

Proliferation assay: Proliferation was assessed using BrdU incorporation (Cell Proliferation ELISA, BrdU (colorimetric); Roche Applied Science) according to the manufacturer recommendations. Briefly, 5000 hCMECs were seeded into each well of a 96 well plate and left to attach for 24 hours. Cells were serum starved for 10 hours and then treated with AngII (1ƒM or 1μM) for 6 hours. Following 6 hours, cells were treated with different combinations of candesartan, antiBDNF, TrkB-Fc, K252a, DMSO or IgG and incubated for 18 hours. The cells were then labeled with BrdU for 4 hours and then processed to quantify BrdU incorporation.

Angiogenesis Assays

***Cell migration*:** Wound recovery assay was used to assess cell migration where hCMECs were cultured in a 12 well plate to confluence and then serum starved for 10

hours followed by 6 hours of AngII (1ƒM or 1μM) treatment. A wound was introduced in the monolayer of endothelial cells and the cells treated with AngII (1ƒM or 1μM) with different combinations of candesartan, antiBDNF, TrkB-Fc, K252a, DMSO or IgG. In some experiments, cells were pretreated with PD-123319 (0.1μM) 30 minutes before AngII treatment. In another set of studies cells were treated with CGP-42112A (0.1μM), candesartan (0.16μM) or their combination. Scratch recovery was assessed by taking images of the scratch at baseline and at 16 hours after scratch introduction and the width of the scratch was measured at both time points using NIH image J software. The percentage wound recovery was calculated as the percent decrease in scratch width at 16 hours and the data was presented as percentage scratch recovery as compared to the control.

Tube formation: 2×10^4 hCMECs were suspended in serum free media and mixed with matrigel (BD Biosciences; San Jose, CA) in a 60:30 ratio and plated in a 96 well plate and then treated with different combinations of AngII (1ƒM or 1μM, sigma), candesartan, antiBDNF, TrkB-Fc, K252a, DMSO, IgG ,or PD-123319. Tube like structure formation was assessed using a digital camera attached to an Olympus microscope. Three images from each well were photographed at 24 hours and the number of tube like structures was quantified.

Statistical analysis: All experiments were repeated three times in triplicate and data was quantified in a blinded manner. Statistical significance was detected by one-way ANOVA for in vitro data followed by post-hoc Tukey test. Unpaired t-test was used to determine the significance of BDNF expression in the right and left brain hemispheres of candesartan treated SHRs as compared to the corresponding hemisphere in saline

treated animals. Statistical analyses were performed using GraphPad prism software (5.1). $P<0.05$ was considered significant.

Results:

Candesartan increases the expression of BDNF in SHR brain: BDNF has been shown to exert a beneficial effect in a variety of CNS pathologies [7]; the direct use of BDNF in therapy is limited by its pharmacokinetic profile [7]. A plausible alternative approach is to use either synthetic BDNF mimetics or agents that can induce BDNF expression in the brain. Treatment with a single dose of candesartan (0.3mg/kg) dramatically reduced the blood pressure from 150 mmHg at baseline to 120 mmHg after treatment (Figure 2-1A). Candesartan treatment significantly increased the expression of BDNF in both right and left hemisphere of SHR animals 24 hours after sham surgery (Figure 2-1B). The effect of candesartan on BDNF expression was maintained in wistar rats (supplemental data S2-3). In contrast, hydralazine treatment did not affect BDNF expression in SHRs (supplemental data S2-4).

Angiotensin II modulates the expression of BDNF in hCMECs. Angiotensin II has been reported to affect the expression of BDNF in the adrenals [15] and brain [16]; but its effect on BDNF expression in endothelial cells has never been reported. In hCMECs treatment with AngII was found to induce a dose and time-dependent modulation of BDNF expression that was maximal at 6 hours after incubation with either AngII 1fM or 1μM (Figure 2-2A). These two concentrations were used in the following experiments.

Candesartan increases BDNF expression in hCMECs: After establishing the effect of ARBs on the expression of BDNF in brain tissue in vivo, the effect of candesartan on

the expression of BDNF in AngII treated cells in vitro was investigated. Candesartan significantly increased the expression of BDNF in hCMECs treated with AngII 1fM in comparison to AngII 1fM alone and in hCMECs treated with both concentrations of AngII as compared to the control as assessed after 16 hours of incubation with candesartan (Figure 2-2B).

Candesartan has a dose dependent modulatory effect on the angiogenic potential of hCMECs. Data from clinical studies in stroke presented conflicting results on whether the hypotensive effect of ARBs is an essential requirement for its reported benefit [17, 18]. Consequently, we were interested in assessing whether a therapeutically relevant concentration of candesartan would have an in vitro effect in hCMECs in the absence of AngII. We calculated the amount of candesartan that can give a concentration similar to that achieved in patients receiving the drug (0.16μM). The angiogenic effect of a range of candesartan concentrations, including the concentration under consideration, was assessed using an in vitro matrigel tube formation assay (Figure 2-2C). The observed response was further confirmed using the wound recovery migration assay (Figure 2-2B). Candesartan induced a dose dependent, bell-shaped modulatory effect on the tube formation rate in hCMECs. The 0.16μM candesartan concentration (therapeutically relevant) significantly increased the tube formation rate, whereas the other concentrations did not affect the rate of tube formation (Figure 2-2C). A similar effect was observed in the wound recovery migration assay, except for an increased migration rate in hCMECs treated with candesartan 1.6μM concentration (Figure 2-2D).This differential effect of the 1.6μM candesartan concentration might be attributed to dose

dependent effects of candesartan on the different processes involved in migration and tube formation.

Candesartan modulates the proliferation of hCMECs: Angiotensin II has been reported to induce the proliferation of cells in vitro [9, 19]. This proliferative effect has been shown to be blocked with ARBs [9, 19]; but the reported concentration of ARBs in these studies was supratherpaeutic [9]. Consequently, we attempted to assess the effect of the therapeutically relevant concentration on the proliferative effect of AngII in hCMECs. Both low and high concentrations of AngII significantly increased the proliferation of hCMECs (Figures 2-3A and D). Treatment with candesartan maintained and further enhanced this proliferative effect (Figures 2-3A and D).

Candesartan modulates the migration rate of hCMECs: Similar to its proliferative effect, AngII has been reported to affect the migration of cells in vitro [20]. Since we have found a proliferative effect of candesartan on AngII treated hCMECs, we were interested in assessing the effect of this dose of candesartan on two critical steps of angiogenesis: migration and tube formation in AngII treated hCMECs. We observed a significant increase in the migration of hCMECs in response to treatment with AngII $1 f$M (Figure 2-4A). Interestingly, the higher AngII concentration did not have an effect on the migration of hCMECs (Figure 2-4D) in our model. While candesartan maintained the increased hCMECs migration in the low dose AngII group (Figure 2-4A), candesartan increased hCMECs migration in the high dose AngII dose treated cells (Figure 2-4D). Cells migration was not affected by K252a, DMSO or IgG (Figures 2-4C and F).

Candesartan has a proangiogenic effect in hCMECs: The pro-angiogenic effect of AngII has been reported previously [20, 21]. In hCMECs there was a significantly increased rate of tube formation in response to AngII 1fM treatment (Figure 2-5A) and a reduction in tube formation in AngII 1μM treated cells (Figure 2-5D). Interestingly, candesartan maintained AngII 1fM induced tube formation and reversed the antiangiogenic effect of AngII 1μM (Figure 2-5A and D).

BDNF mediates the effects of candesartan on hCMECs: Our in vivo results demonstrated the ability of candesartan to increase the expression of BDNF in SHR's brain. Previously, BDNF has been demonstrated to have an angiogenic effect which shifted our interest to assess whether BDNF is involved in the observed effects of candesartan. Upon neutralizing BDNF using three different methods of BDNF neutralization, the observed effects of candesartan were consistently ablated (Figures 2-3B and E; 2-4B and E, 2-5B and E); a finding that identifies BDNF as an essential mediator of candesartan effects in hCMECs. Interestingly, the angiogenic effect of AngII 1fM was significantly inhibited upon BDNF neutralization (Figure 5C), which suggests the involvement of BDNF in AngII induced angiogenesis, which is confirmed by the robust angiogenic response to BDNF treatment (Figures 2-5C and F).

AT2 receptor mediates the angiogenic response to angiotensin II in hCMECs.

Candesartan mediates its effects through AT1 blockade [2, 10], which induces an unopposed stimulation of AT2 receptor [22]. Findings in this investigation suggest a possible involvement of AT2 receptor in mediating candesartan effects. AT2 blockade using PD-123319 significantly inhibited the angiogenic response induced by either angiotensin II or the combination of angiotensin II and candesartan (Figure 2-6 A and B). In addition the AT2 agonist, CGP-24112A, significantly increased the migration of hCMECs. This migratory response was not affected by the concomitant treatment with candesartan (Figure 2-6C). These findings demonstrate an essential role of AT2 in the angiogenic process of hCMECs.

Candesartan induced BDNF expression is mediated through the AT2 receptor.

Findings in this study demonstrated the ability of candesartan to increase the expression of BDNF both in vivo and in vitro; in addition the in vitro effects of candesartan were shown to be mediated through the AT2 receptor. Accordingly, the involvement of AT2 receptor in BDNF expression was evaluated. As has been demonstrated earlier candesartan significantly increased the expression of BDNF in AngII treated cells (Figure 2-2B, 2-7A). Pretreatment with the AT2 antagonist PD-123319 significantly inhibited candesartan induced BDNF expression, which suggests the involvement of AT2 receptor. To confirm this findings hCMECs were treated with the AT2 agonist CGP-42112A (0.1μM) and the expression of BDNF was assessed. CGP-42112A significantly increased t expression of BDNF in hCMECs by about one fold as compared to vehicle treated cells (Figure 2-7B).

AT1 antagonism regulates the expression of AT1 in an AT2 dependent manner.

Candesartan treatment significantly increased the expression of AT1 receptor in cells treated with high dose of AngII as compared to both control and AngII alone. This response was ablated when the AT2 receptor was blocked using PD-123319 (Figure 2-7B). Additionally, the AT2 agonist CGP-24112A induced a significant fourfold increase in AT1 receptor expression in hCMECs (supplementary data). In cells treated with low dose AngII, candesartan significantly increased the expression of AT1 compared to control alone (Figure 2-7A). The expression of AT2 was not changed under the different treatments used (Figure 2-7 C and D).

AT1 antagonism modulates the activity of GSK-3β in an AT2 dependent manner.

GSK-3β was found to modulate BDNF expression [11, 13]. Our results demonstrated the ability of candesartan to increase the expression of BDNF in both in vivo and in vitro settings. Accordingly we were interested in assessing the activity of GSK-3β under the different treatment conditions we were using. Candesartan treatment significantly increased the phosphorylation of GSK-3β at the inhibitory serine 9 residue in cells treated with high dose AngII (Figure 2-8A). The higher phosphorylation was reversed by the concomitant treatment with AT2 blocker PD-123319 (Figure2- 8A).

Discussion: Our results demonstrate, for the first time, the ability of candesartan to promote a proangiogenic state in hCMECs in an AngII concentration independent manner, where candesartan was able to maintain the angiogenic effect of AngII at the low dose, and reverse the antiangiogenic effect of the high dose. The proangiogenic effect of candesartan was found to be mainly dependent on BDNF and is mediated through AngII stimulation of the AT2 receptor. In addition, we have demonstrated the ability of candesartan to induce an angiogenic response in endothelial cells; even in the absence of exogenously added AngII.

Following an ischemic insult in the brain, induction of angiogenesis has been shown to ameliorate the damage and is coupled to neurogenesis leading to enhanced recovery and better outcome [23, 24]. ARBs administration was found to increase vascular density in heart and brain following MI [25] and stroke [1, 5] which was attributed to an increase in VEGF expression in stroke [5]. In contrast to in vivo data, in vitro angiogenesis studies demonstrated an antiangiogenic effect of ARBs in endothelial

cells treated with AngII [9, 21]. This antiangiogenic effect was mediated mainly through inhibiting AngII induced increase in VEGF signaling [9, 26]. The majority of these studies were conducted in either HUVECs [19, 20] or coronary artery endothelial cells [21, 26] and none of them evaluated the effect of AngII or AngII and ARBs in hCMECs which are phenotypically different from other endothelial cells [27]. In addition, the majority of the studies focused on the involvement of VEGF [20], angiopoietins [9] and EGFR transactivation [26] in the angiogenic response to AngII. This ignores the possible role of other angiogenic mediators, like BDNF, which has been shown to induce VEGF expression [6] and produce an angiogenic response comparable to that of VEGF in endothelial cells [28]. Results from this study demonstrate a dose dependent effect of AngII on brain angiogenesis, where low concentrations of AngII induce angiogenesis and higher doses inhibit it. This is consistent with that shown in other vascular beds [19, 20].

The design of this study is unique in many aspects. In a typical in vitro study, cells are initially treated with candesartan followed by AngII treatment. This precludes detection of a possible AngII independent interaction between candesartan and endothelial cells. This design has limited clinical relevance since candesartan will be introduced to the system in response to the effects of AngII or other circulating vasoactive mediators. In our design, we attempted to model the temporal relationship of treatment introduction. Data about the effect of AngII on BDNF expression in endothelial cells were lacking and the only reports addressing the effect of AngII on BDNF expression were in the adrenal cortex [15] and the brain [16]. Accordingly, we did a time and dose response study in hCMECs to determine the incubation time and AngII dose

to be used in the in vitro studies. This dose and time response study was followed by assessing the effect of those AngII on the viability of hCMECs (supplementary data S2-1). Also, the concentration of candesartan used in the in vitro studies was calculated to produce the midpoint therapeutic steady state concentration in humans [29]. The calculated concentration was tested for its angiogenic and migratory effect and compared to other candesartan doses in a dose response curve (Figures 2-2C and D). The in vivo dose was determined based on previously reported data demonstrating neurovascular protection in SHRs [30].

Our findings support and expand our previously reported data on the ability of cerebral spinal fluid (CSF) from candesartan treated non stroked animals to induce angiogenesis in endothelial cells [1]. This proangiogenic effect of candesartan was only partially attributed to VEGF [4], suggesting the involvement of other angiogenic factors. In this study we have identified BDNF as another important mediator of the angiogenic effect of candesartan. BDNF neutralization using receptor inhibitor K252a, antiBDNF antibody or TrkB-Fc almost ablated the effects of candesartan in all reported assays.

The proliferative and angiogenic effects of AngII have been largely attributed to AT1 mediated signaling [9, 20, 21, 26, 31] while AT2 mediated signaling was thought to either counteract AT1 induced angiogenesis [26, 32] or not have an effect on AngII induced angiogenesis [21, 31]. In contrast to this notion, AT2 mediated signaling has been demonstrated to promote angiogenesis in ischemic myocardium [33] and retinal endothelial cells [34]. In this study, AT2 mediated signaling was found to be largely responsible for the angiogenic response in hCMECs. AT2 involvement in angiogenesis was initially suggested by the lack of effect on AngII – mediated angiogenesis when

AT1 was blocked using candesartan and was further confirmed when the angiogenic response was totally prevented upon AT2 blockade using PD-123319. This finding suggests the importance of unopposed AT2 stimulation following stroke [35, 36] and may explain the lack of protective effect of ARBs in the absence of AT2 signaling following cerebral ischemia [37-40] . Our data demonstrates the indispensible role of AT2 for angiogenesis in hCMECs which has been linked to neurogenesis and improving recovery after CNS ischemic insults [14, 23, 24].

The activity of glycogen synthase kinase 3 beta (GSK-3β) has been shown to be involved in the expression of neurotrophins in the brain [12] and in the cross talk between endothelial and neural stem cells through regulating BDNF and VEGF expression [13]. In addition GSK-3β has been recently demonstrated to regulate the angiogenic response in endothelial cells [41]. Data from this study highlights a possible involvement of GSK-3β in mediating the effects of AT1 antagonism in hCMECs in an Ang II dose-dependent manner. Candesartan increased phosphorylation of GSK-3β at the inhibitory serine 9 residue which will inhibit the activity of GSK-3β when used alone or in combination with 1μM AngII the concentration that induced antiangiogenic effects in hCMECs. Concomitantly this increased inhibition of GSK-3β was associated with increased BDNF expression and angiogenic response in hCMECs. These findings are consistent with previously published data about the interaction between GSK-3β and BDNF expression [12, 13].

In this study, candesartan demonstrated the ability to modulate the angiogenic response of hCMECs in the absence of exogenously added AngII. This effect was found to be dose dependent. The therapeutically relevant candesartan concentration induces

a proangiogenic effect; whereas the other two concentrations didn't affect the angiogenic potential of the cells. This finding may help explain the controversial angiogenic effect of ARBs between the in vivo and the in vitro data [10]. Previously published reports on the in vitro antiangiogenic effect of ARBs used supra-therapeutic concentrations [9, 21]. As demonstrated by the findings of this study, the supra-therapeutic doses of ARBs might have direct antiangiogenic effects on endothelial cells.

Another unique aspect of this work is the demonstration of candesartan's ability to increase the expression of BDNF in both hCMECs and the brain tissue of hypertensive animals. Hashikawa-Hobara et al. recently demonstrated the ability of candesartan to stimulate neurite growth in an AT2 dependent manner through Akt signaling [22]. This is consistent with our findings, highlighting the involvement of the AT2 receptor in BDNF expression which is known to stimulate neurite growth [42] and Akt signaling [6]. Interestingly, the effect of candesartan on BDNF expression appears to be independent of blood pressure lowering. Candesartan increased BDNF expression in wistar rats (supplemental data S2-3); whereas, hydralazine had no effect on BDNF expression in SHRs (supplemental data S2-4).

An interesting finding in this study is the involvement of AT2 in AT1 expression. Cross-talk between AngII receptors have been previously reported at both expression and functional levels [43-45]. These reports consistently demonstrated the involvement of AT1 in AT2 expression [45]. In addition, it has been shown that AT2 mediated signaling antagonizes some aspects of AT1 mediated effects [43]. Our results demonstrate for the first time that AT1 expression in hCMECs is positively regulated by AT2 mediated signaling.

In this study all efforts were made to confirm each result using different methods but the following limitations can be identified; the in vitro studies were conducted in an endothelial cell line rather than primary endothelial cells. In addition, BDNF neutralization studies were performed using mainly pharmacologic methods although a genetic approach using RNA interference would provide more power to the study. In addition, in our study we have used human derived cell line in our in vitro study while using a murine model as an in vivo model. In our research the main goal and focus is to model and understand the changes that accompany ischemic stroke in humans. Because of the inability to directly probe human brain samples, we are using rats to study the in vivo changes in response to candesartan or any pharmacologic agent of interest. In addition whenever we had the chance to use human derived tissues or cells we do use them to give a better understanding of what changes are taking place. In this investigation we employed human cerebromicrovascular endothelial cells as an in vitro system and we used SHRs as an in vivo system.

In conclusion, our findings demonstrate the ability of candesartan to modulate the behavior of endothelial cells to promote a proangiogenic state. In hCMECs, this modulatory effect of candesartan can be largely attributed to BDNF and is mediated through the AT2 receptor. In addition, the dose dependent proangiogenic effect of candesartan and even the mechanism involved may help explain the disparate findings on the angiogenic potential of ARBs that prevails in the biomedical literature.

References

1. *Kozak, A., et al., Candesartan augments ischemia-induced proangiogenic state and results in sustained improvement after stroke. Stroke, 2009. 40(5): p. 1870-6.*
2. *Engelhorn, T., et al., The angiotensin II type 1-receptor blocker candesartan increases cerebral blood flow, reduces infarct size, and improves neurologic outcome after transient cerebral ischemia in rats. J Cereb Blood Flow Metab, 2004. 24(4): p. 467-74.*
3. *Björn Dahlöf, R.B.D., Sverre E Kjeldsen, Stevo Julius, Gareth Beevers, Ulf de Faire, Frej Fyhrquist, Hans Ibsen,, O.L.-P. Krister Kristiansson, Lars H Lindholm, Markku S Nieminen, Per Omvik, Suzanne Oparil,, and f.t.L.s.g. Hans Wedel, Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. Lancet, 2002. 359: p. 8.*
4. *Fagan, S.C., et al., Hypertension after experimental cerebral ischemia: candesartan provides neurovascular protection. J Hypertens, 2006. 24(3): p. 535-9.*
5. *Guan, W., et al., Vascular protection by angiotensin receptor antagonism involves differential VEGF expression in both hemispheres after experimental stroke. PLoS One, 2011. 6(9): p. e24551.*
6. *Caporali, A. and C. Emanueli, Cardiovascular actions of neurotrophins. Physiol Rev, 2009. 89(1): p. 279-308.*
7. *Greenberg, M.E., et al., New insights in the biology of BDNF synthesis and release: implications in CNS function. J Neurosci, 2009. 29(41): p. 12764-7.*
8. *Schieffer, B., et al., Comparative effects of chronic angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade on cardiac remodeling after myocardial infarction in the rat. Circulation, 1994. 89(5): p. 2273-82.*
9. *Herr, D., et al., Regulation of endothelial proliferation by the renin-angiotensin system in human umbilical vein endothelial cells. Reproduction, 2008. 136(1): p. 125-30.*
10. *Willis, L.M., et al., Angiotensin receptor blockers and angiogenesis: clinical and experimental evidence. Clin Sci (Lond), 2011. 120(8): p. 307-19.*
11. *Hur, E.M. and F.Q. Zhou, GSK3 signalling in neural development. Nat Rev Neurosci, 2010. 11(8): p. 539-51.*
12. *Wada, A., Lithium and neuropsychiatric therapeutics: neuroplasticity via glycogen synthase kinase-3beta, beta-catenin, and neurotrophin cascades. J Pharmacol Sci, 2009. 110(1): p. 14-28.*

13. *Li, Q., et al., GSK-3beta: a signaling pathway node modulating neural stem cell and endothelial cell interactions. Angiogenesis, 2011. 14(2): p. 173-85.*
14. *Madri, J.A., Modeling the neurovascular niche: implications for recovery from CNS injury. J Physiol Pharmacol, 2009. 60 Suppl 4: p. 95-104.*
15. *Szekeres, M., et al., Angiotensin II-induced expression of brain-derived neurotrophic factor in human and rat adrenocortical cells. Endocrinology, 2010. 151(4): p. 1695-703.*
16. *Chan, S.H., et al., Transcriptional upregulation of brain-derived neurotrophic factor in rostral ventrolateral medulla by angiotensin II: significance in superoxide homeostasis and neural regulation of arterial pressure. Circ Res, 2010. 107(9): p. 1127-39.*
17. *Schrader, J., et al., The ACCESS Study: evaluation of Acute Candesartan Cilexetil Therapy in Stroke Survivors. Stroke, 2003. 34(7): p. 1699-703.*
18. *Schrader, J., et al., Morbidity and Mortality After Stroke, Eprosartan Compared with Nitrendipine for Secondary Prevention: principal results of a prospective randomized controlled study (MOSES). Stroke, 2005. 36(6): p. 1218-26.*
19. *Kou, B., M. Vatish, and D.R. Singer, Effects of angiotensin II on human endothelial cells survival signalling pathways and its angiogenic response. Vascul Pharmacol, 2007. 47(4): p. 199-208.*
20. *Buharalioglu, C.K., et al., Angiotensin II-induced process of angiogenesis is mediated by spleen tyrosine kinase via VEGF receptor-1 phosphorylation. Am J Physiol Heart Circ Physiol, 2011. 301(3): p. H1043-55.*
21. *Hu, C., A. Dandapat, and J.L. Mehta, Angiotensin II induces capillary formation from endothelial cells via the LOX-1 dependent redox-sensitive pathway. Hypertension, 2007. 50(5): p. 952-7.*
22. *Hashikawa-Hobara, N., et al., Candesartan Cilexetil Improves Angiotensin II Type 2 Receptor-Mediated Neurite Outgrowth via the PI3K-Akt Pathway in Fructose-Induced Insulin-Resistant Rats. Diabetes, 2012. 61(4): p. 925-32.*
23. *Xiong, Y., A. Mahmood, and M. Chopp, Angiogenesis, neurogenesis and brain recovery of function following injury. Curr Opin Investig Drugs, 2010. 11(3): p. 298-308.*
24. *Navaratna, D., et al., Mechanisms and targets for angiogenic therapy after stroke. Cell Adh Migr, 2009. 3(2): p. 216-23.*
25. *Sladek, T., et al., The effect of AT1 receptor antagonist on chronic cardiac response to coronary artery ligation in rats. Cardiovasc Res, 1996. 31(4): p. 568-76.*
26. *Fujiyama, S., et al., Angiotensin AT(1) and AT(2) receptors differentially regulate angiopoietin-2 and vascular endothelial growth factor expression and angiogenesis by modulating heparin binding-epidermal growth factor (EGF)-mediated EGF receptor transactivation. Circ Res, 2001. 88(1): p. 22-9.*
27. *Feletou, M., Series on Integrated Systems Physiology: From Molecule to Function to Disease., in The Endothelium: Part 1: Multiple Functions of the Endothelial Cells—Focus on Endothelium-Derived Vasoactive Mediators., G.J. Granger DN, Editor. 2011, Morgan & Claypool Life Sciences: San Rafael (CA).*

28. *Li, Q., et al., Modeling the neurovascular niche: VEGF- and BDNF-mediated cross-talk between neural stem cells and endothelial cells: an in vitro study. J Neurosci Res, 2006. 84(8): p. 1656-68.*
29. *Schulz, M. and A. Schmoldt, Therapeutic and toxic blood concentrations of more than 800 drugs and other xenobiotics. Pharmazie, 2003. 58(7): p. 447-74.*
30. *Kozak, W., et al., Vascular protection with candesartan after experimental acute stroke in hypertensive rats: a dose-response study. J Pharmacol Exp Ther, 2008. 326(3): p. 773-82.*
31. *Otani, A., et al., Angiotensin II potentiates vascular endothelial growth factor-induced angiogenic activity in retinal microcapillary endothelial cells. Circ Res, 1998. 82(5): p. 619-28.*
32. *Javier Carbajo-Lozoya, S.L., Yuxi Feng, Jens Kroll, and T.W. Hans-Peter Hammes, Angiotensin II modulates VEGF-driven angiogenesis by opposing effects of type 1 and type 2 receptor stimulation in the microvascular endothelium. Cellular Signalling, 2012. 24: p. 1261-1269.*
33. *Munk, V.C., et al., Angiotensin II induces angiogenesis in the hypoxic adult mouse heart in vitro through an AT2-B2 receptor pathway. Hypertension, 2007. 49(5): p. 1178-85.*
34. *Sarlos, S., et al., Retinal angiogenesis is mediated by an interaction between the angiotensin type 2 receptor, VEGF, and angiopoietin. Am J Pathol, 2003. 163(3): p. 879-87.*
35. *Oprisiu-Fournier R, F.S., Mazouz H, Boutitie F, Serot JM, Achard JM, Godefroy O, Hanon O, Temmar M, Albu A, Strandgaard S, Wang J, Black SE, Fournier A., Angiotensin AT1-receptor blockers and cerebrovascular protection: do they actually have a cutting edge over angiotensin-converting enzyme inhibitors? Expert Rev Neurother, 2009. 9(9).*
36. *McCarthy, C.A., et al., Angiotensin AT2 receptor stimulation causes neuroprotection in a conscious rat model of stroke. Stroke, 2009. 40(4): p. 1482-9.*
37. *Faure, S., et al., Protective effect of candesartan in experimental ischemic stroke in the rat mediated by AT2 and AT4 receptors. J Hypertens, 2008. 26(10): p. 2008-15.*
38. *Lu, Q., Y.Z. Zhu, and P.T. Wong, Neuroprotective effects of candesartan against cerebral ischemia in spontaneously hypertensive rats. Neuroreport, 2005. 16(17): p. 1963-7.*
39. *Li, J., et al., Angiotensin AT2 receptor protects against cerebral ischemia-induced neuronal injury. FASEB J, 2005. 19(6): p. 617-9.*
40. *Iwai, M., et al., Possible inhibition of focal cerebral ischemia by angiotensin II type 2 receptor stimulation. Circulation, 2004. 110(7): p. 843-8.*
41. *Flugel, D., A. Gorlach, and T. Kietzmann, GSK-3beta regulates cell growth, migration, and angiogenesis via Fbw7 and USP28-dependent degradation of HIF-1alpha. Blood, 2012. 119(5): p. 1292-301.*
42. *Parrish, J.Z., et al., Mechanisms that regulate establishment, maintenance, and remodeling of dendritic fields. Annu Rev Neurosci, 2007. 30: p. 399-423.*

43. Miura, S., et al., Molecular mechanisms of the antagonistic action between AT1 and AT2 receptors. Biochem Biophys Res Commun, 2010. 391(1): p. 85-90.
44. Saavedra, J.M., Emerging features of brain angiotensin receptors. Regul Pept, 1999. 85(1): p. 31-45.
45. De Paolis, P., et al., Modulation of the AT2 subtype receptor gene activation and expression by the AT1 receptor in endothelial cells. J Hypertens, 1999. 17(12 Pt 2): p. 1873-7.

Figure 2-1. Hypertension and AT1 blockade affects the expression of BDNF. Blood pressure transmitters were implanted intraperitoneally in SHR. Animals had sham surgery and received a single dose of candesartan (0.3mg/kg) and the mean arterial blood pressure was monitored (A).Arrow indicates time of candesartan administration; n=6. SHR underwent sham surgery and randomized to receive either candesartan (0.3mg/kg) or saline intravenously (n=6 per group). 24 hours later the animals were sacrificed and the brains were extracted and. Right and left hemispheres were separated and processed for immunoblotting (B).

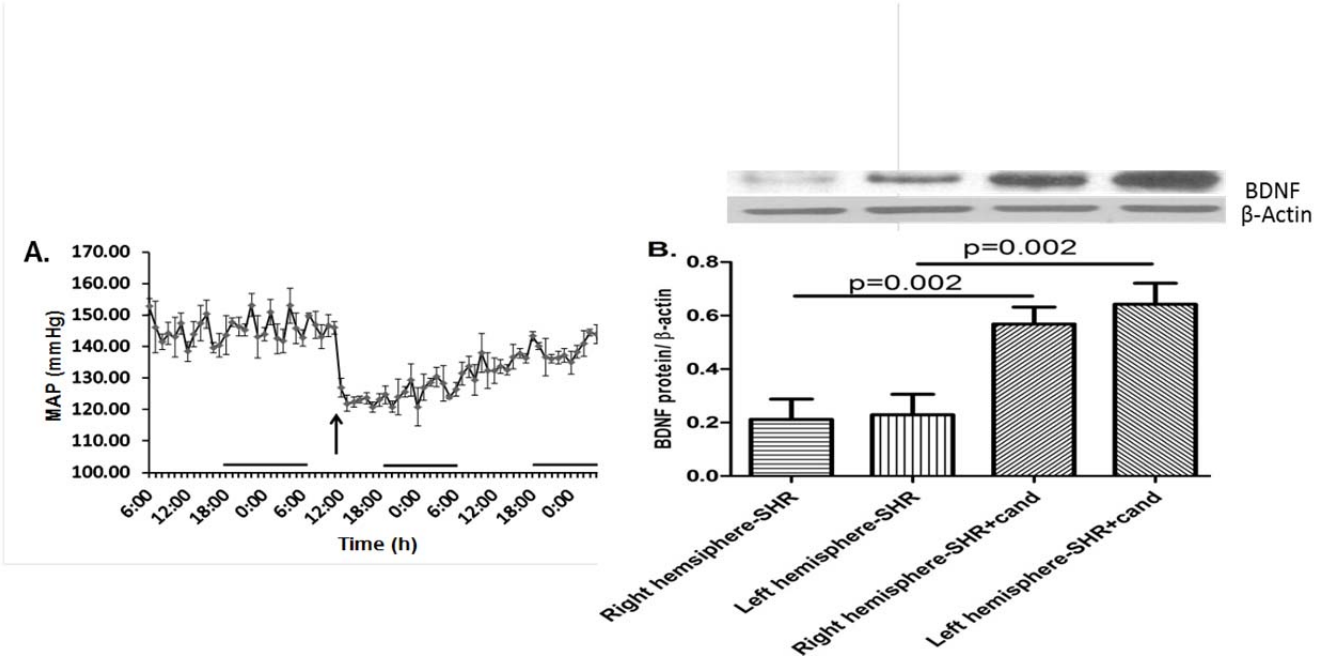

A.
MAP (mmHg)
170.00
160.00
150.00
140.00
130.00
120.00
110.00
100.00
6:00
12:00
18:00
0:00
6:00
12:00
18:00
0:00
6:00
12:00
18:00
0:00
Time (h)
BDNF
β-Actin
B.
BDNF protein/ β-actin
0.8
0.6
0.4
0.2
0.0
p=0.002
p=0.002
Right hemsiphere-SHR
Left hemisphere-SHR
Right hemisphere-SHR+cand
Left hemisphere-SHR+cand

Figure 2-2: Angiotensin II and AT1 blockade affects the expression of BDNF and the angiogenic potential in hCMECs. hCMECs were cultured to confluence followed by serum starvation for 16 hours. Cells were treated with a concentration range of Angiotensin II (AngII) for different time periods. The expression of BDNF was assessed using immunoblotting (A) n=3-5. hCMECs were treated with AngII (1fM or 1μM) for 6 hours followed by treatment with candesartan (0.16μM) for 10 hours and the expression of BDNF was assessed (B) n=3. The ability of candesartan to modulate the angiogenic response of hCMECs was evaluated using in vitro matrigel tube formation assay (C) and wound recovery assay (D) in response to treatment with a concentration range of candesartan (0-1.6μM) n=3. For panels A and B, data presented as mean±SEM, * $p<0.05$. For panels C and D * significantly different from control; $ significantly different from cand 0.16μM. Overall $p=0.0014$, $F=8.19$ (C) and $p<0.0001$; $F=22.44$ (D).

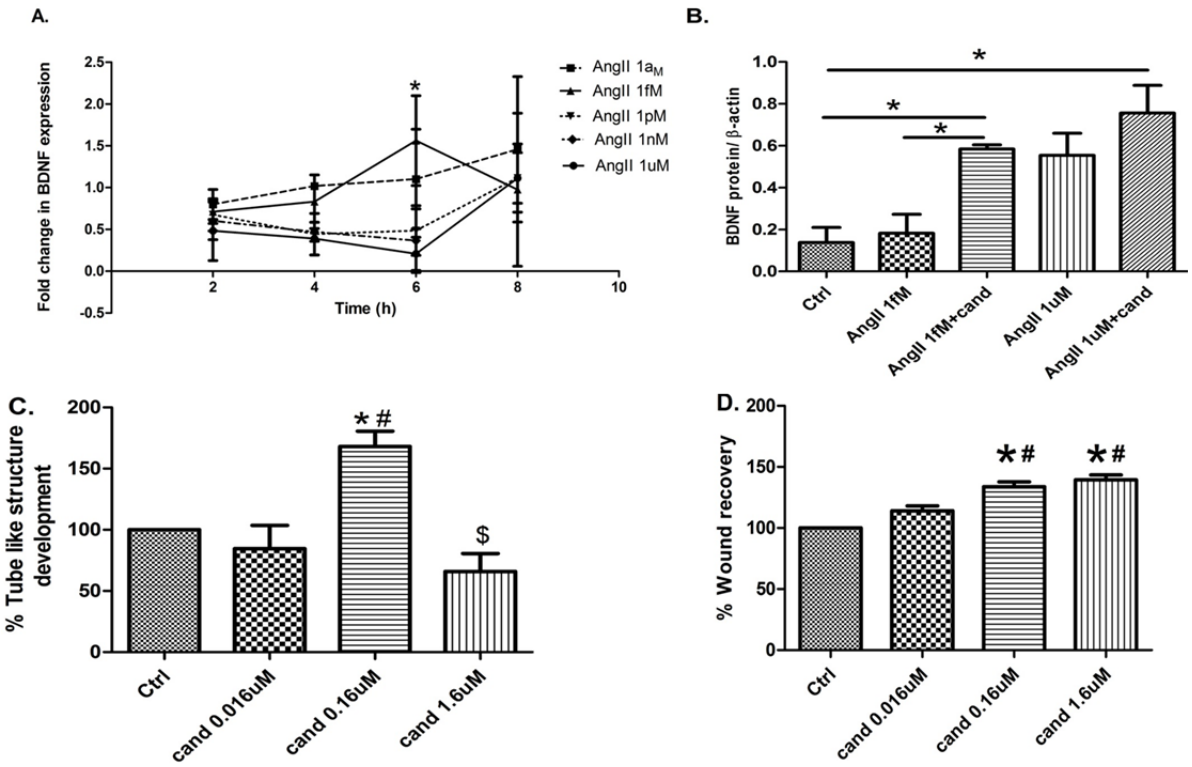

A.
Fold change in BDNF expression
Time (h)
AngII 1a_M
AngII 1fM
AngII 1pM
AngII 1nM
AngII 1uM
B.
BDNF protein/ β-actin
Ctrl
AngII 1fM
AngII 1fM+cand
AngII 1uM
AngII 1uM+cand
C.
% Tube like structure development
Ctrl
cand 0.016uM
cand 0.16uM
cand 1.6uM
D.
% Wound recovery
Ctrl
cand 0.016uM
cand 0.16uM
cand 1.6uM

Figure 2-3: Angiotensin II modulates the proliferation of hCMECs. Proliferative response of HCMECs was evaluated using BrdU incorporation assay. hCMECs were treated with AngII (1ƒM or 1μM) for 6 hours followed by candesartan (0.16μM) alone or in combination with other treatments. The plates were then processed according to the manufacturer recommendations. Data are presented as mean±SEM of 3 different experiments each in triplicate. * Significantly different from control, # significantly different from AngII in the same group, $ significantly different from AngII+cand in the same group; overall $p=0.005$, $F=8.85$ for AngII 10^{-9} and $p<0.0001$, $F=34.97$ for AngII 1μM

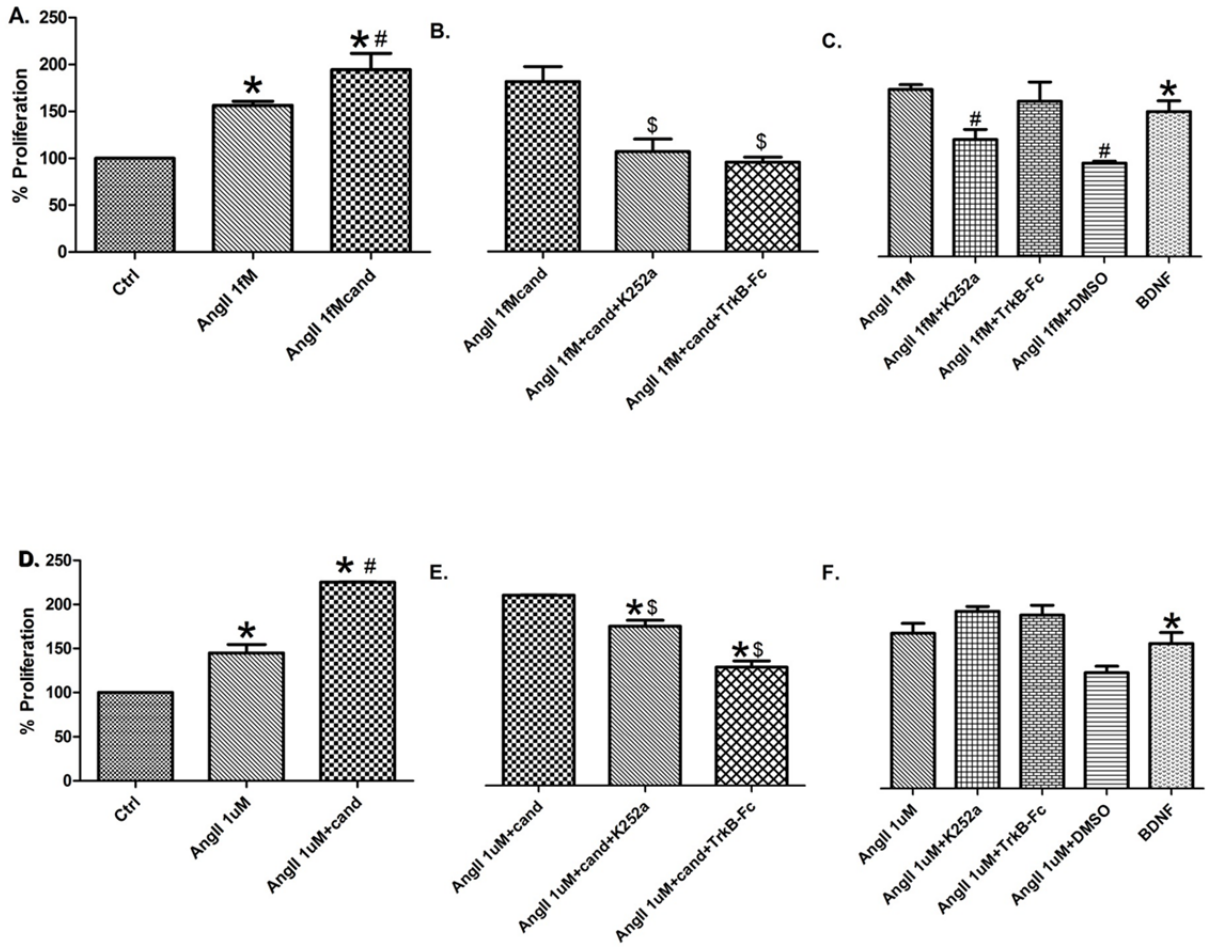

A.
% Proliferation
250
200
150
100
50
0
*
*#
Ctrl
AngII 1fM
AngII 1fMcand
B.
$
$
AngII 1fMcand
AngII 1fM+cand+K252a
AngII 1fM+cand+TrkB-Fc
C.
#
#
*
AngII 1fM
AngII 1fM+K252a
AngII 1fM+TrkB-Fc
AngII 1fM+DMSO
BDNF
D.
% Proliferation
250
200
150
100
50
0
*
* #
Ctrl
AngII 1uM
AngII 1uM+cand
E.
*$
*$
AngII 1uM+cand
AngII 1uM+cand+K252a
AngII 1uM+cand+TrkB-Fc
F.
*
AngII 1uM
AngII 1uM+K252a
AngII 1uM+TrkB-Fc
AngII 1uM+DMSO
BDNF

Figure 2-4: Angiotensin II modulates the migration of hCMECs. Insert; representative image of control, AngII 1μM and AngII+candesartan showing candesartan induced migration of hCMECs. hCMECs were cultured to confluence followed by 10 hours serum starvation. Cells were treated with either AngII 1fM or 1μM for 6 hours and then a scratch was introduced in the monolayer. Cells were then incubated with AngII 1fM or 1μM alone or with candesartan (A and D). The involvement of BDNF was assessed using a number of inhibitors for BDNF functions (B and E) which were added to the media 30 minutes before AngII treatment. Data are presented as mean±SEM of 3 different experiments each in triplicate. * Significantly different from control, # significantly different from AngII in the same group, $ significantly different from AngII+cand in the same group; overall $p<0.0001$, $F=16.08$ for AngII 1fM and $p<0.0001$, $F=22.08$ for AngII 1μM.

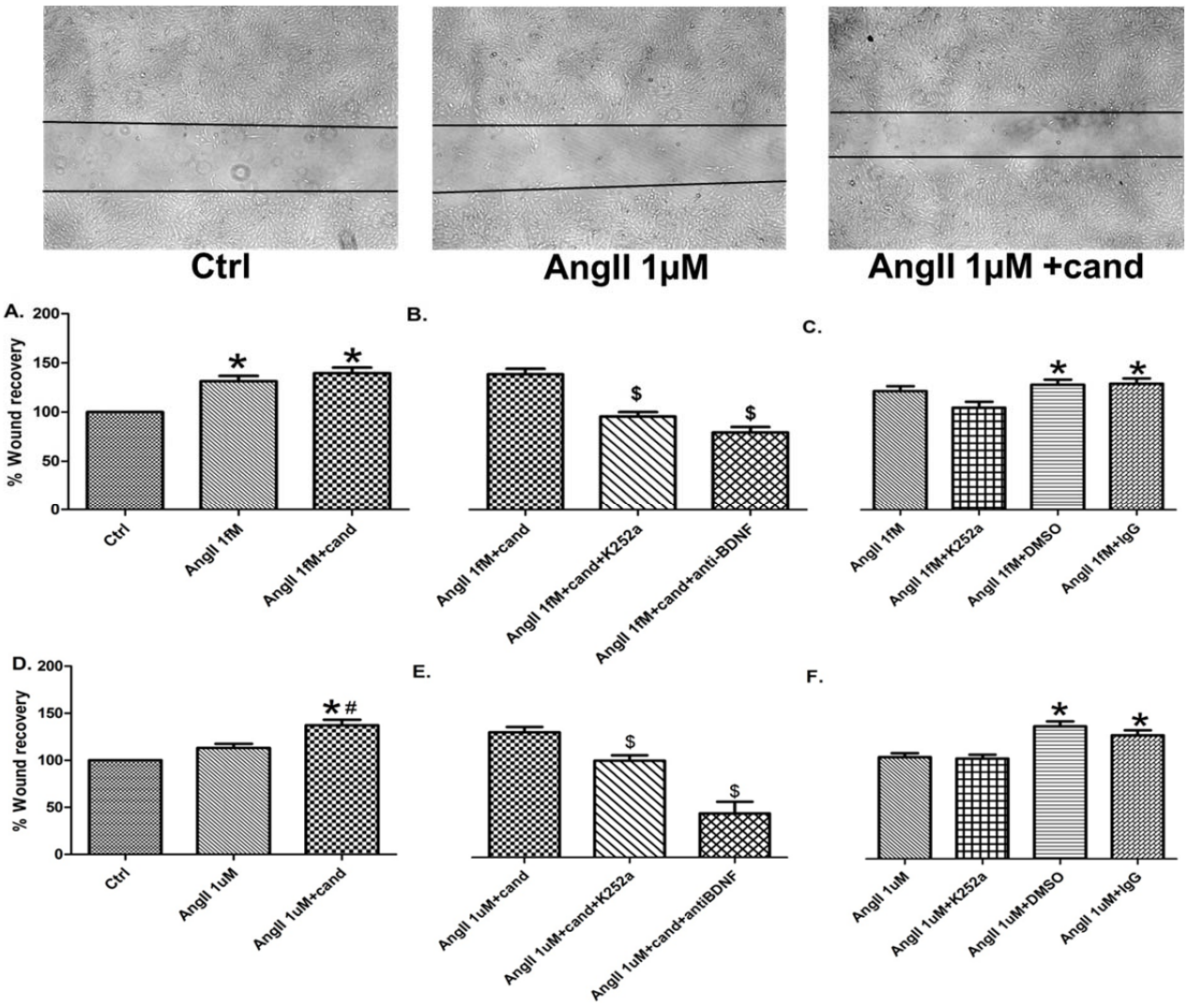

Ctrl
AngII 1μM
AngII 1μM +cand
A.
% Wound recovery
200
150
100
50
0
*
*
Ctrl
AngII 1fM
AngII 1fM+cand
B.
$
$
AngII 1fM+cand
AngII 1fM+cand+K252a
AngII 1fM+cand+anti-BDNF
C.
*
*
AngII 1fM
AngII 1fM+K252a
AngII 1fM+DMSO
AngII 1fM+IgG
D.
% Wound recovery
200
150
100
50
0
*#
Ctrl
AngII 1uM
AngII 1uM+cand
E.
$
$
AngII 1uM+cand
AngII 1uM+cand+K252a
AngII 1uM+cand+antiBDNF
F.
*
*
AngII 1uM
AngII 1uM+K252a
AngII 1uM+DMSO
AngII 1uM+IgG

Figure 2-5: Angiotensin II modulates the angiogenic potential of hCMECs. Insert; representative images of in vitro tube formation (arrows) showing reduced tube formation rate in AngII 1μM treated hCMECs and the reversal of AngII 1μM antiangiogenic effect by candesartan. hCMECS (2×10^4 cells/well) were suspended in a 30:60 solution of matrigel and serum free media. Angiogenic response of hCMECs to AngII 1fM (A) and 1μM (D) in the presence and absence of candesartan was evaluated 24 hours after treatment. The involvement of BDNF was assessed through using K252a (Trk inhibitor) or TrkB-Fc (soluble chimeric receptor) (B and E). Data are presented as mean±SEM of 3 different experiments each in triplicate. * Significantly different from control, # significantly different from AngII in the same group, $ significantly different from AngII+cand in the same group; overall $p<0.0001$, $F=12.74$ for AngII 1fM and $p<0.0001$, $F=9.54$ for AngII 1μM.

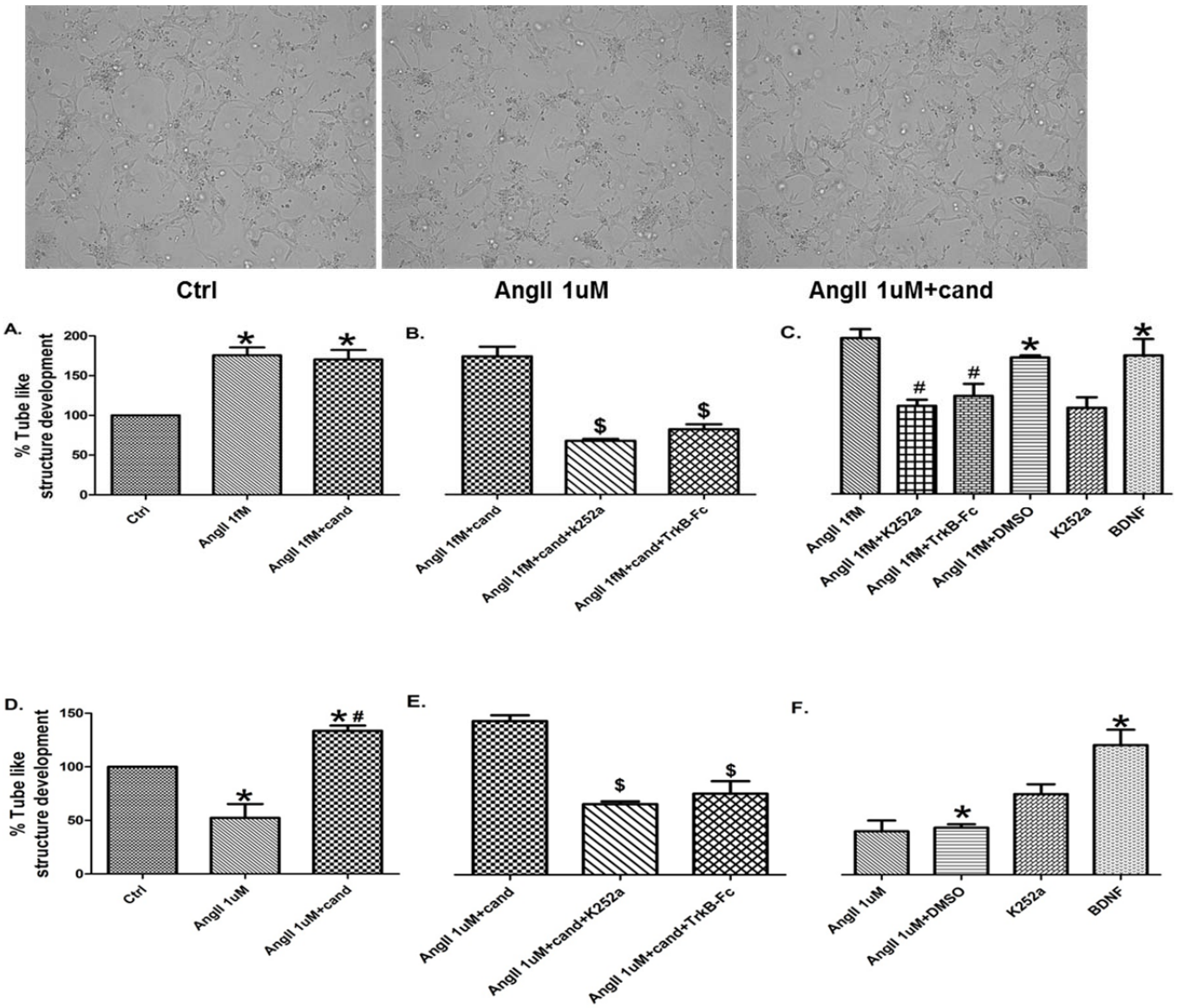

Ctrl
AngII 1uM
AngII 1uM+cand
A.
% Tube like structure development
200
150
100
50
0
Ctrl
AngII 1fM
AngII 1fM+cand
B.
$
$
AngII 1fM+cand
AngII 1fM+cand+k252a
AngII 1fM+cand+TrkB-Fc
C.
#
#
*
*
AngII 1fM
AngII 1fM+K252a
AngII 1fM+TrkB-Fc
AngII 1fM+DMSO
K252a
BDNF
D.
% Tube like structure development
150
100
50
0
*
*#
Ctrl
AngII 1uM
AngII 1uM+cand
E.
$
$
AngII 1uM+cand
AngII 1uM+cand+K252a
AngII 1uM+cand+TrkB-Fc
F.
*
*
AngII 1uM
AngII 1uM+DMSO
K252a
BDNF

Figure 2-6: AT2 receptor mediates the angiogenic response in hCMECs. The involvement of AT2 receptor in the angiogenic response to AngII 1 ƒM alone or in combination with candesartan (A) and to the combination of AngII 1μM and candesartan (B) was assessed using the AT2 antagonist PD-123319 (0.1uM). The angiogenic response was evaluated using matrigel tube formation assay as described in the methods section. Data are presented as mean±SEM of 3 different experiments each in triplicate. * Significantly different from control, # significantly different from AngII in the same group, $ significantly different from AngII+cand in the same group; overall $p<0.0001$, $F=8.779$ for both AngII 1ƒM and AngII 1μM.

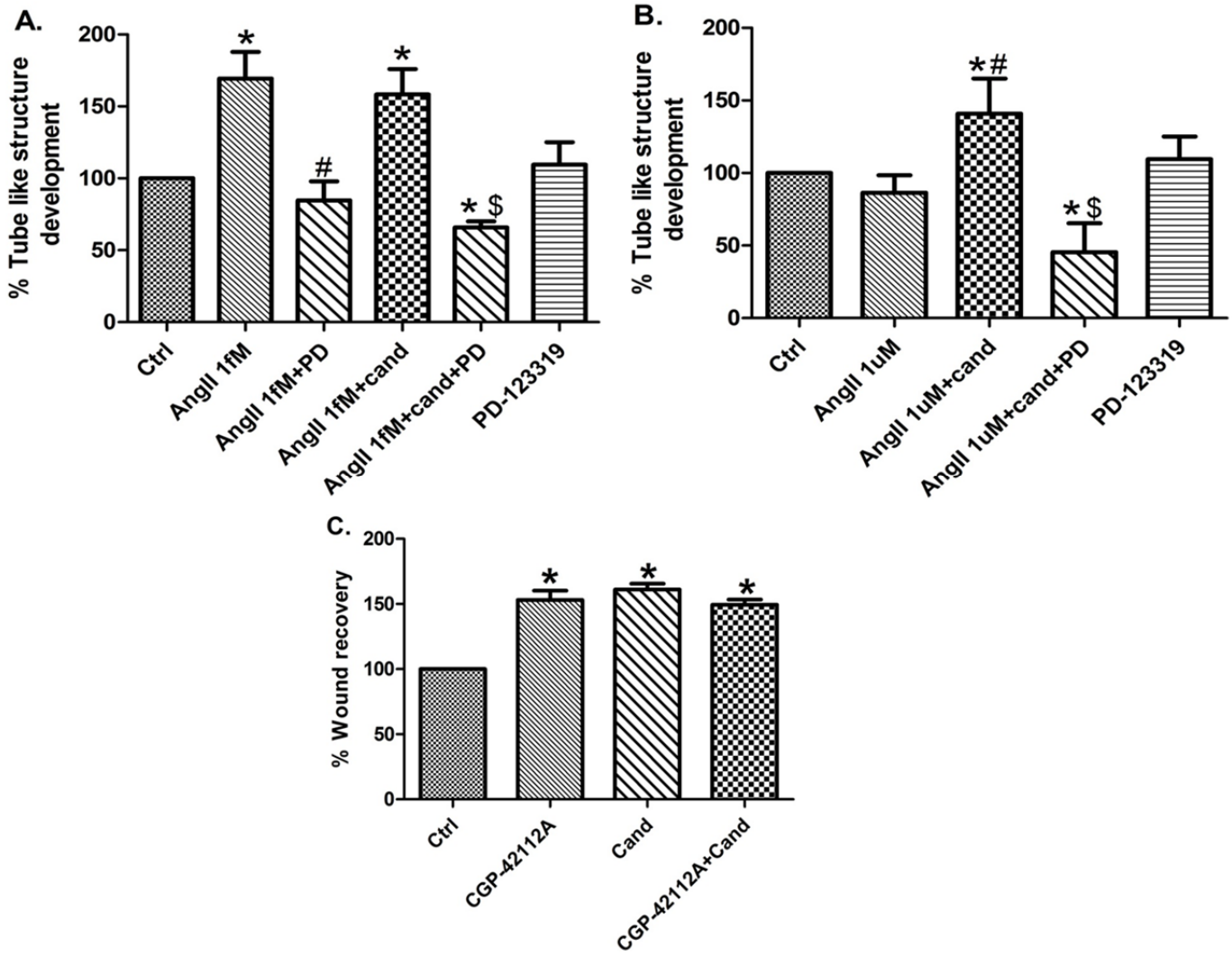
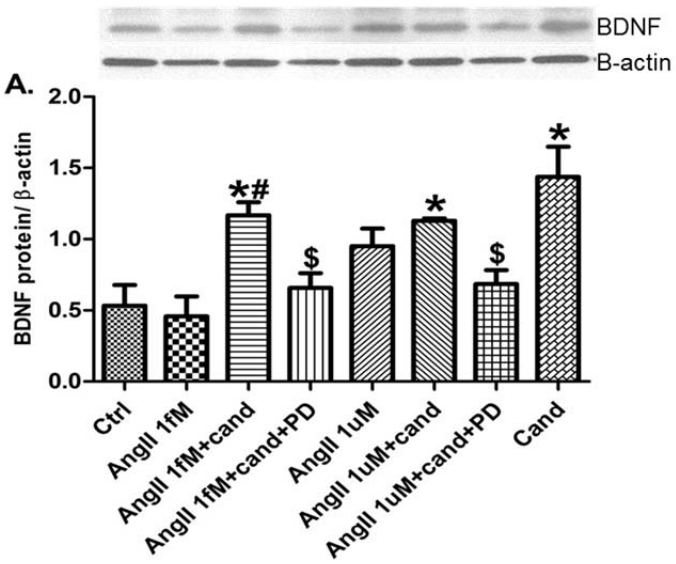

A.
% Tube like structure development
200
150
100
50
0
*
#
*
*$
Ctrl
AngII 1fM
AngII 1fM+PD
AngII 1fM+cand
AngII 1fM+cand+PD
PD-123319
B.
% Tube like structure development
200
150
100
50
0
*#
*$
Ctrl
AngII 1uM
AngII 1uM+cand
AngII 1uM+cand+PD
PD-123319
C.
% Wound recovery
200
150
100
50
0
*
*
*
Ctrl
CGP-42112A
Cand
CGP-42112A+Cand

Figure 2-7: Candesartan induced BDNF expression is mediated through AT2 receptor. To evaluate the involvement of AT2 receptor in candesartan induced BDNF expression hCMECs were pretreated with PD-123319 or vehicle followed by AngII (1ƒM or 1μM) in the presence or absence of candesartan (0.16μM) (Figure 7A). To further confirm the role of AT2 hCMECs were treated with CGP-42112A (0.1μM) or vehicle for 16 hours (Figure 7B). * Significantly different from control, # significantly different from AngII in the same group, $ significantly different from AngII+cand in the same group; overall p=0.02, F=3.086. n=4-6.


A.
BDNF
B-actin
BDNF protein/ β-actin
2.0
1.5
1.0
0.5
0.0
*#
$
*
$
*
Ctrl
AngII 1fM
AngII 1fM+cand
AngII 1fM+cand+PD
AngII 1uM
AngII 1uM+cand
AngII 1uM+cand+PD
Cand

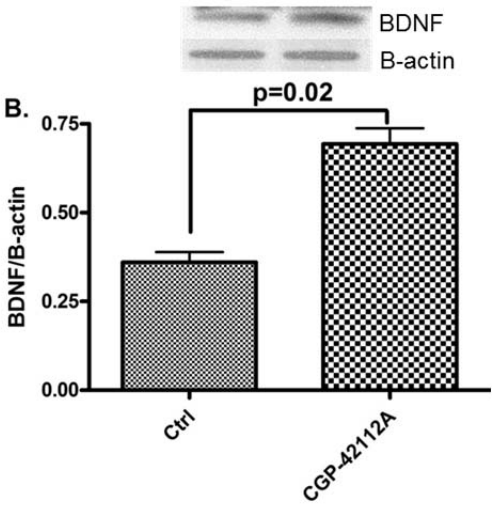
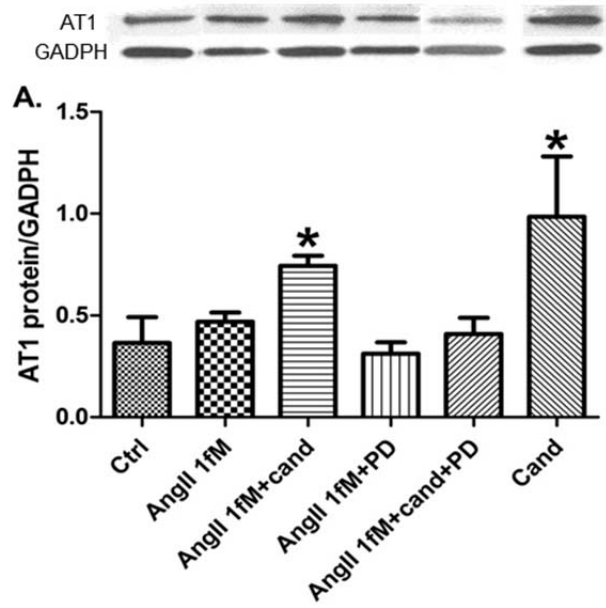
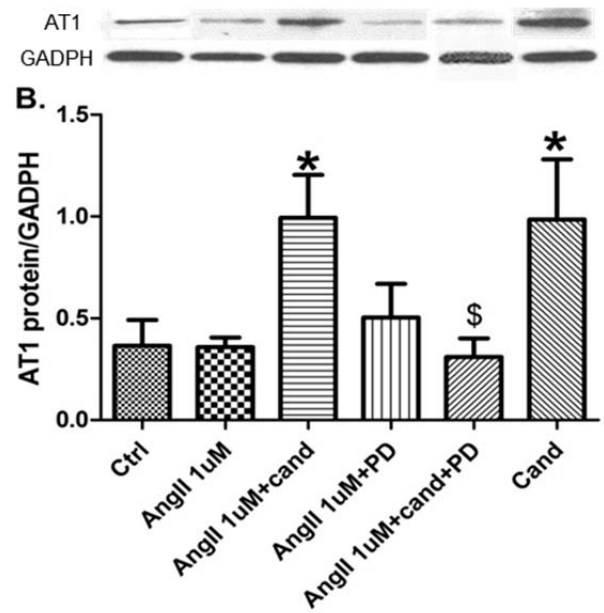
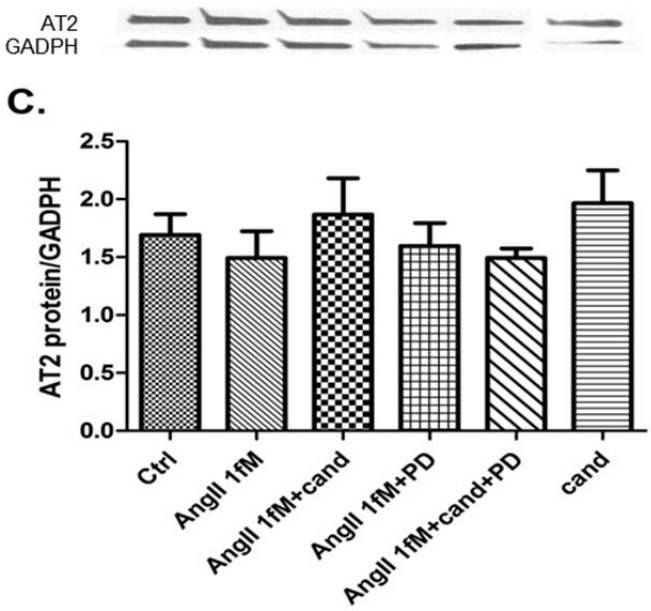

BDNF
B-actin
B.
p=0.02
BDNF/B-actin
0.75
0.50
0.25
0.00
Ctrl
CGP-42112A

Figure 2-8: AT1 antagonism affects the expression of AT1 receptor in an AT2 receptor mediated manner. To assess the expression of AT1 (A and B) and AT2 (Cand D) receptors in response to the different treatments used. Cells were incubated with PD-123319 (0.1uM) or vehicle for 30 minutes followed by 6 hours of AngII 1*f*M (A and C) or 1μM (B and D). After 6 hours of AngII treatment cells were co-incubated with candesartan or vehicle for 10 hours. Receptor expression was assessed using immunoblotting. Data presented as mean ±SEM; n=3-5. * Significantly different from control, # significantly different from AngII in the same group, $ significantly different from AngII+cand in the same group; overall $p<0.0042$, $F=8.742$ for AngII 1μM.


AT1
GADPH
A.
AT1 protein/GADPH
1.5
1.0
0.5
0.0
*
*
Ctrl
AngII 1fM
AngII 1fM+cand
AngII 1fM+PD
AngII 1fM+cand+PD
Cand


AT1
GADPH
B.
AT1 protein/GADPH
1.5
1.0
0.5
0.0
*
*
$
Ctrl
AngII 1uM
AngII 1uM+cand
AngII 1uM+PD
AngII 1uM+cand+PD
Cand


AT2
GADPH
C.
AT2 protein/GADPH
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
AngII 1fM
AngII 1fM+cand
AngII 1fM+PD
AngII 1fM+cand+PD
cand

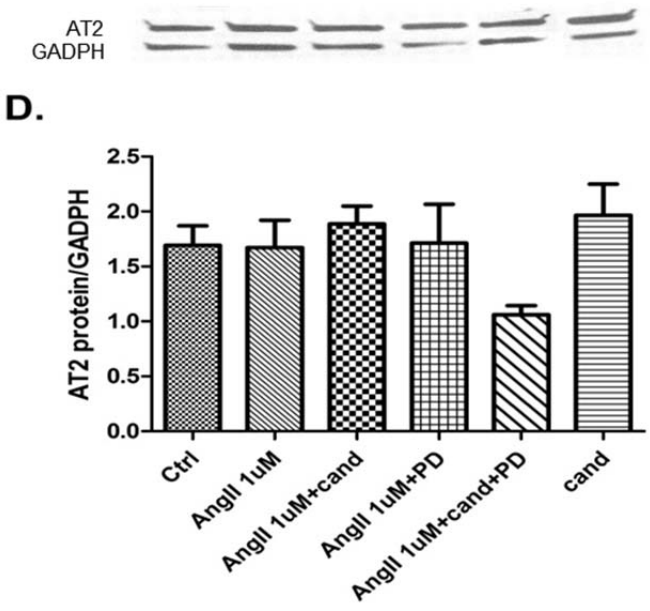

AT2
GADPH
D.
AT2 protein/GADPH
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
AngII 1uM
AngII 1uM+cand
AngII 1uM+PD
AngII 1uM+cand+PD
cand

Figure 2-9: AT1 antagonism modulates the phosphorylation of GSK-3β in an AT2 receptor mediated manner. To assess the phosphorylation of GSK-3β at the inhibitory serine 9 residue, hCMECs were treated as described for AngII receptors expression evaluation. Panel (A) represents the response to AngII 1fM while (B) represents AngII 1μM response. Data presented as mean ±SEM; n=3-5. * Significantly different from control, # significantly different from AngII in the same group, $ significantly different from AngII+cand in the same group; overall p=0.0038, F=6.94 for AngII 1μM.

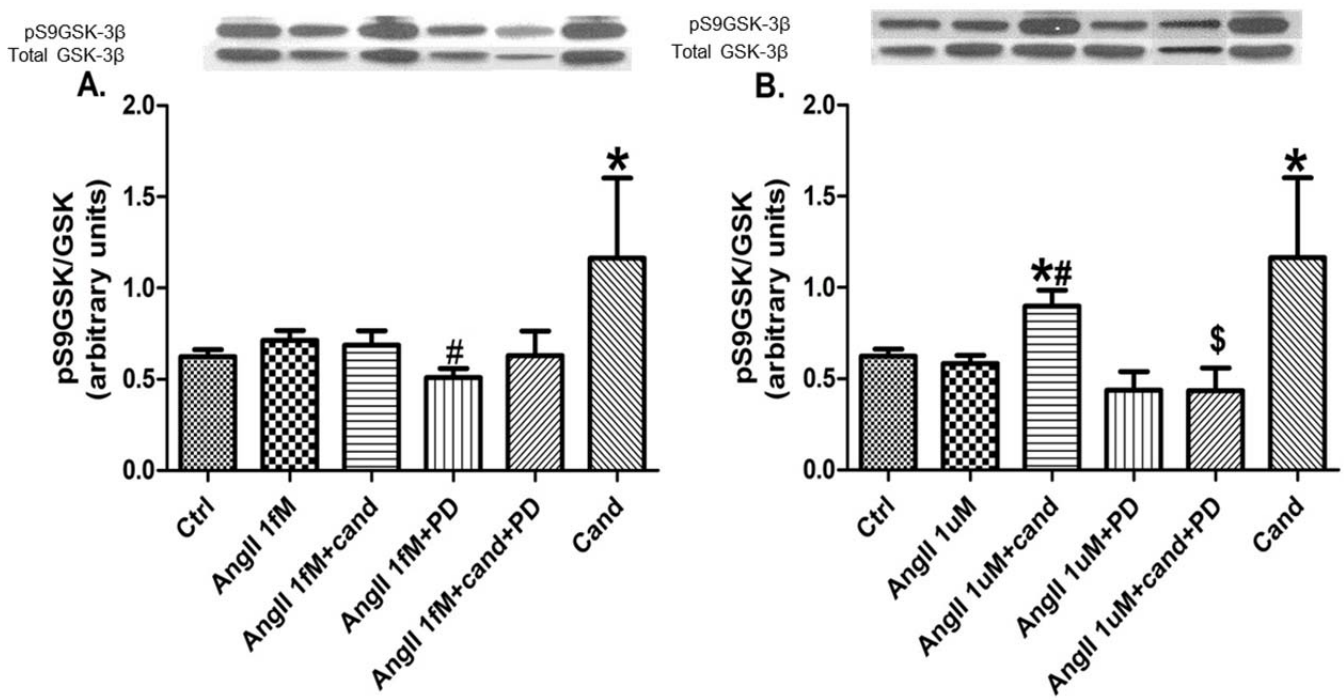
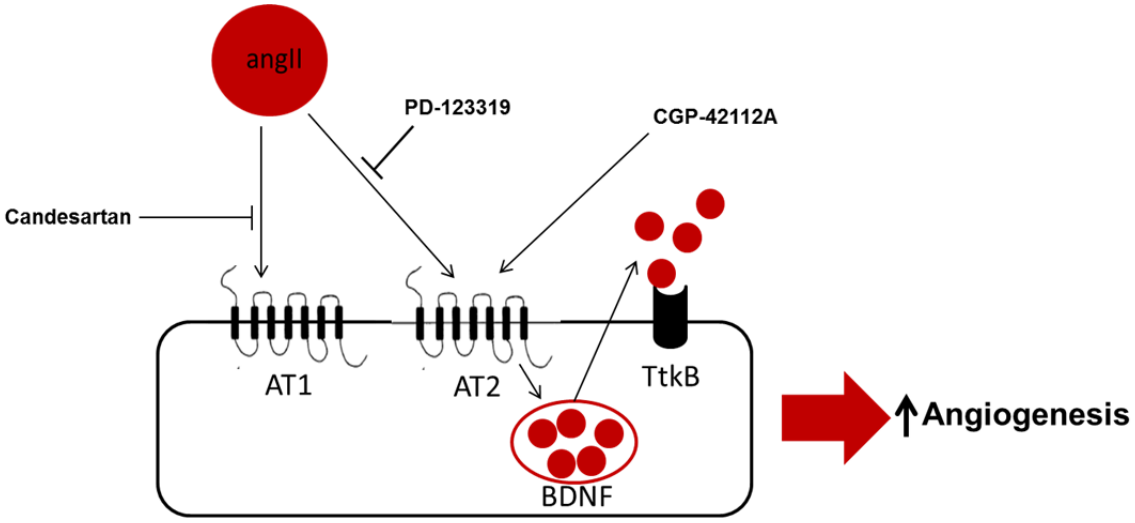
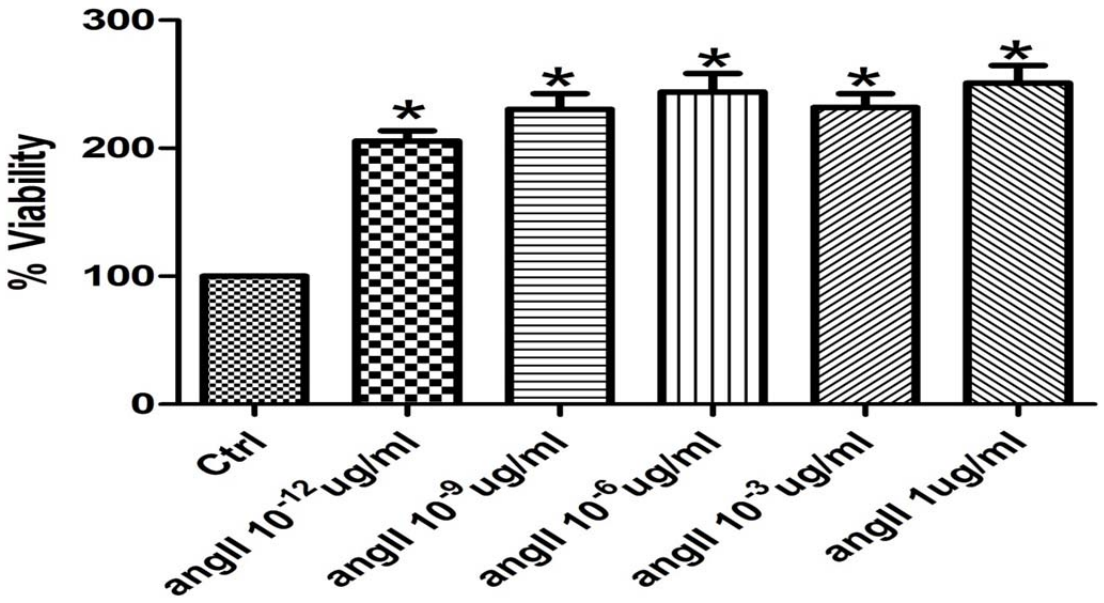

pS9GSK-3β
Total GSK-3β
A.
pS9GSK/GSK (arbitrary units)
2.0
1.5
1.0
0.5
0.0
*
#
Ctrl
AngII 1fM
AngII 1fM+cand
AngII 1fM+PD
AngII 1fM+cand+PD
Cand
pS9GSK-3β
Total GSK-3β
B.
pS9GSK/GSK (arbitrary units)
2.0
1.5
1.0
0.5
0.0
*#
$
*
Ctrl
AngII 1uM
AngII 1uM+cand
AngII 1uM+PD
AngII 1uM+cand+PD
Cand

Figure 2-10: A schematic representation of the results. By blocking AT1 receptors ARBs induce an unopposed stimulation of AT2 receptors. AT2 stimulation induces the expression of BDNF which will bind to its TrkB receptor to promote a proangiogenic state in hCMECs.

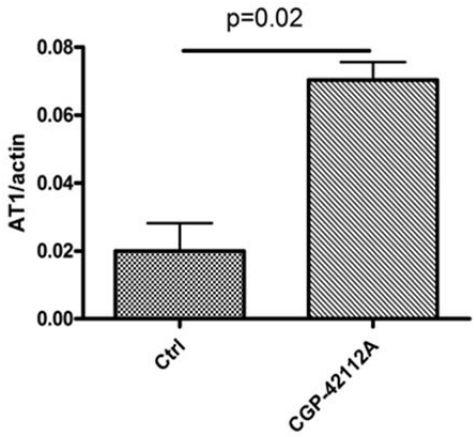

angII
PD-123319
CGP-42112A
Candesartan
AT1
AT2
TtkB
BDNF
Angiogenesis

Supplementary Figure S2-1 Angiotensin II increases the viability of human cerebrovascular endothelial cells (hCMECs). hCMECs were cultured and treated with different doses of AngII and their viability was assessed using MTT metabolism. AngII significantly increased the viability of hCMECs in all doses. * significantly different from ctrl; overall p=0.0001, F=18.36.

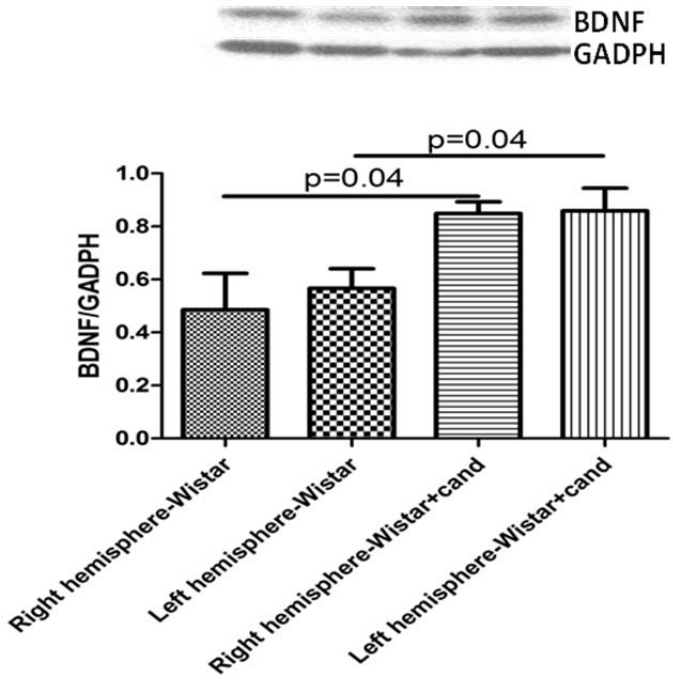

300
200
100
0
% Viability
*
*
*
*
*
Ctrl
angII 10-12 ug/ml
angII 10-9 ug/ml
angII 10-6 ug/ml
angII 10-3 ug/ml
angII 1ug/ml

Supplementary Figure S2-2: AT1 expression is induced through AT2 stimulation. hCMECs were serum starved for 16 hours followed by treatment with either the AT2 agonist CGP-42112A (0.1μM) or vehicle and the expression of AT1 was quantified. Data presented as mean±SEM, n=3.

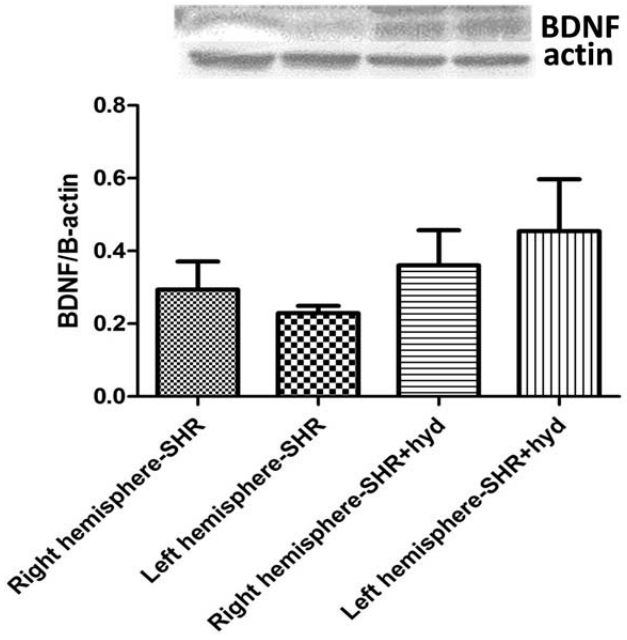

AT1 receptor
actin


p=0.02
0.08
0.06
0.04
0.02
0.00
AT1/actin
Ctrl
CGP-42112A

Supplementary Figure S2-3: AT1 blockade affects the expression of BDNF in wistar rats. Wistar rats were subjected to sham surgery and randomized to receive either saline or candesartan (1mg/kg) 3 hours after surgery. Candesartan significantly increased BDNF expression in both hemispheres as compared to the corresponding hemispheres in saline treated animals. p=0.04; n=4 per group.


BDNF
GADPH
p=0.04
p=0.04
BDNF/GADPH
1.0
0.8
0.6
0.4
0.2
0.0
Right hemisphere-Wistar
Left hemisphere-Wistar
Right hemisphere-Wistar+cand
Left hemisphere-Wistar+cand

Supplementary Figure S2-4: Blood pressure reduction with hydralazine did not affect BDNF expression. Spontaneously hypertensive rats were exposed to sham surgery and randomized to receive either saline or hydralazine (1mg/kg) 3 hours after surgery. Hydralazine treatment resulted in a trend toward increased BDNF expression in the brain; but this trend did not reach statistical significance level. n=4 per group.


BDNF
actin
BDNF/B-actin
0.8
0.6
0.4
0.2
0.0
Right hemisphere-SHR
Left hemisphere-SHR
Right hemisphere-SHR+hyd
Left hemisphere-SHR+hyd

Chapter 3

EARLY AT1 BLOCKADE IMPROVES STROKE OUTCOME BY UP REGULATION OF BDNF IN THE CONTRALESIONAL HEMISPHERE

Ahmed Alhusban, Anna Kozak, Bindu Pillai, Maribeth H. Johnson, Tauheed Ishrat, Adviye Ergul, Susan C. Fagan

To be submitted to Journal of Neurochemistry

Abstract: Stroke is the leading cause of disability worldwide. Despite intensive preclinical and clinical investigations, improving stroke outcome and reducing its overall morbidity is still a daunting task. Improving stroke outcome requires an orchestrated interplay that involves up regulation of pro-survival mediators and a concomitant suppression of pro-apoptotic mediators. In this investigation we aimed at assessing the effect of reactive oxygen species (ROS) scavenging and acute eNOS inhibition on stroke outcome and the protective effects of the AT1 blocker candesartan. To achieve these goals spontaneously hypertensive rats were implanted with blood pressure transmitters, treated with or without tempol for two weeks, and randomized to receive either an eNOS inhibitor (L-NIO) or saline one hour before cerebral ischemia induction. After 3 hours of ischemia animals were randomized to receive either candesartan or saline at the time of reperfusion and sacrificed 24 hours later. Candesartan induced a protective effect that was ablated with eNOS inhibition. This protective effect was associated with protection against ER stress and an eNOS dependent up regulation of BDNF expression in the contralateral hemisphere. Additionally, eNOS inhibition induced a robust increase in nNOS and Nogo-A expression combined with higher levels of proBDNF in the ipsilateral hemisphere.

Key words: stroke, hypertension, NOS, BDNF, ER stress, Nogo-A, neuroprotection.

Introduction:

Data from our lab and others have established the robust neurovascular protective effect of angiotensin II type 1 receptor (AT1) blockers in stroke [1-4]. The exact mechanism by which AT1 blockers exert this neurovascular protective effect is still unknown but a number of possibilities have been identified. These targets include improving cerebral blood flow [3], increased angiogenesis [2], antioxidant effects, antinflammatory effects [5], blood pressure reduction [1], and up regulation of NOS 3 expression [6]. In hypertensive animals, cerebral ischemic susceptibility is increased [7] and this is associated with an increased baseline level of oxidative stress [8].

Our work in spontaneously hypertensive rats (SHRs) demonstrated an improvement in behavioral outcome without a significant reduction in infarct size or bleeding in the injured hemisphere when treated with an AT1 blocker after stroke [9]. We have shown that increased expression of endogenous neurorestorative growth factors (VEGF and BDNF) occurs after AT1 receptor blockade in either normotensive rats with stroke [10] or hypertensive rats without stroke [11]. Importantly, these increases occurred in both hemispheres of the brain [10, 11] and varied based on the region [10].

The purpose of this investigation was to determine the molecular mechanisms of the protective effects of AT1 receptor antagonism in hypertensive rats, particularly focusing on preexisting and acute oxidative stress. We were particularly interested in the contribution of the contralesional hemisphere to the protective effect.

Materials and methods:

Animals: The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Charlie Norwood Veterans Affairs Medical Center (09-04-008). Male spontaneously hypertensive rats (SHRs) (220-250 g; n=6-8 per group) were implanted with blood pressure transmitters as previously reported. After recovery blood pressure was monitored throughout the duration of the experiment. Animals were randomized to receive either a single dose of an eNOS inhibitor N5-(1-iminoethyl) -L-ornithine,hydrochloride (L-NIO) (Cayman chemical; Ann Arbor, MI) or saline one hour before inducing ischemia. Cerebral ischemia was induced through temporary middle cerebral artery occlusion (tMCAO) for 3 hours followed by reperfusion as reported previously [1]. At the time of reperfusion, animals were further randomized to receive saline or 1 mg/kg candesartan IV (Astra-Zeneca). This dose was shown to improve functional outcome in SHRs without reducing infarct size or bleeding [9]. In a subset of animals, we tested whether ameliorating premorbid vascular oxidative stress will restore the ability of this dose of candesartan to reduce the incidence of hemorrhagic transformation. Animals were pretreated with 2 weeks of a superoxide dismutase mimetic, tempol (1mM), in the drinking water (compared to water alone). The dose we used has been shown to prevent cerebrovascular remodeling with only mild blood pressure lowering effects [12]. Animals were followed up for 24 hours and then sacrificed. Brains were harvested; hemispheres separated, and flash frozen in liquid nitrogen.

Behavioral and functional outcome analysis: Functional outcome was evaluated in a blinded manner before sacrifice and at the time of reperfusion using a battery of behavioral tests as reported previously [2].

Western blotting: Brains were homogenized and processed for western blotting as previously described [11]. 30 ug of proteins were loaded in each lane and separated followed by transfer to nitrocellulose membranes. The membranes were blocked using 5% non-fat milk in TBST (1% tween 20 in tris buffered saline) and probed with the following antibodies antiBDNF (1:250; abcam; Cambridge, MA), TrkB (1:500, abcam; Cambridge, MA), p75NTR (1:1000, Millipore; Billerica, MA), Nogo-A (1:1000, Millipore; Billerica, MA), CHOP (1:1000) and nNOS (1:1000, Cell Signaling; Danvers, MA), ATF6 (1:500), pJNK (1:1000)and JNK (1:1000, Santa Cruz biotechnologies ; Santa Cruz, CA). Expression was assessed by quantification of optical density of respective bands normalized to actin using NIH-image J software.

Nitrosative stress: Nitrosative stress was quantified using slot blotting. Briefly, 30ug of proteins of each sample were loaded in each cell of the slot blot apparatus which had pre-wetted nitrocellulose paper. Vacuum was applied to transfer the proteins to the nitrocellulose paper. The membranes were blocked using 5% non-fat milk in TBST (1% tween 20 in tris buffered saline) for 1 hour. Membranes were probed with anti-Nitro-tyrosine antibody (Cayman chemical; Ann Arbor, MI) Nitro-tyrosine levels were quantified by measuring the optic density of the bands using image J software.

Statistical analysis: Data were assessed for normality and a log transformation was used when a distribution was skewed or when the variance increased with the mean.

Transformed variables included hemoglobin excess, BDNF, pro-BDNF, TrkB, p75NTR, Nogo A, and ATF6. Area under the curve (AUC) for blood pressure was calculated for the three hours prior to L-NIO injection (PRE), the three hours of temporary middle cerebral artery occlusion (tMCAO), and the seventeen hours post reperfusion and injection (POST). AUC variables were analyzed using a 2 Candesartan (no vs. yes) by 2 Tempol (no vs. yes) ANOVA. The remaining variables were analyzed using a 2 Candesartan (no vs. yes) by 2 L-NIO (no vs. yes) ANOVA. Interactions were included in all analyses to assess a differential effect of candesartan in the presence of tempol or L-NIO. Paw grasp and beam walk severity score proportions were analyzed for group differences using Fisher's exact test. SAS® version 9.3 (SAS Institute, Inc., Cary, NC) was used for all analyses. Statistical significance was determined at alpha=0.05.

Results:

Acute eNOS inhibition does not affect the hypotensive effect of candesartan:

Previously, we demonstrated the ability of a single injection of candesartan (1mg/kg) to lower ischemia- induced blood pressure increase [9]. eNOS inhibition is known to increase blood pressure [13]. In addition AT1 blockers have been shown to increase eNOS expression and release of the vasorelaxant NO [6]. Acute eNOS inhibition before induction of ischemia did not affect the hypotensive effect of candesartan (Figure 3-1).

Chronic pretreatment with Tempol did not restore the vasculoprotective effect of candesartan: Data from our lab demonstrated a dose dependent vasculoprotective effect of candesartan after tMCAO [9]. Candesartan-induced vasculoprotection (reduced bleeding) was lost at the 1mg/kg dose which was vasculoprotective in Wistar rats [2, 9].

Accordingly, we were interested in assessing whether ameliorating endothelial dysfunction with Tempol would restore the vasculoprotective effect of this dose.

Although tempol reduced BP at baseline (from 141.6 to 130.6 mmHg mean), L-NIO injection minimized this difference (139 to 133 mmHg) transiently (Figure 3-1). However, tempol DID NOT restore the ability of candesartan to reduce hemoglobin excess after stroke. In fact, none of the treatment groups has significantly different vascular damage after tMCAO (S3-1).

Candesartan-induced improved neurobehavioral outcome is mediated through eNOS: Data from our lab and others have demonstrated the ability of candesartan to improve neurobehavioral outcome and recovery after cerebral ischemia in SHRs [4, 9]. Acute eNOS inhibition ablated candesartan induced neuroprotection assessed 24 hours after cerebral ischemia (Figure 3-2B).

Chronic tempol pretreatment did not alter neurobehavioral outcome after stroke: Despite reported amelioration of endothelial dysfunction [12], pretreatment with tempol did not affect neurobehavioral outcome after stroke in SHRs (Figure S3-2).

Acute eNOS inhibition increases nitrosative stress in the contralesional hemisphere: Ischemia/reperfusion is associated with increased oxidative stress in the ipsilateral hemisphere [14]. eNOS inhibition did not affect the levels of nitrosative stress in the ipsilateral hemisphere (Figure 3-3A). In contrast, eNOS inhibition significantly increased the levels of nitrosative stress in the contralesional hemisphere of both candesartan and saline treated animals (Figure 3-3B). Surprisingly, chronic tempol pretreatment did not have any detectable effect on the levels of nitrotyrosine (S3-3).

Chronic pretreatment with tempol did not have any effect on any of the endpoints. Accordingly, the decision was made to limit further analyses to candesartan and L-NIO groups only.

BDNF is modulated by eNOS following stroke: Following cerebral ischemia candesartan significantly increased BDNF expression in the contralesional hemisphere when compared to saline treated animals (Figure 3-4A). Consistent with previous reports on the involvement of eNOS expression in BDNF expression [15], acute eNOS inhibition ablated the candesartan-induced increase in BDNF expression (Figure 4A).

In contrast to the pro-survival effect of mature BDNF, proBDNF is associated with apoptosis and inhibition of neuronal cone extension [16, 17]. In saline-treated animals, acute eNOS inhibition increased proBDNF expression (Figure 3-4B). Consistent with findings in saline-treated animals, acute inhibition of eNOS significantly increased proBDNF levels in the ipsilateral hemisphere of candesartan-treated animals (Figure 3-4B). These findings suggest the involvement of eNOS in different levels of proBDNF expression and processing. BDNF pro-survival effects are mediated through TrkB signaling. Krikov et al. demonstrated the ability of candesartan to increase TrkB levels after cerebral ischemia in normotensive rats [18]. In SHRs, candesartan did not alter the expression of TrkB (Figure3- 4C). Additionally, acute eNOS inhibition did not alter TrkB expression (Figure 3-4C).

Early AT1 blockade reduced p75NTR expression in the ipsilateral hemisphere: Under normal conditions, p75 neurotrophin receptor (p75NTR) expression is very low [16] but upon ischemia, p75NTR expression is upregulated [16]. In agreement with

previous reports, induction of cerebral ischemia resulted in a robust increase of p75NTR expression in the ipsilateral hemisphere (Figure 3-4D). Interestingly, candesartan treatment was associated with reduced p75NTR expression in the ipsilateral hemisphere of SHRs (Figure 3-4D).

Acute eNOS inhibition increases the expression of nNOS in the ipsilateral hemisphere after cerebral ischemia: nNOS expression has been associated with larger infarct size and worsened stroke outcome in experimental models of stroke [19]. Reciprocal regulation of different NOS isoforms has been previously reported [20]. In normotensive rats, MCAO reduced eNOS expression whereas iNOS and nNOS were increased [20]. In contrast, MCAO reduced nNOS level in the ipsilateral hemisphere of both candesartan and saline treated hypertensive rats as compared to the contralesional hemispheres in the same animals. Consistent with data on reciprocal regulation of NOS isoforms, eNOS inhibition induced a robust up regulation of nNOS expression in the ipsilateral hemisphere of both candesartan and saline treated hypertensive animals (Figure 3-5A).

Acute eNOS inhibition increased NOGO-A expression in the ipsilateral hemisphere after cerebral ischemia:

Neurite outgrowth inhibitor-A (NOGO-A) levels and its downstream signaling are associated with worsened outcome and poor recovery after stroke [21]. Induction of cerebral ischemia decreased the level of NOGO-A in the ipsilateral hemisphere of SHRs as compared to contralesional hemisphere (Figure 3-5B). Acute eNOS inhibition upregulated the expression of NOGO-A in the ipsilateral hemisphere of both

candesartan and saline treated SHRs after MCAO (Figure 3-5B). Detrimental effects of NOGO-A signaling are partially mediated through Janus N terminal kinase (JNK) signaling pathway [21]. Consistent with changes in NOGO-A expression; L-NIO treatment significantly increased JNK phosphorylation in the ipsilateral hemispheres of both candesartan and saline treated animals (Figure 3-5C).

AT1 blockade ameliorates ER stress and reduces UPR markers: Endoplasmic reticulum (ER) stress and the resulting unfolded protein response (UPR) have been implicated in stroke pathophysiology [22]. ER stress amelioration has been shown to reduce stroke severity in diabetic animals [23]. Additionally, AngII induced hypertension has been associated with an increase in ER stress and UPR [24], which suggests a possible role of AT1 blockers in ameliorating ER stress. Candesartan administration counteracted the reported ischemia- induced increase in CCAAT-enhancer-binding protein homologous protein(CHOP) levels (Figure 3-6A) [22]. Interestingly, candesartan reduced the cleavage of activating transcription factor 6 (ATF6) as measured by cleaved ATF6 in the brain (Figure 6C). Overexpression of GRP78 has been shown to reverse ER stress associated hypertension [24]. Candesartan significantly increased GRP78 expression in both hemispheres of SHRs (Figure 3-6D).

Discussion: Candesartan is an often-prescribed AT1 blocker with convincing neurovascular protective properties in experimental stroke [1-3]. Clinical development stalled when the Scandinavian Candesartan in Acute Stroke Trial (SCAST) failed to demonstrate a benefit of early, aggressive, blood pressure lowering with candesartan in hypertensive ischemic stroke patients [25]. Therapeutic manipulation of the renin-angiotensin system (RAS) for treatment of brain injury remains very promising,

however, and exploration of the molecular pathways involved continue to reveal a broad range of protective and restorative mediators that may be harnessed. The results of this investigation demonstrate the involvement of eNOS in candesartan-induced activation of BDNF signaling. In addition, we identified some novel mechanisms by which eNOS inhibition might worsen stroke outcome. Interestingly, our data identified, for the first time, the ability of AT1 blockers to attenuate ischemia-induced ER stress to improve stroke outcome in hypertensive animals.

AT1 blockers have been shown to ameliorate stroke outcome through a number of different mechanisms including up regulation of eNOS expression [6], up regulation of growth factor expression [10] and amelioration of oxidative stress [26]. Previously, we demonstrated the ability of candesartan to confer neuroprotection and improve functional outcome in hypertensive rats [9]. Reduced bioavailability of NO and resulting endothelial dysfunction are major pathophysiologic mechanisms of the complications of hypertension [27]. Experimental data from eNOS knockout animals showed a larger infarct size and blunted ischemia-induced up regulation of growth factors [15]. Despite the well-established effect of chronic eNOS inhibition on stroke outcome [28], the effect of acute inhibition of eNOS on stroke outcome in hypertensive animals is still unknown. In addition, the involvement of eNOS in candesartan-induced neuroprotection and growth factor expression remains undetermined.

BDNF has been shown to reduce infarct size and improve functional outcome following cerebral ischemia [29]. Recently, we reported on the ability of candesartan to increase BDNF expression in SHR [11]. Kishi T et al. have also reported the ability of

telmisartan, another AT1 blocker, to ameliorate cognitive dysfunction in SHRSP through the BDNF/TrkB system[30].

Our recent data confirm and further extend data on candesartan's induction of the BDNF/TrkB system [18]. This effect of candesartan was attributed to unopposed stimulation of AT2 receptor in SHRs [11]. Interestingly, we found that candesartan preferentially up regulates BDNF in the contralesional hemisphere which has been shown to be involved in recovery through induction of neuroplasticity [31]. Our data suggest BDNF as a possible mediator of the contralesional hemisphere's involvement in functional recovery.

A unique finding in this work is the possible involvement of eNOS in neurotrophin processing in addition to already reported effects on expression. In both candesartan and saline treated animals, eNOS inhibition resulted in an increase in proBDNF levels in both hemispheres. This finding suggests a possible regulatory role of eNOS in processing of proBDNF to mature BDNF. Additionally, proBDNF accumulation in the ipsilateral hemisphere would adversely affect neuronal survival and neuroplastic changes in the penumbra. This finding identifies a possible mechanism through which eNOS inhibition worsens stroke outcome.

Another interesting finding of this study was the ability of early AT1 blockade to prevent ischemia induced p75NTR expression. This effect was observed in the ipsilateral hemisphere and was not affected by eNOS inhibition.

Reciprocal regulation of different NOS isoforms has been reported previously [20]. Our data supports this concept and identifies the regulation of nNOS expression as

another mechanism by which eNOS activity affects stroke outcome. nNOS expression has been shown to worsen stroke outcome by inhibiting neurogenesis [19]. The exact mechanism of nNOS induced neurogenesis inhibition is still unknown. Our results demonstrated an almost three fold increase in Nogo-A expression after eNOS inhibition. This similar expression pattern between nNOS and NOGO-A suggests a possible mechanistic link between the two proteins. An increase in nNOS might up regulate NOGO-A expression and NOGO-A is known to induce neuronal apoptosis [21]. The concomitant increase in JNK phosphorylation supports this possible link although definitive confirmation requires further mechanistic investigation.

ER stress has been shown to play a role in the pathophysiology of stroke [22] and its alleviation was associated with ameliorated ischemic insult in diabetic animals [23]. Recently, an association between ER stress and hypertension has been suggested [24]. Our results showed a robust increased expression of ER stress markers in the ipsilateral hemisphere of hypertensive animals after ischemia induction. Expression of ER stress markers in the contralesional hemisphere was very low. Accordingly, analysis was limited to the ipsilateral hemisphere. To our knowledge we are the first group to report the ability of AT1 blockers to alleviate ischemia-induced ER stress. Additionally, previous reports on ER stress in stroke did not assess the cleavage of ATF6 that leads to its nuclear localization and induction of its effects. In this work we assessed ATF6 cleavage using the same method that has been used recently by Dromparis et al [32]. These findings identify a novel mechanism by which AT1 blockers improve stroke outcome. In addition, it paves the way for further investigations to assess the possible

implications of this finding in other disease states where ER stress has been shown to be involved in the pathophysiology.

Originally, our working hypothesis was that restoring endothelial function with 2 weeks of tempol treatment would result in neurovascular protection in SHR as was seen with the same dose of candesartan in normotensive animals [1]. This was not the case and although tempol significantly reduced the mean blood pressure prior to stroke, the animals did not achieve normotension and responded in a manner that did not differ from those that did not receive tempol. It is possible that the dose or duration of tempol could have been optimized to achieve the desired effect, but we decided to focus on the effects of acute eNOS inhibition with L-NIO instead.

In this investigation, all attention was made to consider the involvement of different distinct but closely interconnected pathways in stroke outcome. Despite all efforts made, the following limitations should be highlighted. In this work we used a pharmacologic approach to inhibit eNOS. Although not selective for eNOS, L-NIO is widely used as an eNOS inhibitor [33, 34]. In addition, our interest was to assess the role of acute inhibition of eNOS in hypertensive animals rather than the chronic effects. The rationale behind this preference is based on reports of higher eNOS expression in response to increased oxidative stress and endothelial dysfunction, both of which are considered major pathophysiologic mechanisms in hypertension-induced vascular complications [35]. Accordingly, one of our aims was to elucidate the functional role of increased eNOS expression. Another limitation of this work is the correlative nature of some of the analyses. This was most prominent in the discussion of the interaction between eNOS inhibition and Nogo-A expression. This novel finding was exciting for us

especially when considered in lieu of results on the effect of hypertension on Nogo-A expression. Nogo-A expression is limited to neuronal tissue [36] whereas eNOS and nNOS have a wider tissue expression pattern [37]. The almost exact expression pattern of both nNOS and Nogo-A suggests a possible cross-talk between the two proteins. Additionally, this finding identifies Nogo-A and nNOS as a possible link between the cardiovascular and central nervous systems. Confirming this cross talk requires more in depth mechanistic work.

In conclusion, our findings demonstrated the ability of candesartan to confer protection and increase mature BDNF expression in the contralesional hemisphere of hypertensive animals in an eNOS dependent manner. In addition, for the first time, our findings clearly demonstrated candesartan's ability to counteract ischemia-induced ER stress. Finally our data suggests a novel cross talk between NOS isoforms and Nogo-A expression and signaling in hypertensive animals. Therapeutic manipulation of angiotensin receptor signaling remains a promising tactic for enhancing stroke recovery.

References

1. *Fagan, S.C., et al., Hypertension after experimental cerebral ischemia: candesartan provides neurovascular protection. J Hypertens, 2006. 24(3): p. 535-9.*
2. *Kozak, A., et al., Candesartan augments ischemia-induced proangiogenic state and results in sustained improvement after stroke. Stroke, 2009. 40(5): p. 1870-6.*
3. *Engelhorn, T., et al., The angiotensin II type 1-receptor blocker candesartan increases cerebral blood flow, reduces infarct size, and improves neurologic outcome after transient cerebral ischemia in rats. J Cereb Blood Flow Metab, 2004. 24(4): p. 467-74.*
4. *Ito, T., et al., Protection against ischemia and improvement of cerebral blood flow in genetically hypertensive rats by chronic pretreatment with an angiotensin II AT1 antagonist. Stroke, 2002. 33(9): p. 2297-303.*
5. *Lou, M., et al., Sustained blockade of brain AT1 receptors before and after focal cerebral ischemia alleviates neurologic deficits and reduces neuronal injury, apoptosis, and inflammatory responses in the rat. J Cereb Blood Flow Metab, 2004. 24(5): p. 536-47.*
6. *Yamakawa, H., et al., Normalization of endothelial and inducible nitric oxide synthase expression in brain microvessels of spontaneously hypertensive*

rats by angiotensin II AT1 receptor inhibition. J Cereb Blood Flow Metab, 2003. 23(3): p. 371-80.

7. ***Lee, T.H., et al., Effects of aging and hypertension on cerebral ischemic susceptibility: evidenced by MR diffusion-perfusion study in rat. Exp Neurol, 2011. 227(2): p. 314-21.***
8. ***Zhang, X.H., et al., Increased oxidative stress is responsible for severer cerebral infarction in stroke-prone spontaneously hypertensive rats. CNS Neurosci Ther, 2011. 17(6): p. 590-8.***
9. ***Kozak, W., et al., Vascular protection with candesartan after experimental acute stroke in hypertensive rats: a dose-response study. J Pharmacol Exp Ther, 2008. 326(3): p. 773-82.***
10. ***Guan, W., et al., Vascular protection by angiotensin receptor antagonism involves differential VEGF expression in both hemispheres after experimental stroke. PLoS One, 2011. 6(9): p. e24551.***
11. ***Alhusban, A., et al., AT1 Receptor Antagonism Is Proangiogenic in the Brain: BDNF a Novel Mediator. J Pharmacol Exp Ther, 2013. 344(2): p. 348-59.***
12. ***Pires, P.W., et al., Tempol, a superoxide dismutase mimetic, prevents cerebral vessel remodeling in hypertensive rats. Microvasc Res, 2010. 80(3): p. 445-52.***
13. ***Harrison, D.G., Cellular and molecular mechanisms of endothelial cell dysfunction. J Clin Invest, 1997. 100(9): p. 2153-7.***

14. *Chan, P.H., Role of oxidants in ischemic brain damage. Stroke, 1996. 27(6): p. 1124-9.*

15. *Chen, J., et al., Endothelial nitric oxide synthase regulates brain-derived neurotrophic factor expression and neurogenesis after stroke in mice. J Neurosci, 2005. 25(9): p. 2366-75.*

16. *Caporali, A. and C. Emanueli, Cardiovascular actions of neurotrophins. Physiol Rev, 2009. 89(1): p. 279-308.*

17. *Hennigan, A., R.M. O'Callaghan, and A.M. Kelly, Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection. Biochem Soc Trans, 2007. 35(Pt 2): p. 424-7.*

18. *Krikov, M., et al., Candesartan but not ramipril pretreatment improves outcome after stroke and stimulates neurotrophin BNDF/TrkB system in rats. J Hypertens, 2008. 26(3): p. 544-52.*

19. *Sun, Y., et al., Neuronal nitric oxide synthase and ischemia-induced neurogenesis. J Cereb Blood Flow Metab, 2005. 25(4): p. 485-92.*

20. *Koh, P.O., Ferulic acid modulates nitric oxide synthase expression in focal cerebral ischemia. Lab Anim Res, 2012. 28(4): p. 273-8.*

21. *Kilic, E., et al., Role of Nogo-A in neuronal survival in the reperfused ischemic brain. J Cereb Blood Flow Metab, 2010. 30(5): p. 969-84.*

22. *Roussel, B.D., et al., Endoplasmic reticulum dysfunction in neurological disease. Lancet Neurol, 2013. 12(1): p. 105-18.*

23. *Srinivasan, K. and S.S. Sharma, 3-Bromo-7-nitroindazole attenuates brain ischemic injury in diabetic stroke via inhibition of endoplasmic reticulum stress pathway involving CHOP. Life Sci, 2012. 90(3-4): p. 154-60.*

24. *Young, C.N., et al., ER stress in the brain subfornical organ mediates angiotensin-dependent hypertension. J Clin Invest, 2012. 122(11): p. 3960-4.*

25. *Sandset, E.C., et al., The angiotensin-receptor blocker candesartan for treatment of acute stroke (SCAST): a randomised, placebo-controlled, double-blind trial. Lancet, 2011. 377(9767): p. 741-50.*

26. *Hamai, M., et al., Comparison of inhibitory action of candesartan and enalapril on brain ischemia through inhibition of oxidative stress. Neuropharmacology, 2006. 51(4): p. 822-8.*

27. *Briones, A.M. and R.M. Touyz, Oxidative stress and hypertension: current concepts. Curr Hypertens Rep, 2010. 12(2): p. 135-42.*

28. *Sierra, C., A. Coca, and E.L. Schiffrin, Vascular mechanisms in the pathogenesis of stroke. Curr Hypertens Rep, 2011. 13(3): p. 200-7.*

29. *Shi, Q., et al., Adenovirus-mediated brain-derived neurotrophic factor expression regulated by hypoxia response element protects brain from injury of transient middle cerebral artery occlusion in mice. Neurosci Lett, 2009. 465(3): p. 220-5.*

30. *Kishi, T., Y. Hirooka, and K. Sunagawa, Telmisartan protects against cognitive decline via up-regulation of brain-derived neurotrophic factor/tropomyosin-related kinase B in hippocampus of hypertensive rats. J Cardiol, 2012. 60(6): p. 489-94.*

31. *Liu, Z., et al., Bone marrow stromal cells promote skilled motor recovery and enhance contralesional axonal connections after ischemic stroke in adult mice. Stroke, 2011. 42(3): p. 740-4.*

32. *Dromparis, P., et al., Attenuating endoplasmic reticulum stress as a novel therapeutic strategy in pulmonary hypertension. Circulation, 2013. 127(1): p. 115-25.*

33. *de la Torre, J.C. and G. Aliev, Inhibition of vascular nitric oxide after rat chronic brain hypoperfusion: spatial memory and immunocytochemical changes. J Cereb Blood Flow Metab, 2005. 25(6): p. 663-72.*

34. *Jiang, M.H., et al., Different effects of eNOS and nNOS inhibition on transient forebrain ischemia. Brain Res, 2002. 946(1): p. 139-47.*

35. *Schulz, E., T. Gori, and T. Munzel, Oxidative stress and endothelial dysfunction in hypertension. Hypertens Res, 2011. 34(6): p. 665-73.*

36. *Teng, F.Y. and B.L. Tang, Cell autonomous function of Nogo and reticulons: The emerging story at the endoplasmic reticulum. J Cell Physiol, 2008. 216(2): p. 303-8.*

37. *Forstermann, U. and W.C. Sessa, Nitric oxide synthases: regulation and function. Eur Heart J, 2012. 33(7): p. 829-37, 837a-837d.*

Figure 3-1: Early AT1 blockade and Tempol treatment reduced blood pressure after the induction of cerebral ischemia. Blood pressure telemetry showing the hypotensive effect of Candesartan and Tempol following cerebral ischemia. Tempol treatment reduced baseline blood pressure, blood pressure during tMCAO and the acute increase after reperfusion; $ $p<0.001$,* $p=0.038$. a, b, c Pairs of blood pressure telemetry means with different letters are significantly different from each other. Data presented as mean±SEM; n=5-6 animals per group.

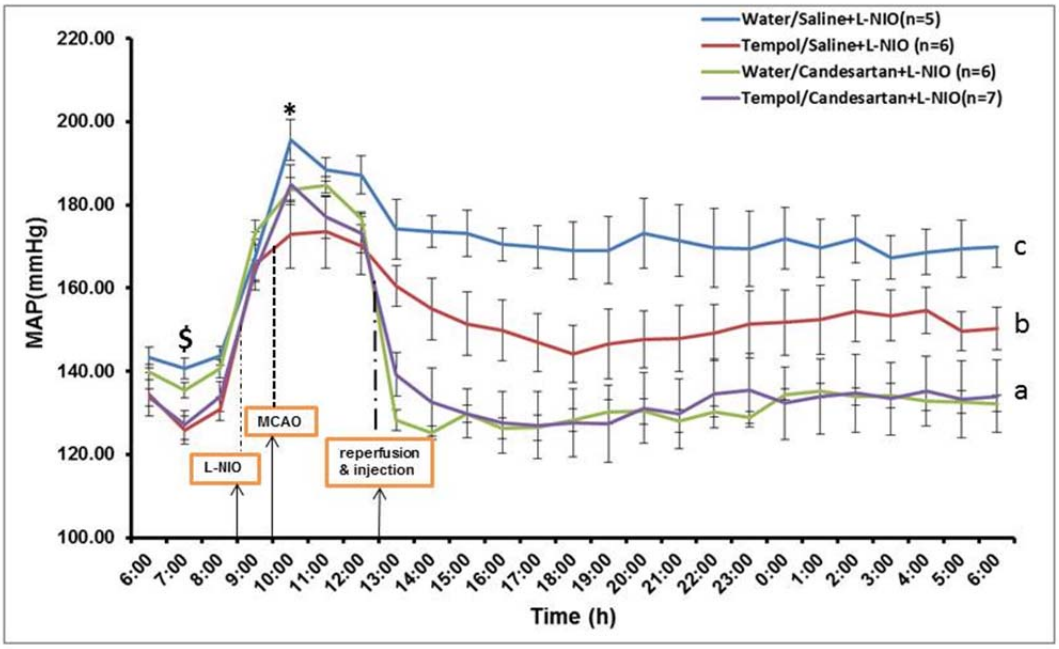

Water/Saline+L-NIO(n=5)
Tempol/Saline+L-NIO (n=6)
Water/Candesartan+L-NIO (n=6)
Tempol/Candesartan+L-NIO(n=7)
MAP(mmHg)
220.00
200.00
180.00
160.00
140.00
120.00
100.00
$
*
MCAO
L-NIO
reperfusion
& injection
c
b
a
6:00
7:00
8:00
9:00
10:00
11:00
12:00
13:00
14:00
15:00
16:00
17:00
18:00
19:00
20:00
21:00
22:00
23:00
0:00
1:00
2:00
3:00
4:00
5:00
6:00
Time (h)

Figure 3-2: Early AT1 blockade induced neuroprotection is eNOS mediated. Candesartan improved neurological outcome after stroke as assessed by modified bederson (B). a, b Pairs of means with different letters are significantly different from each other. Data presented as mean±SEM; n=6-8 per group.

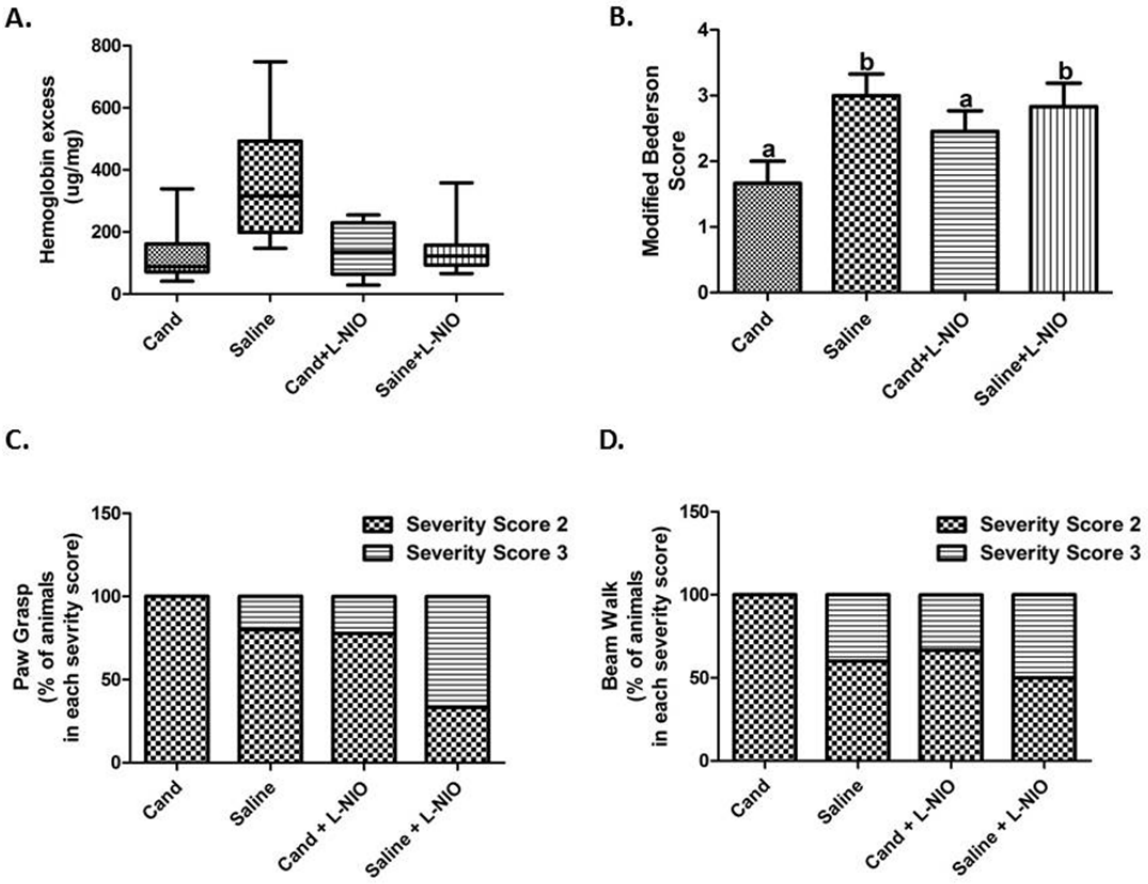
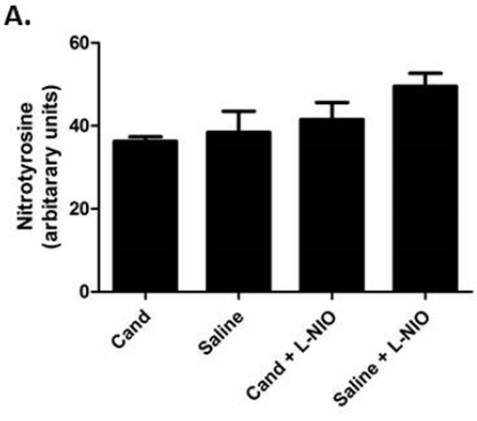

A.
Hemoglobin excess (ug/mg)
800
600
400
200
0
Cand
Saline
Cand+L-NIO
Saine+L-NIO
B.
Modified Bederson Score
4
3
2
1
0
a
b
a
b
Cand
Saline
Cand+L-NIO
Saline+L-NIO
C.
Paw Grasp (% of animals in each sevrity score)
150
100
50
0
Severity Score 2
Severity Score 3
Cand
Saline
Cand + L-NIO
Saline + L-NIO
D.
Beam Walk (% of animals in each severity score)
150
100
50
0
Severity Score 2
Severity Score 3
Cand
Saline
Cand + L-NIO
Saline + L-NIO

Figure 3-3: eNOS inhibition alters nitrosative stress levels after stroke. Acute L-NIO treatment did not affect the nitrosative stress levels in the ipsilateral hemisphere (A). In contrast, L-NIO induced an increased nitrosative stress in the contralateral hemispheres of both candesartan and saline treated animals (B). a, b Pairs of means with different letters are significantly different from each other. Data presented as mean±SEM; n=6-8 per group.


A.
Nitrotyrosine (arbitrary units)
60
40
20
0
Cand
Saline
Cand + L-NIO
Saline + L-NIO

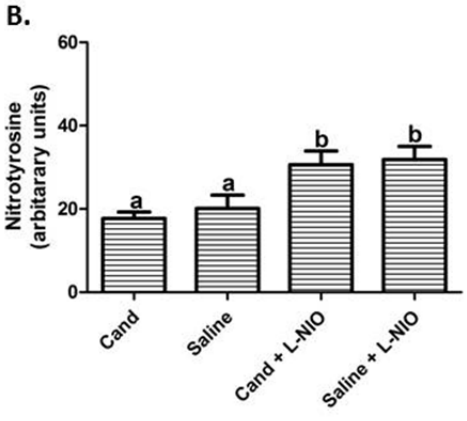

B.
Nitrotyrosine (arbitrary units)
60
40
20
0
a
a
b
b
Cand
Saline
Cand + L-NIO
Saline + L-NIO

Figure 3-4: Early AT1 blockade upregulated BDNF expression in the contralesional hemisphere. Candesartan increased BDNF expression in an eNOS dependent manner (A). eNOS inhibition increased proBDNF expression in both hemispheres (B). eNOS inhibition increased p75NTR in the ipsilateral hemisphere (D). TrkB expression was not altered by any of the used interventions (C). Data presented as mean±SEM. Solid columns represent ipsilateral hemisphere, striped columns represent contralateral hemisphere. a, b or A, B Pairs of means with different letters are significantly different from each other. n=4 animals per group.

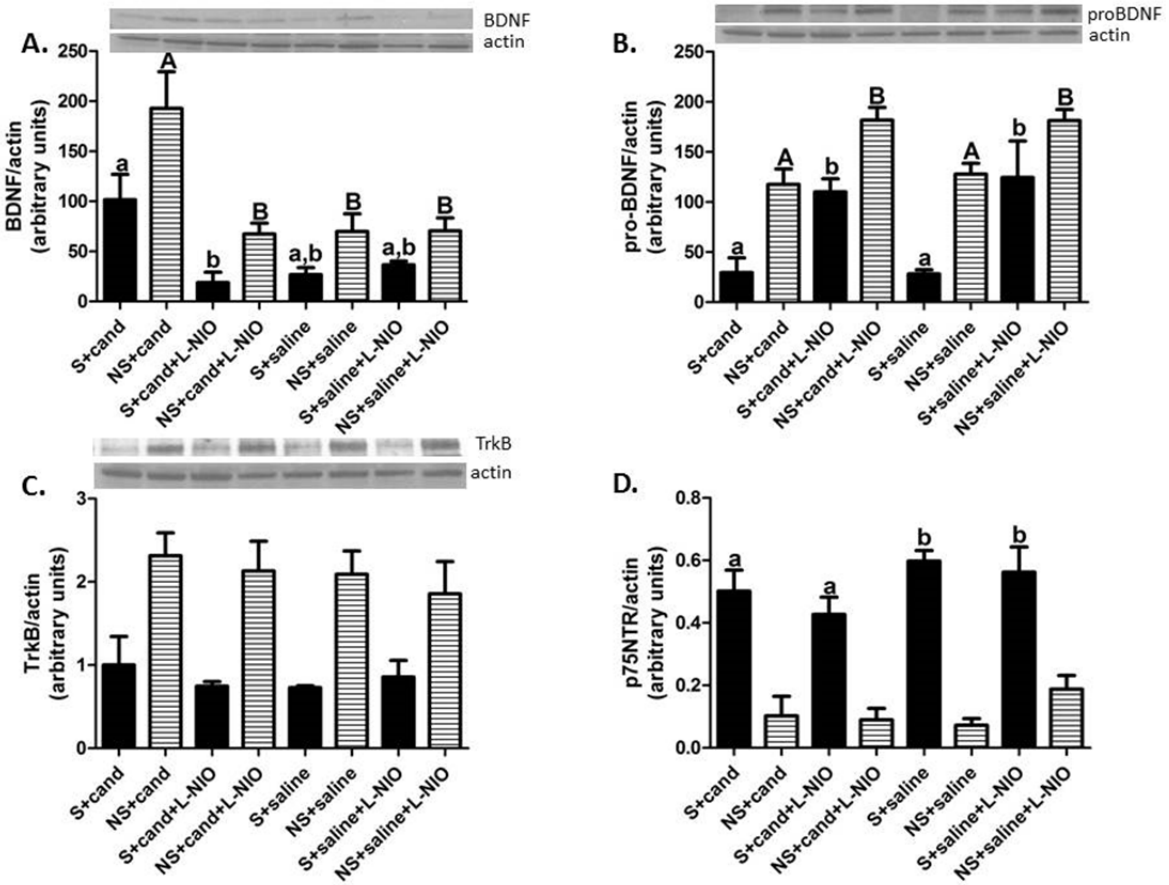
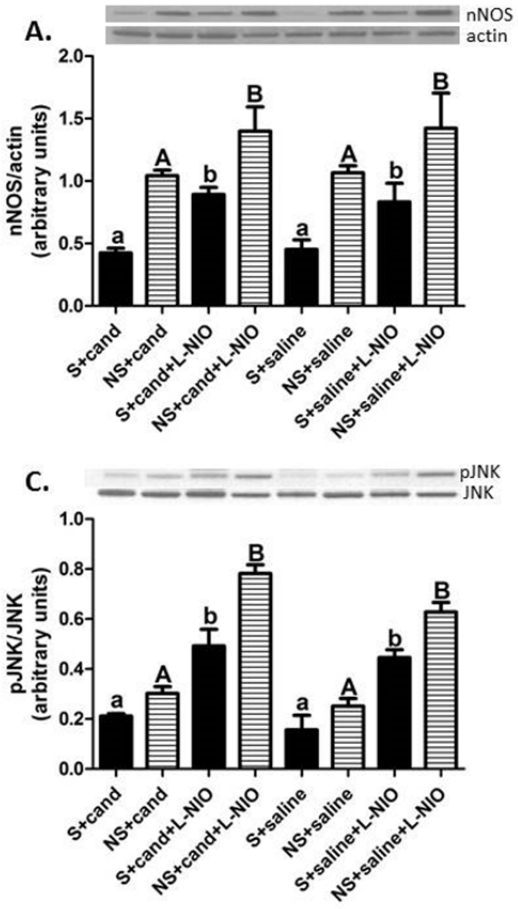
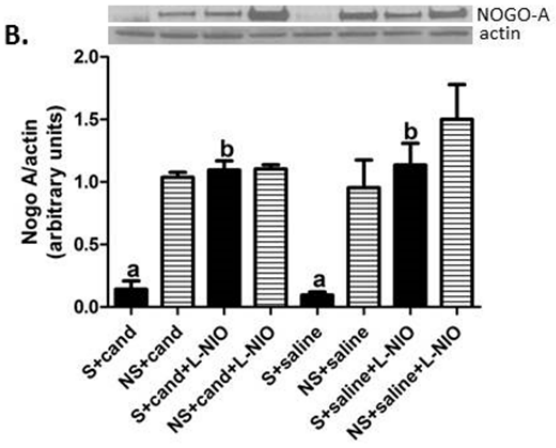

A.
BDNF
actin
BDNF/actin (arbitrary units)
250
200
150
100
50
0
a
A
b
B
a,b
B
a,b
B
S+cand
NS+cand
S+cand+L-NIO
NS+cand+L-NIO
S+saline
NS+saline
S+saline+L-NIO
NS+saline+L-NIO
B.
proBDNF
actin
pro-BDNF/actin (arbitrary units)
250
200
150
100
50
0
a
A
b
B
a
A
b
B
S+cand
NS+cand
S+cand+L-NIO
NS+cand+L-NIO
S+saline
NS+saline
S+saline+L-NIO
NS+saline+L-NIO
C.
TrkB
actin
TrkB/actin (arbitrary units)
3
2
1
0
S+cand
NS+cand
S+cand+L-NIO
NS+cand+L-NIO
S+saline
NS+saline
S+saline+L-NIO
NS+saline+L-NIO
D.
p75NTR/actin (arbitrary units)
0.8
0.6
0.4
0.2
0.0
a
a
b
b
S+cand
NS+cand
S+cand+L-NIO
NS+cand+L-NIO
S+saline
NS+saline
S+saline+L-NIO
NS+saline+L-NIO

Figure 3-5: eNOS inhibition worsens stroke outcome. Acute L-NIO treatment increased nNOS (A) and Nogo-A (B) expression in the ipsilateral hemisphere. This increase was associated with a concomitant increase in JNK phosphorylation (C). Acute eNOS inhibition and candesartan combination had an additive effect on JNK phosphorylation. Data presented as mean±SEM. Solid columns represent ipsilateral hemisphere, columns with stripes represent contralateral hemisphere. a, b or A, B Pairs of means with different letters are significantly different from each other. n=6-8 animals per group.


A.
nNOS
actin
nNOS/actin (arbitrary units)
2.0
1.5
1.0
0.5
0.0
a
A
b
B
a
A
b
B
S+cand
NS+cand
S+cand+L-NIO
NS+cand+L-NIO
S+saline
NS+saline
S+saline+L-NIO
NS+saline+L-NIO


B.
NOGO-A
actin
Nogo A/actin (arbitrary units)
2.0
1.5
1.0
0.5
0.0
a
b
a
b
S+cand
NS+cand
S+cand+L-NIO
NS+cand+L-NIO
S+saline
NS+saline
S+saline+L-NIO
NS+saline+L-NIO

C.
pJNK
JNK
pJNK/JNK (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
a
A
b
B
a
A
b
B
S+cand
NS+cand
S+cand+L-NIO
NS+cand+L-NIO
S+saline
NS+saline
S+saline+L-NIO
NS+saline+L-NIO

Figure 3-6: Early AT1 blockade ameliorates ischemia induced increase in ER stress. Candesartan treatment at time of reperfusion reduced the expression of CHOP (A), cleaved ATF6 (C) and increased GRP78 expression in an eNOS dependent manner. eNOS inhibition increased the expression of full length ATF6 (B) Data presented as mean±SEM. Solid columns represent ipsilateral hemisphere, columns with stripes represent contralateral hemisphere. a, b Pairs of means with different letters are significantly different from each other * n=4 per group.

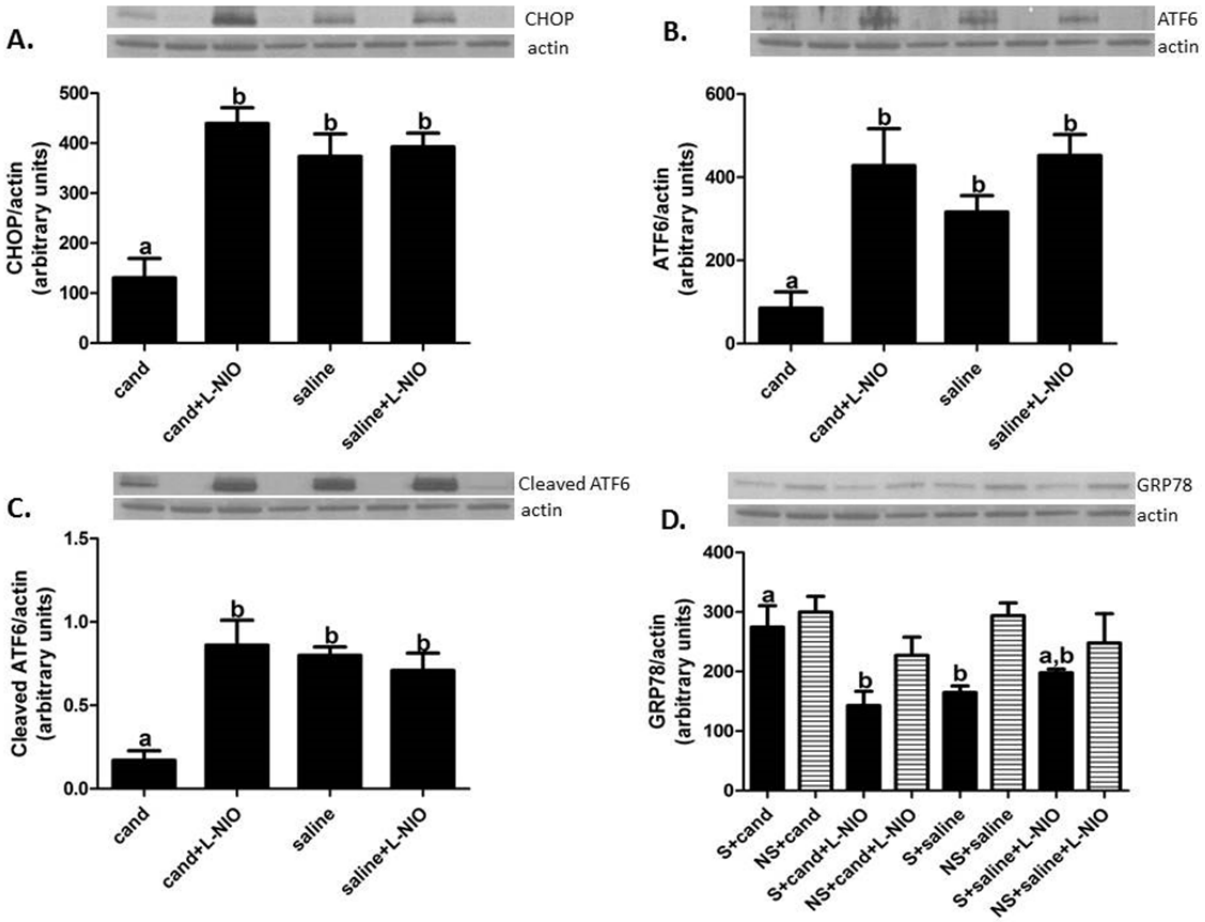
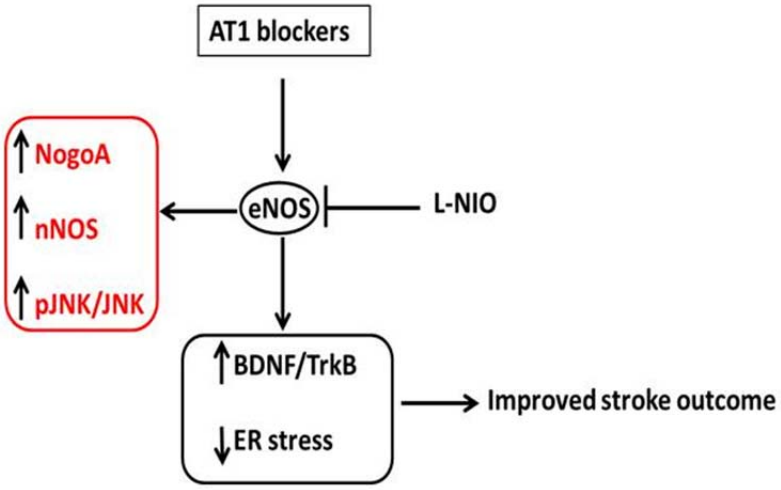
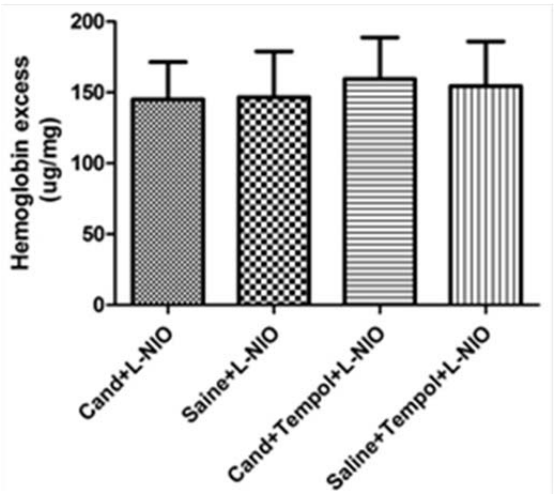

A.
CHOP
actin
CHOP/actin (arbitrary units)
500
400
300
200
100
0
a
b
b
b
cand
cand+L-NIO
saline
saline+L-NIO
B.
ATF6
actin
ATF6/actin (arbitrary units)
600
400
200
0
a
b
b
b
cand
cand+L-NIO
saline
saline+L-NIO
C.
Cleaved ATF6
actin
Cleaved ATF6/actin (arbitrary units)
1.5
1.0
0.5
0.0
a
b
b
b
cand
cand+L-NIO
saline
saline+L-NIO
D.
GRP78
actin
GRP78/actin (arbitrary units)
400
300
200
100
0
a
b
b
a,b
S+cand
NS+cand
S+cand+L-NIO
NS+cand+L-NIO
S+saline
NS+saline
S+saline+L-NIO
NS+saline+L-NIO

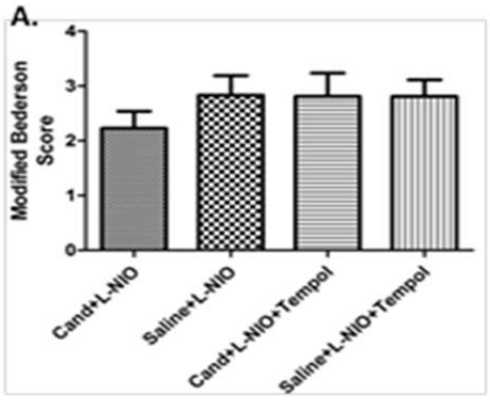
Figure 3-7: A schematic representation of the results. AT1 blockers enhances BDNF/TrkB system and reduced ER stress through the activity of eNOS in SHRs. Acute eNOS inhibition increases NogoA, nNOS and pJNK/JNK after stroke in SHRs.

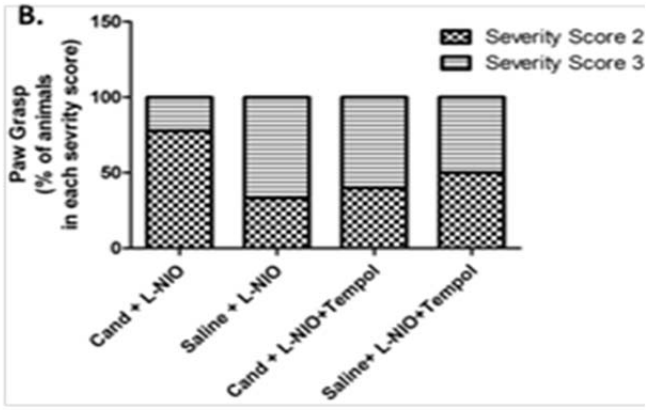

AT1 blockers
NogoA
nNOS
pJNK/JNK
eNOS
L-NIO
BDNF/TrkB
ER stress
Improved stroke outcome

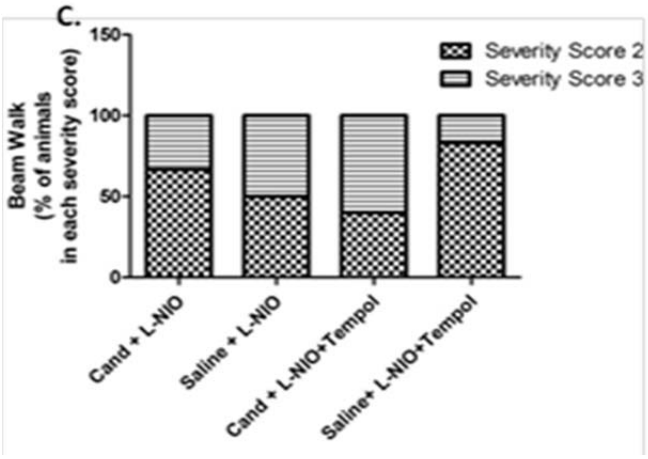
Supplementary Figure S3-1: Chronic Tempol treatment did not restore candesartan induced vasculoprotective effect. Animals were randomized to receive either water or tempol for 2 weeks and then received L-NIO injection one hour before MCAO. At the time of reperfusion they were randomized to either candesartan (1mg/kg) or saline. n=6-8 per group.


Hemoglobin excess (ug/mg)
200
150
100
50
0
Cand+L-NIO
Saine+L-NIO
Cand+Tempol+L-NIO
Saline+Tempol+L-NIO

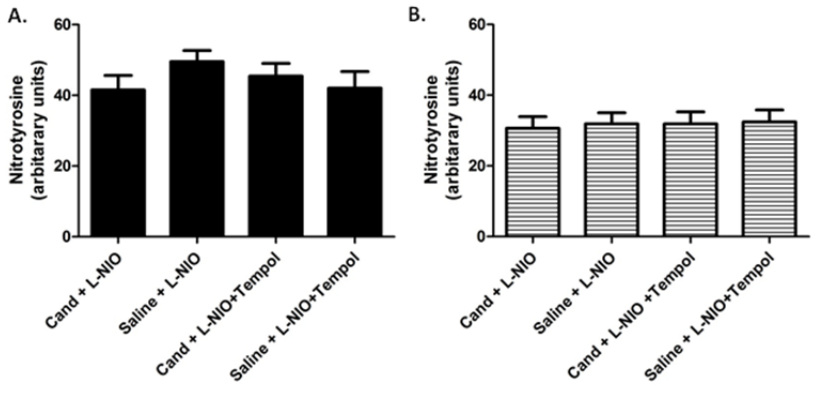
Supplementary Figure S3-2: Chronic Tempol treatment did not affect neurobehavioral outcome. Animals were randomized to receive either water or tempol for 2 weeks and then received L-NIO injection one hour before MCAO. At the time of reperfusion they were randomized to either candesartan (1mg/kg) or saline. n=6-8 per group.


A.
Modified Bederson Score
0
1
2
3
4
Cand+L-NIO
Saline+L-NIO
Cand+L-NIO+Tempol
Saline+L-NIO+Tempol


B.
Paw Grasp (% of animals in each sevrity score)
0
50
100
150
Severity Score 2
Severity Score 3
Cand + L-NIO
Saline + L-NIO
Cand + L-NIO+Tempol
Saline+ L-NIO+Tempol


C.
Beam Walk (% of animals in each severity score)
0
50
100
150
Severity Score 2
Severity Score 3
Cand + L-NIO
Saline + L-NIO
Cand + L-NIO+Tempol
Saline+ L-NIO+Tempol

Supplementary Figure S3-3: Chronic Tempol treatment did not affect the levels of nitrosative stress after stroke. Animals were randomized to receive either water or tempol for 2 weeks and then received L-NIO injection one hour before MCAO. At the time of reperfusion they were randomized to either candesartan (1mg/kg) or saline. Solid columns represent ipsilateral hemisphere, striped columns represent contralateral hemisphere. n=6-8 per group.


A.
Nitrotyrosine (arbitrary units)
60
40
20
0
Cand + L-NIO
Saline + L-NIO
Cand + L-NIO+Tempol
Saline + L-NIO+Tempol
B.
Nitrotyrosine (arbitrary units)
60
40
20
0
Cand + L-NIO
Saline + L-NIO
Cand + L-NIO +Tempol
Saline + L-NIO+Tempol

Chapter 4

REPERFUSION MODULATES THE NEUROPROTECTIVE EFFECT OF CANDESARTAN

Ahmed Alhusban, Anna Kozak, Adviye Ergul, and Susan C Fagan

To be submitted to the Journal of Hypertension

Abstract

Background: Blood flow restoration is essential to salvage at risk neurons after stroke. Angiotensin receptor blockers have been demonstrated to improve stroke outcome in models of permanent and temporary occlusion, but whether their effect is modulated by reperfusion is unknown.

Methods: Normotensive male wistar rats were implanted with blood pressure transmitters and subjected to middle cerebral artery occlusion. Animals were randomized to receive reperfusion or not followed by further randomization to receive candesartan (0.3mg/kg) or saline. Functional outcome, infarct size, and biochemical changes were assessed 24 hours after ischemia induction.

Results: Candesartan reduced infarct size and improved functional outcome after stroke. Lack of reperfusion blunted candesartan induced neuroprotection ($p<0.05$) and reduced the induced improvement of functional outcome ($p<0.05$). Candesartan increased mature BDNF expression in the contralateral hemisphere ($p<0.05$) and activated Akt-GSK3-β signaling ($p<0.05$). These effects were ablated by lack of reperfusion. Lack of reperfusion reduced TrkB expression whereas Nogo-A expression was significantly increased ($p<0.05$).

Conclusion: Candesartan induced pro-recovery effects are dependent on the presence of reperfusion.

Introduction:

High blood pressure is a well-known risk factor for both primary and secondary strokes [1]. Interventions directed toward reducing blood pressure have been demonstrated to reduce stroke incidence and recurrence [2-4]. The effect of blood pressure reduction during the acute period following stroke is still questionable[5]. Recently, a large randomized trial suggested no benefit with a trend toward worse outcome when candesartan was used during the acute period [6]. Despite being criticized for the rapid up titration of candesartan dose and aggressive blood reduction, SCAST is the only large randomized clinical trial that evaluated acute administration of candesartan in stroke [6]. In contrast to results from SCAST, ACCESS trial demonstrated an impressive improvement in stroke outcome [7]. ACCESS's reported positive results were associated with minimal changes in blood pressure [7]. This discrepancy makes it interesting to test whether a sub-hypotensive dose can improve stroke outcome.

Following stroke re-establishing blood flow to the ischemic area is essential to salvage metabolically stunted neurons [8, 9]. In fact, the only FDA approved drug for the management of stroke is intended to resolve clots and restore perfusion [10, 11]. Despite this importance, restoring blood flow after stroke is associated with a secondary injury in the ischemic tissue [8, 9, 12]. The pathophysiology of ischemia/reperfusion injury involves multiple pathologic mechanisms including vascular leakage, endoplasmic stress cell death and no reflow phenomenon [8, 9, 12]. In the latter mechanism, although the lumen of the blood vessel is patent, blood flow through the blood vessel and consequent perfusion are absent [12]. This phenomenon has been attributed to

plastic changes in the pericytes covering blood vessel after stroke [13]. These changes result in constant vasoconstriction of the affected blood vessels and are associated with activation of p38MAPK signaling [14]. Angiotensin Receptor Blockers (ARBs) have been demonstrated to have vasculoprotective effects [15-17].

In this investigation our aim is to assess neuroprotective potential of candesartan at sub-hypotensive doses and also to assess whether reperfusion would affect candesartan induced neuroprotection after stroke.

Materials and Methods:

Animals: All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Charlie Norwood Veterans Affairs Medical Center (09-04-008). Male wistar rats (280-300g) were subjected to middle cerebral artery occlusion (MCAO) as described earlier [15, 18]. Briefly, the ventral side of the neck was shaved and probed with iodine and 70% ethanol. A midline incision was made to expose neck blood vessels. The common carotid artery (CCA) and the external carotid artery (ECA) were isolated. After being isolated, the ECA was ligated, cauterized and a small incision was introduced in the ECA stub. A silicone coated filament was then introduced into the internal carotid artery (ICA) through the ECA stub and was pushed all the way to block the origin of the middle cerebral artery (MCA). After three hours of occlusion, animals were randomized to have reperfusion by withdrawal of the filament or to have permanent occlusion. At the same time, these animals were further randomized to receive either candesartan (0.3mg/kg) or saline. Animals were followed up for 24 hours after occlusion when they were sacrificed by decapitation. In a subset of animals brains

were harvested, sliced and stained with 5% Triphenyl Tetrazolium Chloride (TTC) to assess infarct size and edema volume. In another subset of animals, brains were harvested and flash frozen in liquid nitrogen for biochemical analysis.

Blood Pressure Telemetry: To follow up blood pressure changes, male wistar rats (160-180 g) were implanted with blood pressure transmitters. Briefly, a midline abdominal incision was introduced and the abdominal aorta was isolated. The abdominal aorta was temporarily occluded and small incision was introduced in the vascular wall to introduce the blood pressure transmitter probe. The transmitter was then secured to the abdominal by sutures and the incision was sutured and the skin closed using surgical clips. The animals were allowed 7-10 days to recover after surgery and then they were exposed to MCAO and randomized as described above.

Behavioral outcome: Behavioral outcome were evaluated 24 hours after MCAO using modified bederson score as described previously [15].

Immunoblotting: Brains were homogenized using 1X RIPA buffer supplemented with protease inhibitor cocktail, PMSF, and sodium orthovanidate. Protein content was determined using bicinchonic acid (BCA) method (Thermo-Scientific) and 30ug proteins from each samples were loaded and separated on 4-20% ready-made criterion gel (Bio-Rad). Proteins were transferred to nitrocellulose membranes and membranes were blocked with 5% low fat milk in TBST (1% tween in Tris- Buffered Saline). The membranes were probed with antiBDNF (1:250; Santa Cruz biotechnologies ; Santa Cruz, CA), TrkB (1:500, abcam; Cambridge, MA), anti pTrkB (1:250; abcam; Cambridge, MA), pGSK3-β (1:1000, Cell Signaling; Danvers, MA), total GSK3-β

(1:1000, Cell Signaling; Danvers, MA), pAkt-473(1:1000, Cell Signaling; Danvers, MA), pan Akt (1:1000, Cell Signaling; Danvers, MA), ATF6 (1:500, Santa Cruz biotechnologies ; Santa Cruz, CA), CHOP (1:1000, Cell Signaling; Danvers, MA). Expression was quantified by measuring the optic density of the band relative to its cognate actin band using image j software.

Statistical analysis: Statistical significance was detected using student t test and two way ANOVA as appropriate. Statistical analyses were performed using GraphPad prism software (5.1). $P<0.05$ was considered significant.

Results:

Candesartan 0.3mg/kg reduces moderately reduces blood pressure: Administration of 0.3mg/kg candesartan at the time of reperfusion reduces blood pressure moderately as compared to 1mg/kg dose (Figure 5-1).

Candesartan induces neuroprotection and improves functional outcome at sub-hypotensive doses: Data from our lab and others have demonstrated the neuroprotective effect of candesartan [18-20]. Recently, we have shown that candesartan improves stroke outcome and induces a proangiogenic state after stroke [18]. This proangiogenic effect was demonstrated to be independent of the hypotensive effect of candesartan under normoxic conditions [18]. Accordingly, our interest shifted to assess whether sub-hypotensive doses of candesartan might induce similar response in normotensive animals. Candesartan at sub-hypotensive doses reduced infract size by 23% (Figure 5-2A). Additionally, acute administration of the sub-hypotensive dose of

candesartan improved the functional outcome as measured by modified bederson score (Figure 5-2B).

Reperfusion is essential for ARBs induced neuroprotection but not for improving functional outcome: Despite being essential for salvaging neurons in the penumbra, reperfusion has been demonstrated to induce an additional injury in the ischemic tissue [8, 9]. Our previous data demonstrated the effects of candesartan administered at the time of reperfusion on stroke outcome [17, 21]. In this investigation we aimed at assessing whether reperfusion is necessary for candesartan induced neuroprotection and functional outcome improvement. Lack of reperfusion blunted candesartan induced neuroprotection (Figure 5-2A). Surprisingly, candesartan induced improvement in functional outcome was maintained even in non reperfused animals (Figure 5-2B).

Acute candesartan administration up-regulates the expression of BDNF: BDNF has been demonstrated to improve stroke outcome [22-24]. Candesartan has been shown to up-regulate BDNF expression in both wistar and SHR rats that are not stroked [27]. Accordingly, we were interested to check whether candesartan in sub-hypotensive doses can up-regulate BDNF expression after stroke. Candesartan significantly increased mature BDNF in the contralateral hemisphere (Figure 5-3A). This increase in mature BDNF was accompanied by an increase in the mature to proBDNF ratio (Figure 5-3B).

Candesartan modulates the expression of BDNF in a reperfusion dependent manner: In contrast to its effect in reperfused animals, candesartan reduced mature BDNF both hemispheres of non reperfused animals (Figure 5-3A). Candesartan

induced reduction in mature BDNF was also observed in proBDNF levels in the brain (Figure 5-3B). On the other hand candesartan did not alter the levels of proBDNF in reperfused animals (Figure 5-3B).

Candesartan modulates the expression of TrkB in a reperfusion dependent manner: BDNF induced neuroprotection is mediated through TrkB signaling [28, 29]. In reperfused brains candesartan did not alter the expression of TrkB after stroke (Figure 5-3D). Interestingly, candesartan administration significantly inhibited TrkB expression in the absence of reperfusion (Figure 5-3D).

Candesartan induced neuroprotection involves up-regulation of Akt-GSK3-β axis: Recently, Guo et al. demonstrated the involvement of Akt-GSK3-β activity in BDNF mediated neuroprotection conferred by endothelial cells [30]. Previously, our data suggested the involvement of GSK3-β in candesartan induced up-regulation of BDNF expression in endothelial cells [27]. Candesartan administration significantly activated the Akt -GSK3-β axis in both ipsilateral and contralateral hemispheres (Figures 5-4A and B, respectively).

Candesartan induced activation of Akt-GSK3-β signaling is reperfusion dependent: Similar to our findings on candesartan induced neuroprotection, the activity of Akt-GSK3-β was reperfusion dependent. Lack of reperfusion blunted candesartan induced activation of Akt signaling (Figure 5-4A) and GSK-3β inhibition (Figure 5-4B).

Candesartan reduces ER stress in the contralateral hemisphere of non-reperfused animals: The involvement of Endoplasmic Reticulum (ER) stress has been demonstrated in the pathophysiology of stroke[31]. In reperfused brain candesartan

reduced CHOP expression by about 50% in the ipsilateral hemisphere (Figure 5-5), but this reduction was not statistically significant. Surprisingly, in the non-reperfused brain candesartan reduced CHOP expression by more than 7 folds in the contralesional hemisphere (Figure 5-5).

Candesartan increases Nogo-A expression in non-reperfused brain after stroke: To verify whether the observed effects of candesartan in non-reperfused brain are not due to reduced protein expression, the expression of Nogo-A is quantified. Candesartan increased the expression of Nogo-A expression in non-reperfused brain (Figure5-6).

Discussion: Our results demonstrate the ability of a sub-hypotensive dose of candesartan to improve stroke outcome and confer neuroprotection. In addition our results demonstrate the essential requirement of reperfusion for the neuroprotective effects of candesartan. The currently reported neuroprotective effect of candesartan involves increased mature BDNF expression and activation of the Akt-GSK3-β signaling. The effect of reperfusion is bimodal on both de novo expression of BDNF and TrkB expression.

Angiotensin II receptor Blockers (ARBS) have been demonstrated to improve stroke outcome [15, 19, 20]. This effect was evident even at doses that had minimal effect on blood pressure [19]. In conformity with these data, our results demonstrate the ability of candesartan to decrease infract size and improve stroke functional outcome in doses that had a moderate effect on blood pressure [19, 32]. The dose we have used in the current study has been previously used in a number of studies [19, 33-36]. These studies were either conducted in hypertensive animals or have administered the drug

via chronic subcutaneous administration [19, 33-37]. In this study we have used a single I.V. injection in a normotensive model of stroke.

In support of our previous data on the ability of candesartan to increase BDNF expression in the brain [17, 27], candesartan increased the levels of mature BDNF after stroke. This increase was limited to the contralateral hemisphere only. Involvement of the contralateral hemisphere in stroke recovery and neuroplasticity has been previously reported [38, 39]. BDNF is a well-known mediator of neuroplasticity [28, 29]. Additionally, changes in the contralateral content of BDNF detected as early as 2 days post ischemia have been shown to be associated with improved functional outcome[39].

Interestingly, candesartan induced neuroprotection was totally ablated in the absence of reperfusion. This finding is also consistently demonstrated in the activity of Akt-GSK-3β signaling axis which has been demonstrated to be involved in neuroprotection and ameliorating functional outcome after stroke[30]. In reperfused brains, candesartan administration significantly increased Akt and GSK3-β phosphorylation. In contrast, candesartan administration in permanent stroke model significantly reduced Akt and GSK3-β activity.

Consistent with findings on Akt-GSk3-β activity, candesartan effect on mature BDNF content was completely reversed in non reperfused brain. Mature BDNF content in the brain is determined by both de novo expression and proBDNF processing into the mature form[29]. Our results suggest that in non reperfused brain, AT1 blockade reduces de novo expression of BDNF as detected by a reduction in both pro and mature

forms of BDNF. Similarly, candesartan significantly reduced the expression TrkB- BDNF receptor- in non reperfused brain.

In contrast to candesartan induced neuroprotection, candesartan improved stroke outcome in both reperfused and non reperfused brain when compared to saline treated animals in both models. Interestingly, lack of reperfusion reduced candesartan induced improvement in functional outcome.

A plausible explanation of these interesting findings is suggested by studies on penumbra development and cellular bioenergetics after ischemia. In an elegant work Mies et al. reported a 55ml/100gm/min cerebral blood flow threshold for protein synthesis in the brain[40]; below this threshold protein synthesis in the brain ceases. To account for this possibility, our interest shifted to quantify the expression of proteins known to be involved in worsening stroke outcome. One candidate protein in this setting is Nogo-A, which has been shown to worsen stroke outcome and also to antagonize the effects of BDNF in neurons [41, 42]. If the observed reduction in BDNF and TrkB expression is due to stunted transcriptional machinery in non reperfused brain, Nogo-A expression would also be reduced. Interestingly, Candesartan administration induced a robust increase in Nogo-A expression in non reperfused brain. This finding suggests that candesartan induced reduction in BDNF and TrkB expression is not due to a mere synthetic machinery failure induced by long duration of ischemia. in contrast, it suggests that lack of reperfusion reduces the expression or the bioavailability of an essential mediator for candesartan induced neuroprotection. Identifying this protein may positively affect stroke management by serving as surrogate biomarker to identify patients that will positively respond to ARBs treatment. In addition it might help in understanding the

discrepancy between ACCESS [7] and SCAST [6] trials with regard to candesartan effect on stroke outcome.

In conclusion, candesartan confers neuroprotection and improves stroke outcome at sub-hypotensive doses. This neuroprotective effect is mediated through an up-regulation of BDNF expression and the resulting activation of Akt-GSK3-β signaling. Additionally, candesartan induced neuroprotection is dependent on the presence of reperfusion.

References

1. *Go, A.S., et al., Heart disease and stroke statistics--2013 update: a report from the American Heart Association. Circulation, 2013. 127(1): p. e6-e245.*
2. *Randomised trial of a perindopril-based blood-pressure-lowering regimen among 6,105 individuals with previous stroke or transient ischaemic attack. Lancet, 2001. 358(9287): p. 1033-41.*
3. *Yusuf, S., et al., Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. N Engl J Med, 2000. 342(3): p. 145-53.*
4. *Schrader, J., et al., Morbidity and Mortality After Stroke, Eprosartan Compared with Nitrendipine for Secondary Prevention: principal results of a prospective randomized controlled study (MOSES). Stroke, 2005. 36(6): p. 1218-26.*
5. *Luders, S., Drug therapy for the secondary prevention of stroke in hypertensive patients: current issues and options. Drugs, 2007. 67(7): p. 955-63.*
6. *Sandset, E.C., et al., The angiotensin-receptor blocker candesartan for treatment of acute stroke (SCAST): a randomised, placebo-controlled, double-blind trial. Lancet, 2011. 377(9767): p. 741-50.*
7. *Schrader, J., et al., The ACCESS Study: evaluation of Acute Candesartan Cilexetil Therapy in Stroke Survivors. Stroke, 2003. 34(7): p. 1699-703.*
8. *Pundik, S., K. Xu, and S. Sundararajan, Reperfusion brain injury: focus on cellular bioenergetics. Neurology, 2012. 79(13 Suppl 1): p. S44-51.*
9. *Heiss, W.D., The ischemic penumbra: how does tissue injury evolve? Ann N Y Acad Sci, 2012. 1268: p. 26-34.*
10. *Hacke, W., et al., Randomised double-blind placebo-controlled trial of thrombolytic therapy with intravenous alteplase in acute ischaemic stroke (ECASS II). Second European-Australasian Acute Stroke Study Investigators. Lancet, 1998. 352(9136): p. 1245-51.*
11. *Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. N Engl J Med, 1995. 333(24): p. 1581-7.*
12. *Eltzschig, H.K. and T. Eckle, Ischemia and reperfusion--from mechanism to translation. Nat Med, 2011. 17(11): p. 1391-401.*
13. *Yemisci, M., et al., Pericyte contraction induced by oxidative-nitrative stress impairs capillary reflow despite successful opening of an occluded cerebral artery. Nat Med, 2009. 15(9): p. 1031-7.*

14. *Edvinsson, L.I. and G.K. Povlsen, Vascular plasticity in cerebrovascular disorders. J Cereb Blood Flow Metab, 2011. 31(7): p. 1554-71.*
15. *Fagan, S.C., et al., Hypertension after experimental cerebral ischemia: candesartan provides neurovascular protection. J Hypertens, 2006. 24(3): p. 535-9.*
16. *Kozak, W., et al., Vascular protection with candesartan after experimental acute stroke in hypertensive rats: a dose-response study. J Pharmacol Exp Ther, 2008. 326(3): p. 773-82.*
17. *Guan, W., et al., Vascular protection by angiotensin receptor antagonism involves differential VEGF expression in both hemispheres after experimental stroke. PLoS One, 2011. 6(9): p. e24551.*
18. *Kozak, A., et al., Candesartan augments ischemia-induced proangiogenic state and results in sustained improvement after stroke. Stroke, 2009. 40(5): p. 1870-6.*
19. *Ito, T., et al., Protection against ischemia and improvement of cerebral blood flow in genetically hypertensive rats by chronic pretreatment with an angiotensin II AT1 antagonist. Stroke, 2002. 33(9): p. 2297-303.*
20. *Dai, W.J., et al., Blockade of central angiotensin AT(1) receptors improves neurological outcome and reduces expression of AP-1 transcription factors after focal brain ischemia in rats. Stroke, 1999. 30(11): p. 2391-8; discussion 2398-9.*
21. *Guan, W., et al., Acute Treatment with Candesartan Reduces Early Injury After Permanent Middle Cerebral Artery Occlusion. Transl Stroke Res, 2011. 2(2): p. 179-185.*
22. *Muller, H.D., et al., Brain-derived neurotrophic factor but not forced arm use improves long-term outcome after photothrombotic stroke and transiently upregulates binding densities of excitatory glutamate receptors in the rat brain. Stroke, 2008. 39(3): p. 1012-21.*
23. *Schabitz, W.R., et al., Effect of brain-derived neurotrophic factor treatment and forced arm use on functional motor recovery after small cortical ischemia. Stroke, 2004. 35(4): p. 992-7.*
24. *Schabitz, W.R., et al., Intravenous brain-derived neurotrophic factor enhances poststroke sensorimotor recovery and stimulates neurogenesis. Stroke, 2007. 38(7): p. 2165-72.*
25. *Ploughman, M., et al., Brain-derived neurotrophic factor contributes to recovery of skilled reaching after focal ischemia in rats. Stroke, 2009. 40(4): p. 1490-5.*
26. *Mahmood, A., D. Lu, and M. Chopp, Intravenous administration of marrow stromal cells (MSCs) increases the expression of growth factors in rat brain after traumatic brain injury. J Neurotrauma, 2004. 21(1): p. 33-9.*
27. *Alhusban, A., et al., AT1 receptor antagonism is proangiogenic in the brain: BDNF a novel mediator. J Pharmacol Exp Ther, 2013. 344(2): p. 348-59.*
28. *Waterhouse, E.G. and B. Xu, New insights into the role of brain-derived neurotrophic factor in synaptic plasticity. Mol Cell Neurosci, 2009. 42(2): p. 81-9.*

29. *Marini, A.M., et al., Role of brain-derived neurotrophic factor and NF-kappaB in neuronal plasticity and survival: From genes to phenotype. Restor Neurol Neurosci, 2004. 22(2): p. 121-30.*
30. *Guo, S., et al., Vascular neuroprotection via TrkB- and Akt-dependent cell survival signaling. J Neurochem, 2012. 123 Suppl 2: p. 58-64.*
31. *Roussel, B.D., et al., Endoplasmic reticulum dysfunction in neurological disease. Lancet Neurol, 2013. 12(1): p. 105-18.*
32. *Nishikawa, K., Angiotensin AT1 receptor antagonism and protection against cardiovascular end-organ damage. J Hum Hypertens, 1998. 12(5): p. 301-9.*
33. *Yamakawa, H., et al., Normalization of endothelial and inducible nitric oxide synthase expression in brain microvessels of spontaneously hypertensive rats by angiotensin II AT1 receptor inhibition. J Cereb Blood Flow Metab, 2003. 23(3): p. 371-80.*
34. *Zhou, J., et al., AT1 receptor blockade regulates the local angiotensin II system in cerebral microvessels from spontaneously hypertensive rats. Stroke, 2006. 37(5): p. 1271-6.*
35. *Lu, Q., Y.Z. Zhu, and P.T. Wong, Neuroprotective effects of candesartan against cerebral ischemia in spontaneously hypertensive rats. Neuroreport, 2005. 16(17): p. 1963-7.*
36. *Zhou, J., et al., Angiotensin II AT1 receptor antagonism downregulates stress-related gene expression in brain microvessels from spontaneously hypertensive and normotensive rats. Ann N Y Acad Sci, 2004. 1018: p. 480-6.*
37. *Brdon, J., et al., Comparison between early and delayed systemic treatment with candesartan of rats after ischaemic stroke. J Hypertens, 2007. 25(1): p. 187-96.*
38. *Liu, Z., et al., Bone marrow stromal cells promote skilled motor recovery and enhance contralesional axonal connections after ischemic stroke in adult mice. Stroke, 2011. 42(3): p. 740-4.*
39. *Kim, M.W., et al., Exercise increased BDNF and trkB in the contralateral hemisphere of the ischemic rat brain. Brain Res, 2005. 1052(1): p. 16-21.*
40. *Mies, G., et al., Ischemic thresholds of cerebral protein synthesis and energy state following middle cerebral artery occlusion in rat. J Cereb Blood Flow Metab, 1991. 11(5): p. 753-61.*
41. *Kilic, E., et al., Role of Nogo-A in neuronal survival in the reperfused ischemic brain. J Cereb Blood Flow Metab, 2010. 30(5): p. 969-84.*
42. *Pernet, V. and M.E. Schwab, The role of Nogo-A in axonal plasticity, regrowth and repair. Cell Tissue Res, 2012. 349(1): p. 97-104.*

Figure 4-1: Candesartan modulates blood pressure levels. Candesartan administered at the time of reperfusion reduced mean arterial blood pressure in a dose dependent manner. Candesartan at a 0.3mg/kg dose reduced blood pressure moderately as compared to 1mg/kg. n=9-18 per group.

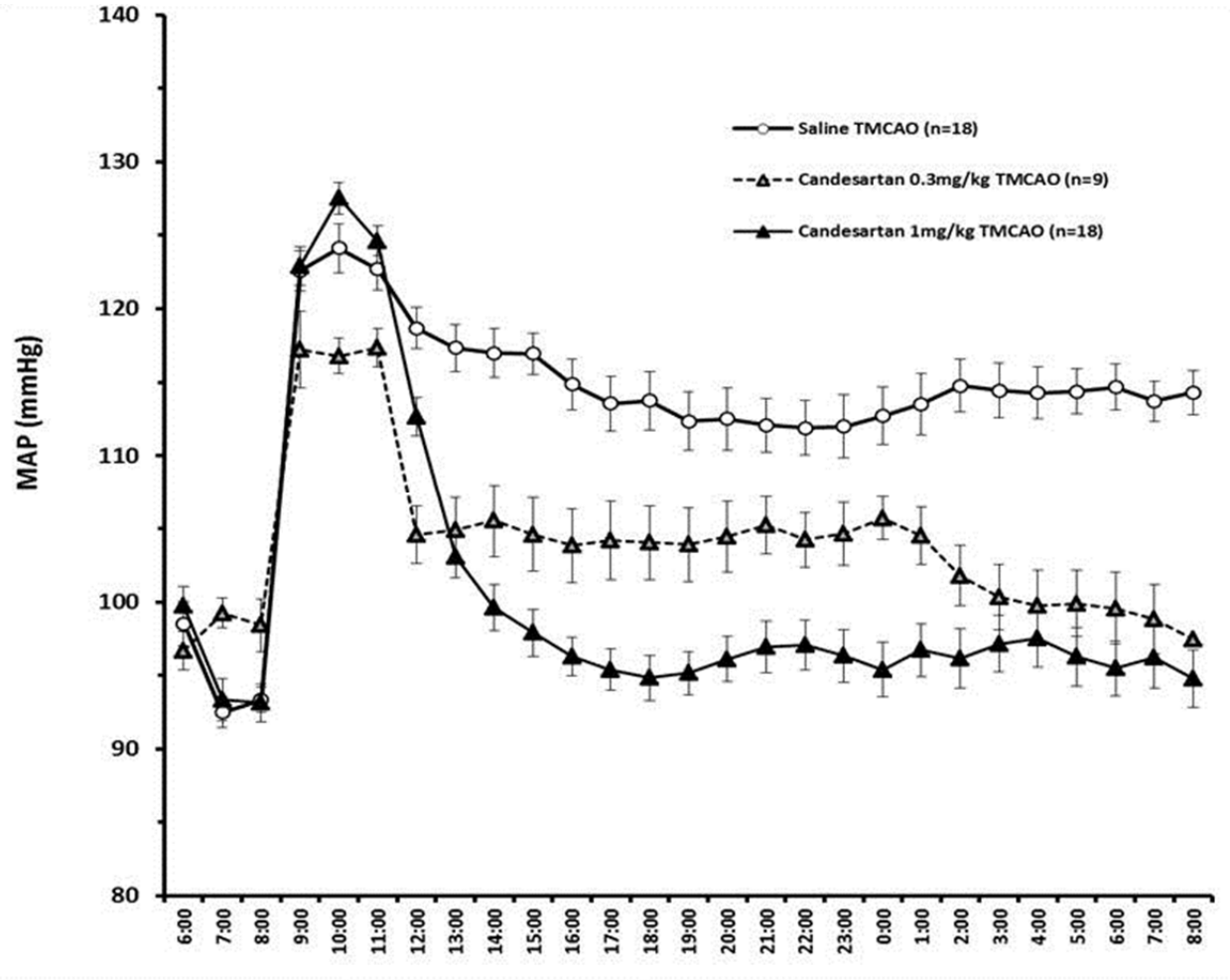

Saline TMCAO (n=18)
Candesartan 0.3mg/kg TMCAO (n=9)
Candesartan 1mg/kg TMCAO (n=18)
MAP (mmHg)
140
130
120
110
100
90
80
6:00
7:00
8:00
9:00
10:00
11:00
12:00
13:00
14:00
15:00
16:00
17:00
18:00
19:00
20:00
21:00
22:00
23:00
0:00
1:00
2:00
3:00
4:00
5:00
6:00
7:00
8:00

Figure 4-2: Reperfusion is essential for candesartan induced functional outcome improvement. Sub-hypotensive dose of candesartan conferred neuroprotective effect after stroke in a reperfusion dependent manner (A). In addition, the ability of candesartan to improve functional outcome is also reperfusion dependent (B). * $p<0.05$, a, b pairs of means with different letters are significantly different from each other. n=8-14 per group.

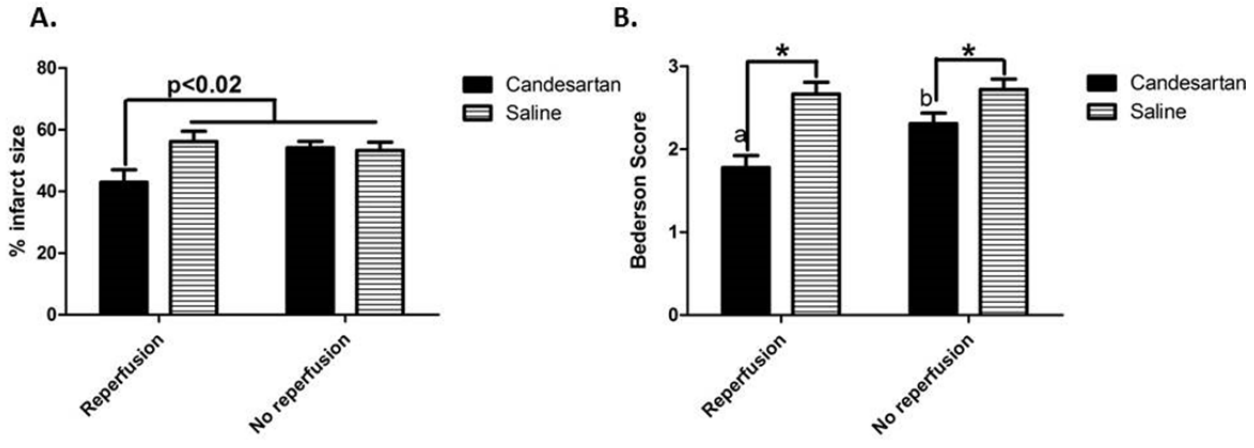

A.
p<0.02
% infarct size
80
60
40
20
0
Reperfusion
No reperfusion
Candesartan
Saline
B.
Bederson Score
3
2
1
0
a
b
*
*
Reperfusion
No reperfusion
Candesartan
Saline

Figure 4-3: Reperfusion modulates the ability of candesartan to affect the expression of BDNF/TrkB system components. Candesartan increased mature BDNF expression in the contralateral hemisphere in a reperfusion dependent manner (A). The effect of reperfusion is most prominent in proBDNF (B), BDNF/proBDNF ratio (C), and TrkB (D) expression. Groups connected by line are significantly different from each other. a, b pairs of means with different letters are significantly different from each other. n=4 per group.

Figure 5-3

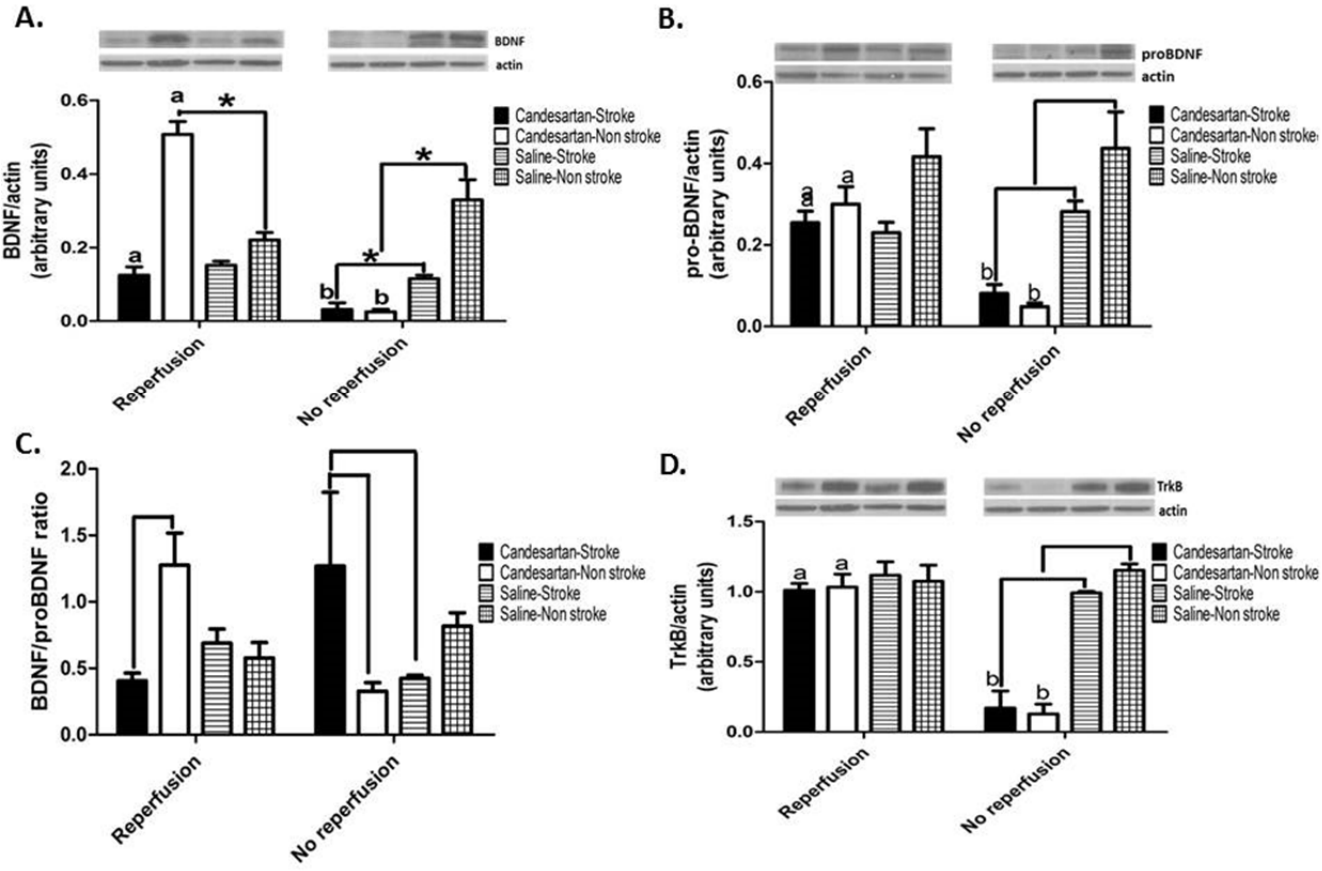

A.
BDNF
actin
BDNF/actin (arbitrary units)
0.6
0.4
0.2
0.0
a
a
*
b
b
*
*
Candesartan-Stroke
Candesartan-Non stroke
Saline-Stroke
Saline-Non stroke
Reperfusion
No reperfusion
B.
proBDNF
actin
pro-BDNF/actin (arbitrary units)
0.6
0.4
0.2
0.0
a
a
b
b
Candesartan-Stroke
Candesartan-Non stroke
Saline-Stroke
Saline-Non stroke
Reperfusion
No reperfusion
C.
BDNF/proBDNF ratio
2.0
1.5
1.0
0.5
0.0
Candesartan-Stroke
Candesartan-Non stroke
Saline-Stroke
Saline-Non stroke
Reperfusion
No reperfusion
D.
TrkB
actin
TrkB/actin (arbitrary units)
1.5
1.0
0.5
0.0
a
a
b
b
Candesartan-Stroke
Candesartan-Non stroke
Saline-Stroke
Saline-Non stroke
Reperfusion
No reperfusion

Figure 4-4: Reperfusion is necessary for candesartan induced activation of survival signaling. Candesartan induced neuroprotection involves the activation of Akt (A) and GSK3-β signaling (B). Lack of reperfusion blunts the ability of candesartan to activate intracellular survival signals. Groups connected by line are significantly different from each other. a, b pairs of means with different letters are significantly different from each other. n=4 per group.

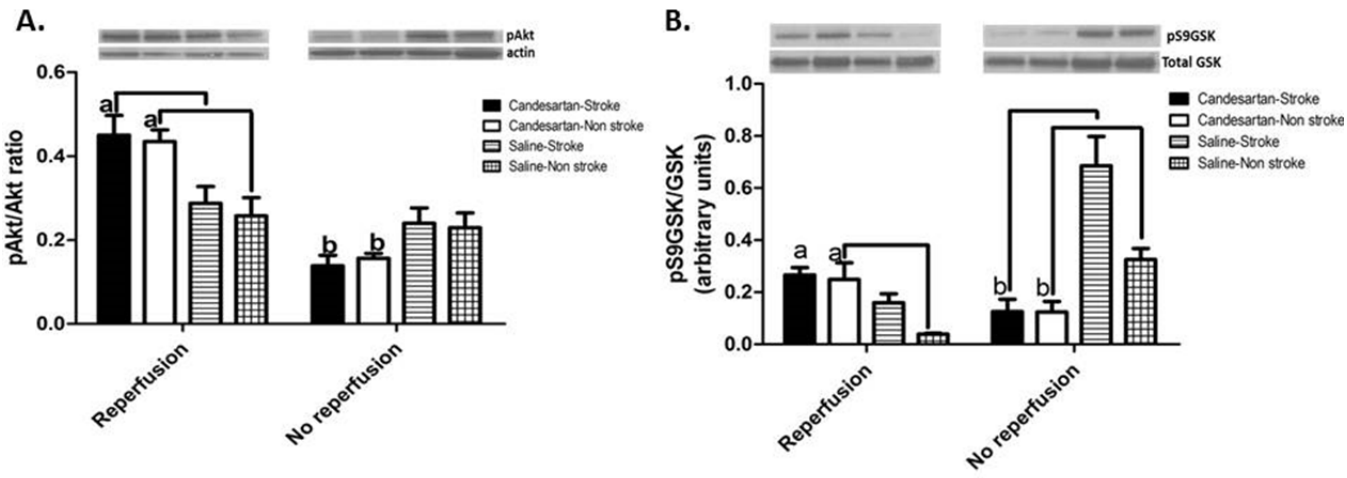

A.
pAkt
actin
0.6
0.4
0.2
0.0
pAkt/Akt ratio
a
a
b
b
Candesartan-Stroke
Candesartan-Non stroke
Saline-Stroke
Saline-Non stroke
Reperfusion
No reperfusion
B.
pS9GSK
Total GSK
1.0
0.8
0.6
0.4
0.2
0.0
pS9GSK/GSK
(arbitrary units)
a
a
b
b
Candesartan-Stroke
Candesartan-Non stroke
Saline-Stroke
Saline-Non stroke
Reperfusion
No reperfusion

Figure 4-5: Reperfusion modulates the ability of candesartan to ameliorate endoplasmic reticulum stress. Candesartan significantly reduced ER stress in the ipsilateral hemisphere in animals with temporary MCAO. Although lack of reperfusion blunted this effect in the ipsilateral hemisphere, it reduced ER stress in the contralateral hemisphere. Groups connected by line are significantly different from each other. a, b pairs of means with different letters are significantly different from each other. n=4 per group.

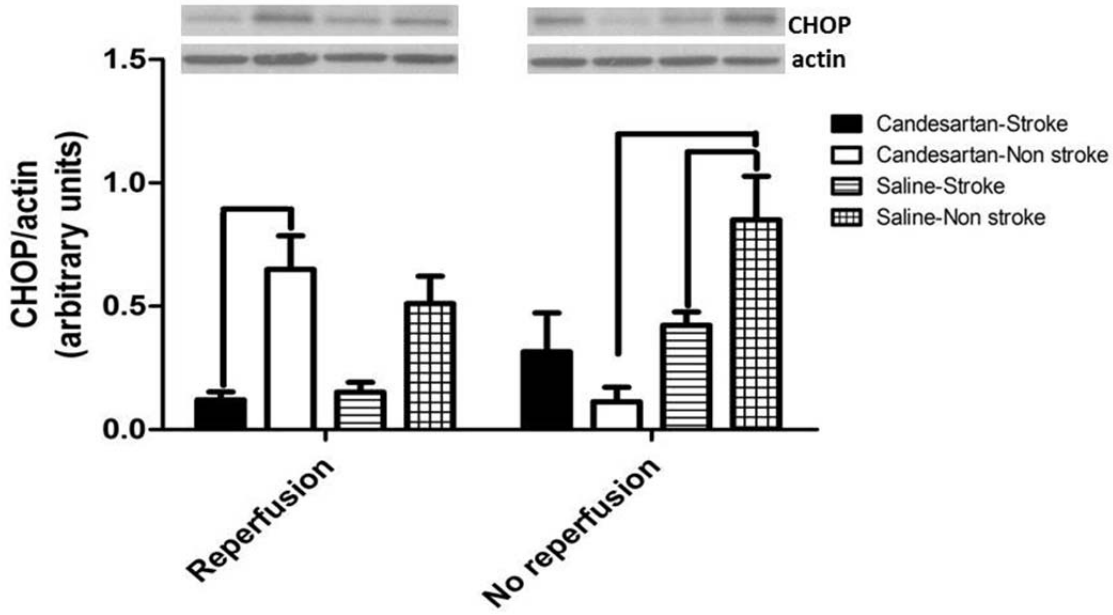
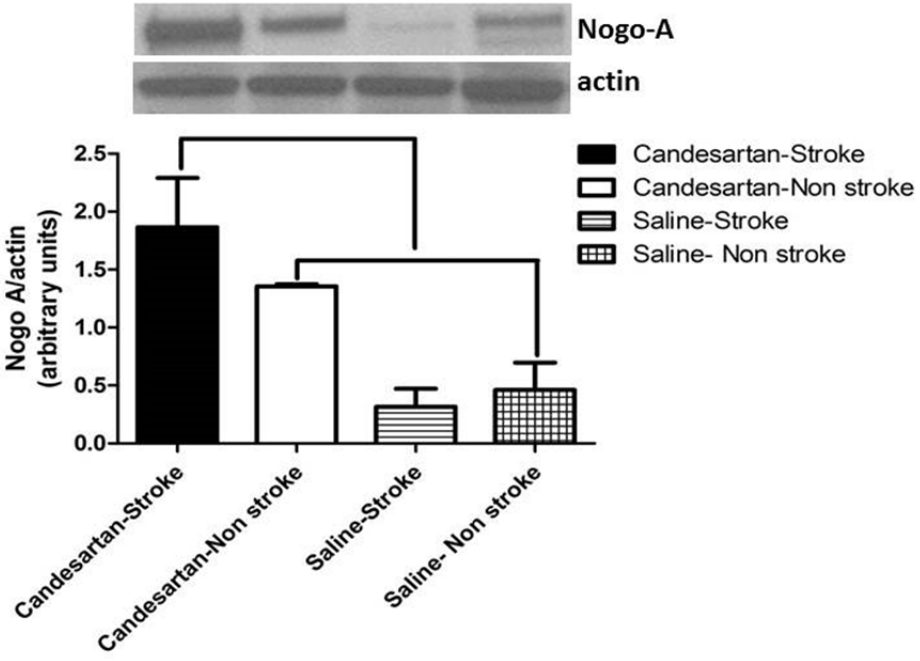

CHOP
actin
1.5
1.0
0.5
0.0
CHOP/actin
(arbitrary units)
Candesartan-Stroke
Candesartan-Non stroke
Saline-Stroke
Saline-Non stroke
Reperfusion
No reperfusion

Figure 4-6: candesartan increases the expression of Nogo-A in non-reperfused brains. Candesartan administration in animals exposed to permanent middle cerebral artery occlusion increased Nogo-A expression in both hemispheres. Groups connected by line are significantly different from each other. a, b pairs of means with different letters are significantly different from each other. n=4 per group.


Nogo-A
actin
Nogo A/actin (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
Candesartan-Stroke
Candesartan-Non stroke
Saline-Stroke
Saline- Non stroke
Candesartan-Stroke
Candesartan-Non stroke
Saline-Stroke
Saline- Non stroke

Chapter 5

CANDESARTAN INDUCED FUNCTIONAL OUTCOME IMPROVEMENT IS BLUNTED BY KNOCKING DOWN BDNF EXPRESSION

Ahmed Alhusban, Abdelrahman Fouda, Bindu Pillai, Adviye Ergul, Susan C Fagan

To be submitted to stroke

Abstract:

Background: Brain derived neurotrophic factor (BDNF) has been demonstrated to improve stroke outcome. Data from our lab and others have demonstrated the ability of angiotensin receptor blockers to improve stroke outcome. Previously, we demonstrated the ability of candesartan to increase the expression of BDNF.

Methods: lentivirus particles expressing BDNF shRNA or empty vector were injected in the right and left cerebral ventricles. Animals were then exposed to 90 minutes of middle cerebra artery occlusion and randomized to receive either candesartan or saline at the time of reperfusion. Functional improvement was assessed over 2 weeks after ischemia induction.

Results: Bilateral injection of BDNF shRNA significantly inhibited the expression of BDNF by about 70% as compared to control ($p<0.05$). Candesartan induced functional improvement was blunted by BDNF knockdown ($p<0.05$).

Conclusion: Candesartan induced improvement in long-term functional outcome might be mediated by BDNF/TrkB signaling.

Introduction:

Angiotensin II receptor blockers (ARBs) have been demonstrated to improve stroke outcome [1-4]. Data from our lab has demonstrated the ability of a single dose of candesartan to improve long term stroke outcome [2]. This intriguing finding suggests that candesartan induced functional outcome improvement may be independent of the hypotensive effect of the drug.

Brain derived neurotrophic factor (BDNF) is a member of the neurotrophins family [5-7]. It has been demonstrated to play an important role in both physiology and pathophysiology of multiple disease states [7]. In stroke BDNF has been found to be involved in functional recovery after stroke [8-12]. In addition BDNF has been implicated in neuroplasticity [5, 13, 14] and angiogenesis [6, 15, 16] which are also vital to functional recovery [17-19]. Krikov *et al.* suggested the involvement of the BDNF/TrkB in candesartan induced neuroprotection after stroke [20]. In their study they have reported the ability of candesartan to increase TrkB rather than BDNF after stroke [20]. In contrast, data from our lab and others have demonstrated the ability of candesartan and other ARBs to increase BDNF expression in both normal animals [21] and in animals after stroke [22, 23]. It remains controversial whether BDNF is involved in ARBs induced improvement in functional outcome.

In this investigation our aim is to assess feasibility of using shRNA as a tool in dissecting the role of certain proteins in stroke research. In addition, we are interested in assessing the involvement of BDNF in candesartan induced pro-recovery effect.

Materials and methods:

Animals: all animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Charlie Norwood Veterans Affairs Medical Center (09-04-008).

In vivo BDNF knockdown: Rats were placed in the stereotaxic frame and the head shaved and scrubbed with betadine. A midline incision was made and the skull exposed. To deliver lentivirus particles either one or two small holes were made in the skull using a hand-held drill. Stereotaxic coordinates used were anterioposterior −1 mm, lateral 2 mm and dorso–ventral−3 mm relative to bregma and ventral from dura. Using a 28-gauge Hamilton® syringe 5µl of lentivirus BDNF shRNA (Dharmacon) or empty vector were slowly injected into the lateral ventricles.

Cerebral ischemia: Following stereotaxic injection animals were followed for two weeks before being subjected to MCAO for 90 minutes as has been described previously [1, 24]. At the time of reperfusion animals were randomized to receive either candesartan (1mg/kg) or saline. After two weeks animals were euthanized and perfused using ice cold phosphate buffered saline (PBS). Brains were harvested and fixed in 4% paraformaldehyde (4%PFA) overnight and cut into 7µm sections using a microtome.

Behavioral outcome analysis: Functional outcome was evaluated using a battery of functional tests on days 1, 4, 7, 10, and 14 after MCAO:

Modified bederson test: animals were placed on the floor and allowed to freely move and explore the surrounding environment. Functional outcome was evaluated using a 4 point scale with higher score indicating worst outcome. During the test a point was given

to each of the following parameters: 1. Forelimb flexion. 2. Reduced resistance. 3. Circling while moving. Points were then summed and a final score was given to each animal.

Beam walk test: animals were placed on a 1 m beam placed 20 cm above the floor. Animals were observed and a score was given to each animal based on the following criteria: 0= balances and walks over the beam, 1=balances over the beam but not moving; 2= balances over the beam with one limb falling; 3=balances over the beam with two limbs falling; 4= balances over the beam >40 seconds before falling; 5= balances over the beam 20-40 seconds before falling; 6= attempts to balance over the beam but falls <20 seconds.

Paw grasp test: animals were held by tail and approached to a metallic pole. A score was given to each animal based on the following criteria: 1= animal hugging the pole with both forelimbs; 2= animal hugs the pole with one limb and touches the limb with the paretic limb; 3=animal is not showing any interest in hugging the pole.

Grid walking test: animals were placed on a grid composed of 3.8×3.8 cm cells, elevated 1m from the floor. A video camera was placed at a 45° angle below the grid to tape animal movement. Two blinded investigators observed the animals during the test and the number of time the forelimb falls was counted in addition to the total number of steps. For each animal the percentage of fault steps was then calculated.

Statistical analysis: statistical significance was detected using one way ANOVA and two way ANOVA tests followed by benferroni or tuckey post-hoc analysis of difference.

Statistical analyses were performed using GraphPad prism software (5.1). $P<0.05$ was considered significant.

Results:

Short hairpin RNA knocks down BDNF gene expression in dose dependent manner: BDNF is an important protein during both development and later during adult life [5-7]. BDNF gene deletion is lethal [7]. Accordingly, assessing the involvement of BDNF in stroke outcome requires knocking down BDNF in mature animals. To achieve this we assessed the efficiency of lentiviruses expressing BDNF shRNA delivered into the lateral ventricle to knockdown BDNF gene expression. BDNF shRNA single injection inhibited BDNF expression by about 30%, whereas bilateral injection achieved about 70% gene expression knockdown in whole brain homogenate (Figure 6-1). Accordingly, the decision was made to use bilateral intracerebroventricular injection of lentivirus of the remaining experiments.

Single dose candesartan administration improves long-term functional outcome: Data from our lab and others have demonstrated the ability of ARBs to improve stroke outcome [1, 2, 4, 25]. Previously, we have demonstrated the ability of a single dose of candesartan to improve stroke outcome when assessed 7 days after stroke [2]. Our results demonstrate the ability of a single dose of candesartan to long-term functional outcome as assessed 14 days after the induction of ischemia (Figure 6-2). This improvement was evident as early as one day after stroke (Figure 6-2).

BDNF may mediate candesartan induced improvement in the functional outcome: Data from our lab demonstrated the ability of candesartan to increase BDNF expression

after stroke [23]. BDNF has been demonstrated to play a vital role in functional recovery after stroke [8-12]. Accordingly, we aimed at assessing the involvement of BDNF in candesartan induced improvement in long-term functional outcome. Knocking down BDNF ablated candesartan induced functional outcome improvement (Figures 6-2). The effect of knocking down BDNF on functional outcome was observed immediately after stroke and was maintained during the whole period of follow up.

Discussion: Our data demonstrate the feasibility of using lentiviruses expressing shRNA as a tool to study the involvement of vital proteins in stroke research. In addition, we demonstrated the possible involvement of BDNF in candesartan induced improvement in long-term functional outcome.

Brain derived neurotrophic factor (BDNF) has been demonstrated to play a vital role in the development of the cardiovascular [7] and central nervous system [5, 13]. BDNF knockout animals were demonstrated to have serious cardiovascular defects and their survival was limited to only weeks after birth [7]. This notion makes it difficult to assess the involvement of similar proteins in studies that require long-term survival. To overcome this, the adoption of heterozygotes (BDNF +/-) [26] or TrkB knockout [27] animals has been suggested. Despite valuable, these models cannot accurately dissect the role of BDNF in long term studies. This limitation is due to the complex nature of BDNF signaling [5, 13]. In the normal brain BDNF exists with two different isoforms a pro and a mature form which have opposing effects [13]. In addition, it's unknown whether the presence of a single allele of BDNF in heterozygotes would compensate for the lack of the other allele. Another level of complexity in studying the effects of BDNF is the presence of two receptors to which pro and mature BDNF can bind [5]. Lack of TrkB

may result in the unopposed stimulation of the other BDNF receptor (p75NTR). This fact makes it difficult to know with certainty if any observed effect is due to lack of BDNF or to over activation of p75NTR. Taking these factors into account, it's essential to develop models that would enable us to study the functions of proteins essential for survival. In this study we have demonstrated the feasibility of using shRNA expressing lentiviruses to knockdown proteins and assess their functional roles in mechanistic studies.

Candesartan mediated effects has been shown to involve different mechanisms. After stroke we demonstrated that candesartan induced up regulation of vascular endothelial growth factor (VEGF) partially accounts for stroke improvement [2]. Besides VEGF, we have demonstrated the ability of candesartan to up regulate the expression of a number of genes among which BDNF received special interest [23]. In this study we aimed at assessing the involvement of BDNF in candesartan induced effects. In this study we demonstrated that in animals that received BDNF shRNA injections, the effects of candesartan were not detected. This effect was confirmed using different functional outcome assessment tools that cover different aspects of recovery.

A major limitation of this study is the lack of BDNF shRNA control group. BDNF has been suggested to play an important role in recovery after stroke. In an interesting paper Ploughman et al. demonstrated the essential role of BDNF in recovery [12]. Despite the novelty of their work, they missed the use of non-targeting sequence of oligonucleotides as a negative control in the study [12]. Similar to RNA interference techniques, oligonucleotides might have off target effects that might affect the parameter of interest [28]. Accordingly, it would be not possible to ascertain whether the observed effects were due to BDNF knockdown or due to off target effects of the used

oligonucleotides. In our results, knocking down BDNF in candesartan treated animals resulted in a behavior similar to saline in some tests, and an intermediate behavior in others. Based on this discussion, it remains essential to assess the effect of BDNF shRNA on stroke outcome before making a conclusion about the involvement of BDNF in candesartan induced effects.

In conclusion, intracerebroventricular injection of BDNF shRNA expressing lentiviruses offers a feasible tool in understanding the role of some vital proteins in stroke. Additionally, BDNF might play a role in candesartan induced improvement of long-term functional outcome.

References

1. *Fagan, S.C., et al., Hypertension after experimental cerebral ischemia: candesartan provides neurovascular protection. J Hypertens, 2006. 24(3): p. 535-9.*
2. *Kozak, A., et al., Candesartan augments ischemia-induced proangiogenic state and results in sustained improvement after stroke. Stroke, 2009. 40(5): p. 1870-6.*
3. *Ito, T., et al., Protection against ischemia and improvement of cerebral blood flow in genetically hypertensive rats by chronic pretreatment with an angiotensin II AT1 antagonist. Stroke, 2002. 33(9): p. 2297-303.*
4. *Engelhorn, T., et al., The angiotensin II type 1-receptor blocker candesartan increases cerebral blood flow, reduces infarct size, and improves neurologic outcome after transient cerebral ischemia in rats. J Cereb Blood Flow Metab, 2004. 24(4): p. 467-74.*
5. *Mocchetti, I. and M. Brown, Targeting neurotrophin receptors in the central nervous system. CNS Neurol Disord Drug Targets, 2008. 7(1): p. 71-82.*
6. *Kermani, P. and B. Hempstead, Brain-derived neurotrophic factor: a newly described mediator of angiogenesis. Trends Cardiovasc Med, 2007. 17(4): p. 140-3.*
7. *Caporali, A. and C. Emanueli, Cardiovascular actions of neurotrophins. Physiol Rev, 2009. 89(1): p. 279-308.*
8. *Muller, H.D., et al., Brain-derived neurotrophic factor but not forced arm use improves long-term outcome after photothrombotic stroke and transiently upregulates binding densities of excitatory glutamate receptors in the rat brain. Stroke, 2008. 39(3): p. 1012-21.*
9. *Schabitz, W.R., et al., Effect of brain-derived neurotrophic factor treatment and forced arm use on functional motor recovery after small cortical ischemia. Stroke, 2004. 35(4): p. 992-7.*
10. *Schabitz, W.R., et al., Intravenous brain-derived neurotrophic factor enhances poststroke sensorimotor recovery and stimulates neurogenesis. Stroke, 2007. 38(7): p. 2165-72.*
11. *Mahmood, A., D. Lu, and M. Chopp, Intravenous administration of marrow stromal cells (MSCs) increases the expression of growth factors in rat brain after traumatic brain injury. J Neurotrauma, 2004. 21(1): p. 33-9.*
12. *Ploughman, M., et al., Brain-derived neurotrophic factor contributes to recovery of skilled reaching after focal ischemia in rats. Stroke, 2009. 40(4): p. 1490-5.*

13. *Marini, A.M., et al., Role of brain-derived neurotrophic factor and NF-kappaB in neuronal plasticity and survival: From genes to phenotype. Restor Neurol Neurosci, 2004. 22(2): p. 121-30.*
14. *Waterhouse, E.G. and B. Xu, New insights into the role of brain-derived neurotrophic factor in synaptic plasticity. Mol Cell Neurosci, 2009. 42(2): p. 81-9.*
15. *Sun, C., et al., The effect of brain-derived neurotrophic factor on angiogenesis. J Huazhong Univ Sci Technolog Med Sci, 2009. 29(2): p. 139-43.*
16. *Wang, Y.D., et al., Brain derived neurotrophic factor induces endothelial cells angiogenesis through AKT and ERK1/2 signal pathway. Zhongguo Shi Yan Xue Ye Xue Za Zhi, 2008. 16(1): p. 175-80.*
17. *Madri, J.A., Modeling the neurovascular niche: implications for recovery from CNS injury. J Physiol Pharmacol, 2009. 60 Suppl 4: p. 95-104.*
18. *Li, Q., et al., Modeling the neurovascular niche: VEGF- and BDNF-mediated cross-talk between neural stem cells and endothelial cells: an in vitro study. J Neurosci Res, 2006. 84(8): p. 1656-68.*
19. *Xiong, Y., A. Mahmood, and M. Chopp, Angiogenesis, neurogenesis and brain recovery of function following injury. Curr Opin Investig Drugs, 2010. 11(3): p. 298-308.*
20. *Krikov, M., et al., Candesartan but not ramipril pretreatment improves outcome after stroke and stimulates neurotrophin BNDF/TrkB system in rats. J Hypertens, 2008. 26(3): p. 544-52.*
21. *Alhusban, A., et al., AT1 receptor antagonism is proangiogenic in the brain: BDNF a novel mediator. J Pharmacol Exp Ther, 2013. 344(2): p. 348-59.*
22. *Kishi, T., Y. Hirooka, and K. Sunagawa, Telmisartan protects against cognitive decline via up-regulation of brain-derived neurotrophic factor/tropomyosin-related kinase B in hippocampus of hypertensive rats. J Cardiol, 2012. 60(6): p. 489-94.*
23. *Guan, W., et al., Vascular protection by angiotensin receptor antagonism involves differential VEGF expression in both hemispheres after experimental stroke. PLoS One, 2011. 6(9): p. e24551.*
24. *Guan, W., et al., Acute Treatment with Candesartan Reduces Early Injury After Permanent Middle Cerebral Artery Occlusion. Transl Stroke Res, 2011. 2(2): p. 179-185.*
25. *Randomised trial of a perindopril-based blood-pressure-lowering regimen among 6,105 individuals with previous stroke or transient ischaemic attack. Lancet, 2001. 358(9287): p. 1033-41.*
26. *Gerecke, K.M., et al., Exercise does not protect against MPTP-induced neurotoxicity in BDNF haploinsufficient mice. PLoS One, 2012. 7(8): p. e43250.*
27. *Dorsey, S.G., et al., Genetic deletion of trkB.T1 increases neuromuscular function. Am J Physiol Cell Physiol, 2012. 302(1): p. C141-53.*
28. *Jackson, A.L. and P.S. Linsley, Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. Nat Rev Drug Discov, 2010. 9(1): p. 57-67.*

Figure 5-1: Intracerebroventricular delivery of BDNF shRNA expressing lentiviruses inhibits BDNF expression. Wistar rats were injected with either unilateral or bilateral BDNF shRNA expressing lentiviruses in the lateral ventricle. shRNA injection reduced BDNF expression in a dose dependent manner. * Significantly different from control; # significantly different from unilateral injection group. n=3 per group.

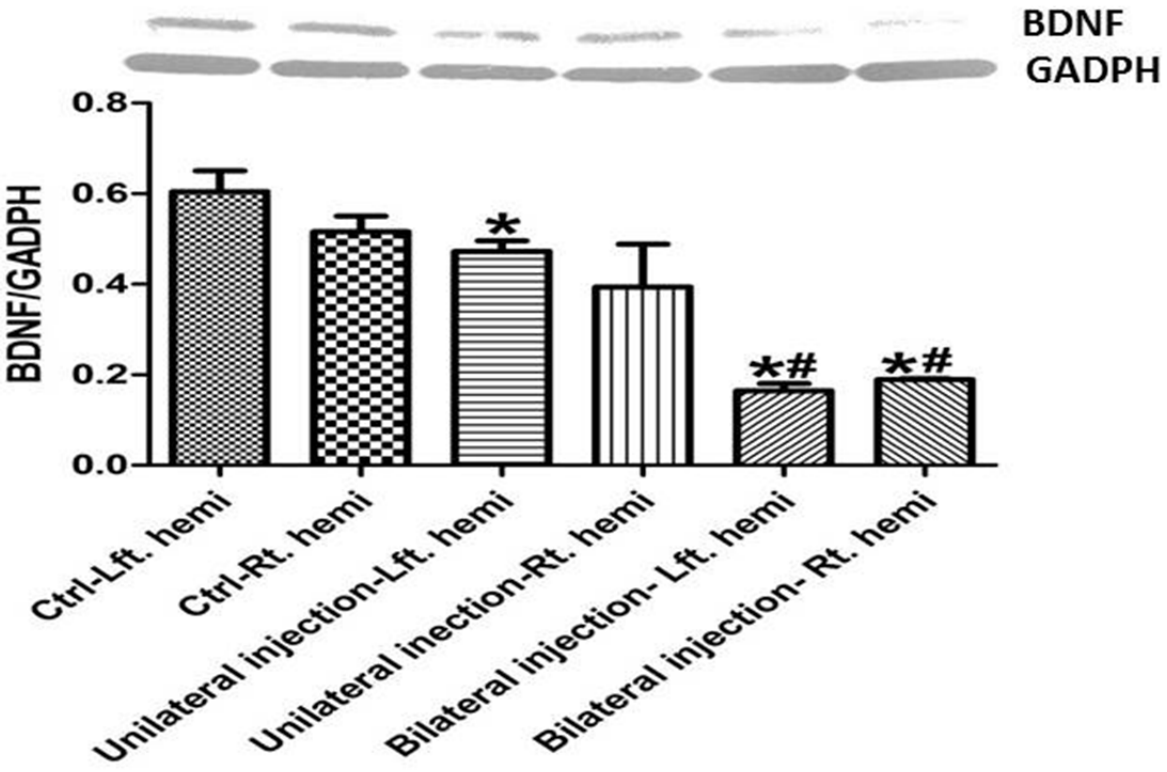
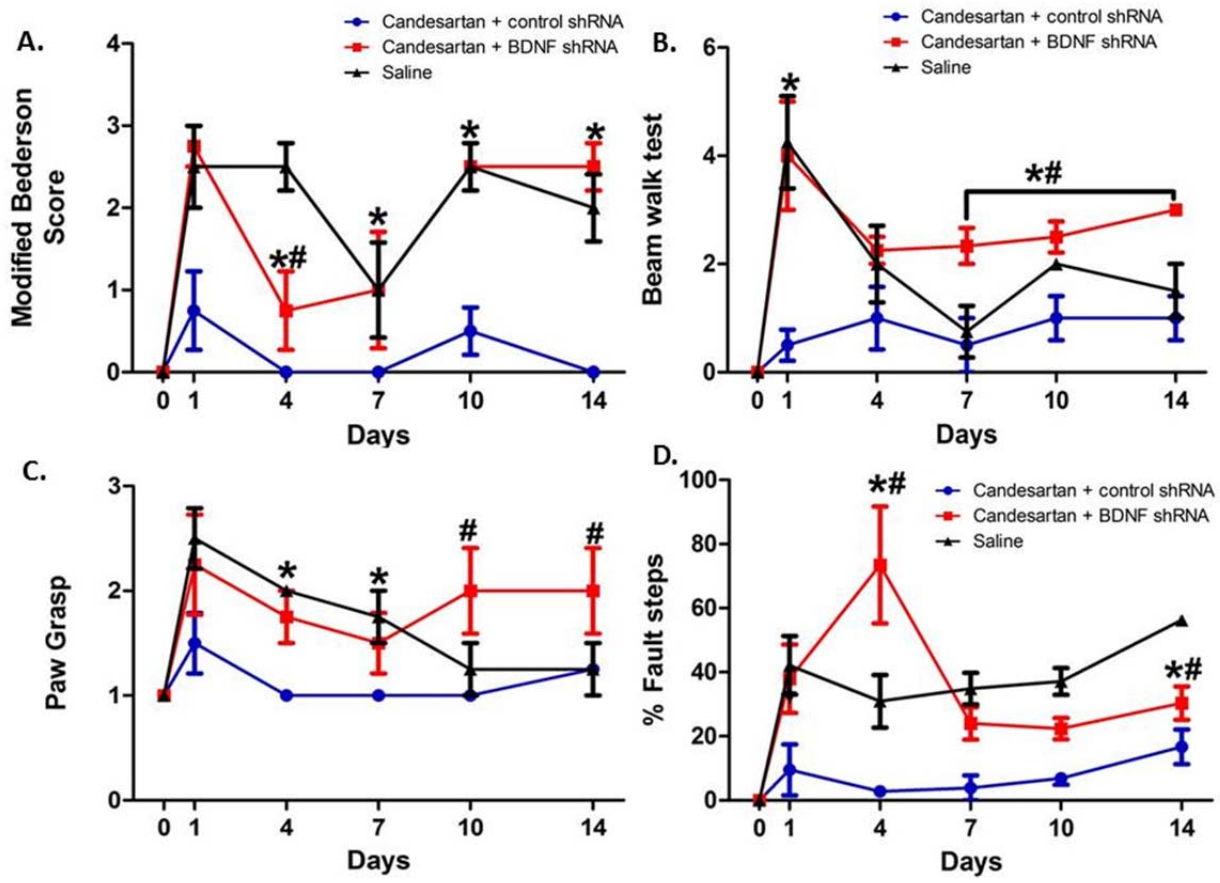

BDNF
GADPH
BDNF/GADPH
0.8
0.6
0.4
0.2
0.0
*
*#
*#
Ctrl-Lft. hemi
Ctrl-Rt. hemi
Unilateral injection-Lft. hemi
Unilateral inection-Rt. hemi
Bilateral injection- Lft. hemi
Bilateral injection- Rt. hemi

Figure 5-2: BDNF might be involved in candesartan induced improvement in stroke outcome. BDNF inhibition ablated candesartan induced improvement in stroke outcome as assessed using modified bederson test (A), beam walk test (B), paw grasp test (C), and grid walking test (D). * Significantly different from candesartan group; # significantly different from candesartan and saline groups n=4 per group.


A.
Modified Bederson Score
Candesartan + control shRNA
Candesartan + BDNF shRNA
Saline
Days
B.
Beam walk test
Candesartan + control shRNA
Candesartan + BDNF shRNA
Saline
Days
C.
Paw Grasp
Days
D.
% Fault steps
Candesartan + control shRNA
Candesartan + BDNF shRNA
Saline
Days

Chapter 6

INTEGRATED DISCUSSION

The aim of this dissertation is to assess the interaction between blood pressure reduction with candesartan and BDNF/TrkB mediated improvement in functional outcome after stroke. Previously, we demonstrated the ability of a single dose candesartan administered at the time of reperfusion to improve long-term functional outcome after stroke [1]. This finding highlights the possibility that the candesartan induced neurovascular protection may not be attributed solely to it hypotensive effect since it was administered once only. This notion was supported by the work of Nishikawa [2] and Ito et al. [3]. Furthermore, data from clinical trials suggested similar findings [4, 5]. In clinical practice, blood pressure reduction is generally avoided during the acute stage after stroke [6, 7]. Additionally, the findings of SCAST [8] where an aggressive approach to reduce blood pressure was adopted, demonstrated no benefit of acute administration of candesartan after stroke. If the blood pressure lowering effect of candesartan is demonstrated to be non-essential for improving stroke outcome; acute administration of sub-hypotensive of candesartan or similarly functioning agents might offer a plausible tactic to improve stroke outcome. Accordingly, we hypothesize that blood pressure reduction improves functional outcome and recovery after cerebral ischemia by increasing the expression of brain derived neurotrophic factor (BDNF) in the brain.

BDNF is a member of the neurotrophins family that is widely expressed and has been shown to have angiogenic, neurogenic, neuroprotective and to induce neuroplasticity [9-11]. Following experimental cerebral ischemia higher levels of BDNF in the brain were found to limit the injury and improve functional outcome [12-15]. The applicability of experimentally used approaches in humans is limited by the invasive nature and the associated risks. BDNF itself is a poor candidate for systematic administration because of its kinetic profile [16, 17]. Accordingly attention should be directed toward agents having the ability to stimulate the BDNF/TrkB signaling [17].

Data from our lab demonstrated that candesartan induced pro-recovery effect was associated with a proangiogenic state in the brain [1]. This proangiogenic sate was found to be partially mediated by VEGF, which suggests the ability of candesartan to upregualte the expression of other angiogenic mediators after stroke. In a follow up paper Guan *et al.* demonstrated the ability of candesartan to upregualte expression of a number of genes that have angiogenic potential [18]. Additionally, they demonstrated the ability of candesartan to upregualte the expression of BDNF at the mRNA level after stroke [18].

The angiogenic potential of ARBs is highly controversial especially in in vitro studies, where the majority of them suggest an anti-angiogenic effect [19-21]. in an extensive review, Willis et al. demonstrated a tissue and context dependent pro-angiogenic effect of ARBs [22]. In this work, we demonstrated an angiogenic potential of candesartan in human cerebromicrovascular endothelial cells (hCMECs) (Figures 2-2C and 2-5). In addition, our data suggests an intrinsic pro-angiogenic effect of candesartan (Figure 2-2C and D).

Available data on the interaction between RAAS system and BDNF expression is limited [23, 24]. Szekeres et al. demonstrated the ability of angiotensin II to upregualte BDMF expression in the adrenal medulla [24]. Similarly, Chan et al. demonstrated the ability of angiotensin II to upregualte the expression of BDNF in the rostroventral medulla in the brain [23]. There work suggested that blocking AT1 blunts this reported effect of angiotensin II. In contrast, we demonstrated the ability of candesartan to increase the expression of the mature form of BDNF in both normotensive and hypertensive animals that were not exposed to cerebral ischemia (Figure 2-1B). Interestingly, we demonstrated that this effect was not related to the hypotensive effect of candesartan (Figure 2-S3). Furthermore we demonstrated the ability of candesartan to increase BDNF expression in hCMECs (Figure 2-2B) which has been shown to be a major source of BDNF expression [25, 26].

BDNF has been demonstrated to have an angiogenic potential [10, 27]. In our work, we demonstrated the essential role of BDNF in candesartan mediated angiogenic effect (Figures 2-4 and 5). This finding might explain our previous finding on the partial involvement of VEGF in candesartan induced proangiogenic effect [1].

Iwai et al demonstrated the essential role of AT2 mediated signaling in the neuroprotective role of ARBs [28]. This has been further supported by the work of McCarthy et al. [29]. In contrast to the general notion that AT2 signaling is anti-angiogenic we demonstrated an essential role of AT2 receptor in mediating the angiogenic effect of candesartan in hCMECs (Figures 2-6 A and B). Furthermore, we confirmed the involvement of AT2 mediated signaling in the angiogenic process of hCMECs (Figure 2-6C).

Our data has demonstrated the essential role of BDNF and AT2 receptor in mediating candesartan induced proangiogenic effect. These findings suggest a possible link between AT2 receptor mediated signaling and BDNF expression. Our work tested this possible link and demonstrated that blocking AT2 receptor blunts candesartan induced BDNF expression (Figure 2-7A). In addition, we demonstrated the ability of AT2 stimulation to increase BDNF expression in hCMECs (Figure 2-7B). our findings in hCMECs has been recently replicated by the work of Namsolleck et al. [30] in neurons. Accordingly, we proposed that candesartan induced blockade of AT1 receptor results in unopposed stimulation of AT2 receptor (Figure 2-10). Unopposed stimulation of AT2 upregulates BDNF expression which will induce a proangiogenic state in endothelial cells, which will ultimately lead to improved recovery after stroke (Figure 2-10).

Our findings on the beneficial effects of AT2 stimulation are in conformity with multiple recent reports about the beneficial effects of compound 21 (C21), a recently developed water soluble AT2 agonist [30-35]. Interestingly, the administration of the compound does not affect blood pressure after stroke (personal communication). C21's minimum effect on blood pressure and its possible ability to increase BDNF expression highlights the promising potential of this drug in stroke management. Preclinical testing of C21 has been recently initiated in Dr. Fagan lab.

To understand the mechanisms behind candesartan induced BDNF expression, we assessed the involvement of eNOS and reperfusion in candesartan induced BDNF expression. In hypertensive animals we demonstrated the essential role eNOS in mediating candesartan inductive effect on BDNF expression. This finding is in line with the work of Chen et al. about the role of eNOS in BDNF expression [36]. Interestingly,

we demonstrated the ability of candesartan to ameliorate ischemia induced endoplasmic reticulum stress. This finding offers a novel mechanism that can explain the candesartan induced neurovascular protection. Furthermore, we assessed the involvement of reperfusion in candesartan induced BDNF/TrkB signaling. Our findings suggest a bimodal effect of reperfusion on BDNF/TrkB signaling. Lack of reperfusion was found to blunt the ability of candesartan to increase BDNF expression after stroke (Figure 5-3A). Additionally, lack of reperfusion was found to inhibit the expression of TrkB receptor (Figure 5-3D).

To assess the role of blood pressure reduction in candesartan mediated improvement in stroke outcome, two approaches were adopted: a) a direct approach to override the hypotensive effect of candesartan using continuous infusion of vasoconstrictors. b) An indirect approach by using sub-hypotensive effects of candesartan. Results from the direct approach were surprising. Candesartan administration in animals receiving hypertensive concentrations of angiotensin II, α-phenylephrine, and vasopressin resulted in mortality observed in all tested animals (APPENDIX 1.1). This unexpected finding urged us to halt pursuing this approach, until we would understand the basics behind these findings. In the indirect approach, we demonstrated the ability of candesartan administered in sub-hypotensive doses (Figure 5-1) to improve stroke outcome (Figures 5-2A and B). This improvement was also associated with an up regulation of BDNF expression (Figure 5-3A). These findings are further supported by our findings in hypertensive animals when candesartan and hydralazine were compared (Figure 2-1B and Figure 2-S3).

To assess the involvement of BDNF in candesartan mediated neurovascular protection, we adopted shRNA mediated BDNF knockdown. BDNF shRNA expressing lentiviruses were injected into the cerebral ventricles followed by exposing the animals to 90 minutes of MCAO. Candesartan administration improved functional outcome as assessed using multiple validated assessment tools (Figure 5-2). Despite the observed blunting of candesartan induced protection by knocking down BDNF, a definitive conclusion cannot be made with our current results due to the lack of a positive control.

In conclusion, our data demonstrates that lowering blood pressure is not essential for activating BDNF/TrkB activity. In addition, the ability of candesartan to activate the BDNF/TrkB system is mediated through unopposed AT2 receptor stimulation.

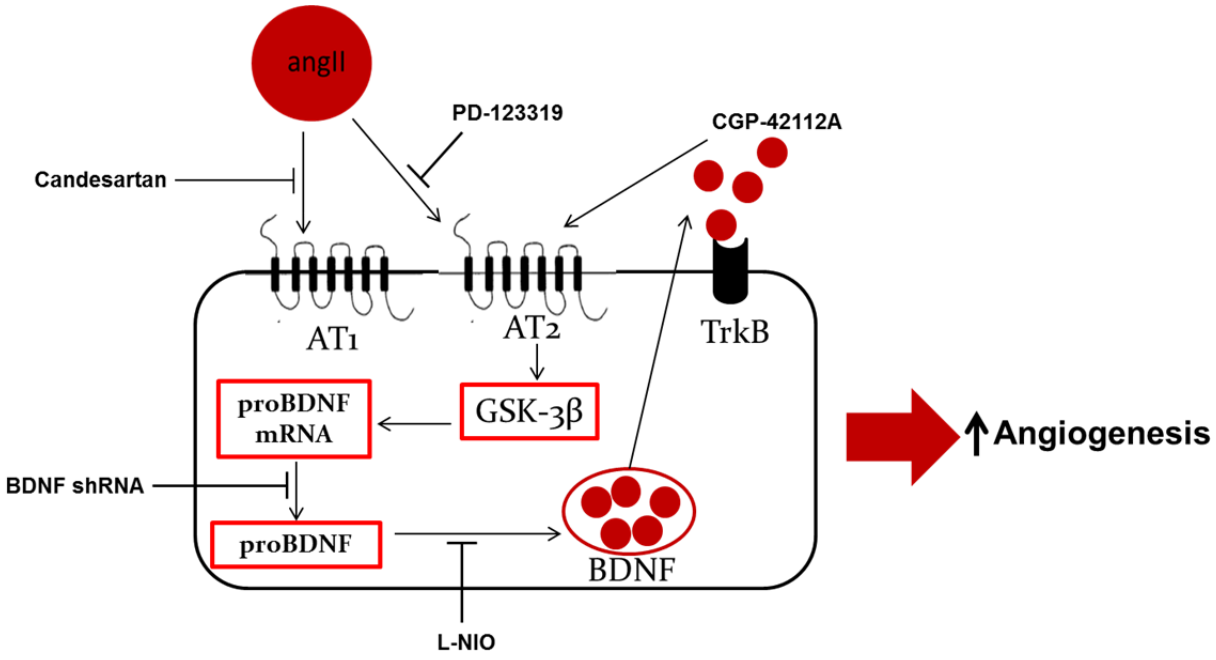


Figure 6-1: A schematic diagram of the findings.

References

1. **Kozak, A., et al., *Candesartan augments ischemia-induced proangiogenic state and results in sustained improvement after stroke.* Stroke, 2009. 40(5): p. 1870-6.**
2. **Nishikawa, K., *Angiotensin AT1 receptor antagonism and protection against cardiovascular end-organ damage.* J Hum Hypertens, 1998. 12(5): p. 301-9.**
3. **Ito, T., et al., *Protection against ischemia and improvement of cerebral blood flow in genetically hypertensive rats by chronic pretreatment with an angiotensin II AT1 antagonist.* Stroke, 2002. 33(9): p. 2297-303.**
4. **Schrader, J., et al., *The ACCESS Study: evaluation of Acute Candesartan Cilexetil Therapy in Stroke Survivors.* Stroke, 2003. 34(7): p. 1699-703.**
5. **Schrader, J., et al., *Morbidity and Mortality After Stroke, Eprosartan Compared with Nitrendipine for Secondary Prevention: principal results of a prospective randomized controlled study (MOSES).* Stroke, 2005. 36(6): p. 1218-26.**
6. **Luders, S., *Drug therapy for the secondary prevention of stroke in hypertensive patients: current issues and options.* Drugs, 2007. 67(7): p. 955-63.**
7. **Jauch, E.C., et al., *Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association.* Stroke, 2013. 44(3): p. 870-947.**
8. **Sandset, E.C., et al., *The angiotensin-receptor blocker candesartan for treatment of acute stroke (SCAST): a randomised, placebo-controlled, double-blind trial.* Lancet, 2011. 377(9767): p. 741-50.**
9. **Caporali, A. and C. Emanueli, *Cardiovascular actions of neurotrophins.* Physiol Rev, 2009. 89(1): p. 279-308.**
10. **Kermani, P. and B. Hempstead, *Brain-derived neurotrophic factor: a newly described mediator of angiogenesis.* Trends Cardiovasc Med, 2007. 17(4): p. 140-3.**
11. **Greenberg, M.E., et al., *New insights in the biology of BDNF synthesis and release: implications in CNS function.* J Neurosci, 2009. 29(41): p. 12764-7.**
12. **Ploughman, M., et al., *Brain-derived neurotrophic factor contributes to recovery of skilled reaching after focal ischemia in rats.* Stroke, 2009. 40(4): p. 1490-5.**
13. **Mahmood, A., D. Lu, and M. Chopp, *Intravenous administration of marrow stromal cells (MSCs) increases the expression of growth factors in rat brain after traumatic brain injury.* J Neurotrauma, 2004. 21(1): p. 33-9.**

14. **Schabitz, W.R., et al., *Effect of brain-derived neurotrophic factor treatment and forced arm use on functional motor recovery after small cortical ischemia.* Stroke, 2004. 35(4): p. 992-7.**
15. **Schabitz, W.R., et al., *Intravenous brain-derived neurotrophic factor enhances poststroke sensorimotor recovery and stimulates neurogenesis.* Stroke, 2007. 38(7): p. 2165-72.**
16. **Mocchetti, I. and M. Brown, *Targeting neurotrophin receptors in the central nervous system.* CNS Neurol Disord Drug Targets, 2008. 7(1): p. 71-82.**
17. **Jang, S.W., et al., *A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone.* Proc Natl Acad Sci U S A, 2010. 107(6): p. 2687-92.**
18. **Guan, W., et al., *Vascular protection by angiotensin receptor antagonism involves differential VEGF expression in both hemispheres after experimental stroke.* PLoS One, 2011. 6(9): p. e24551.**
19. **Fujiyama, S., et al., *Angiotensin AT(1) and AT(2) receptors differentially regulate angiopoietin-2 and vascular endothelial growth factor expression and angiogenesis by modulating heparin binding-epidermal growth factor (EGF)-mediated EGF receptor transactivation.* Circ Res, 2001. 88(1): p. 22-9.**
20. **Herr, D., et al., *Regulation of endothelial proliferation by the renin-angiotensin system in human umbilical vein endothelial cells.* Reproduction, 2008. 136(1): p. 125-30.**
21. **Hu, C., A. Dandapat, and J.L. Mehta, *Angiotensin II induces capillary formation from endothelial cells via the LOX-1 dependent redox-sensitive pathway.* Hypertension, 2007. 50(5): p. 952-7.**
22. **Willis, L.M., et al., *Angiotensin receptor blockers and angiogenesis: clinical and experimental evidence.* Clin Sci (Lond), 2011. 120(8): p. 307-19.**
23. **Chan, S.H., et al., *Transcriptional upregulation of brain-derived neurotrophic factor in rostral ventrolateral medulla by angiotensin II: significance in superoxide homeostasis and neural regulation of arterial pressure.* Circ Res, 2010. 107(9): p. 1127-39.**
24. **Szekeres, M., et al., *Angiotensin II-induced expression of brain-derived neurotrophic factor in human and rat adrenocortical cells.* Endocrinology, 2010. 151(4): p. 1695-703.**
25. **Guo, S., et al., *Neuroprotection via matrix-trophic coupling between cerebral endothelial cells and neurons.* Proc Natl Acad Sci U S A, 2008. 105(21): p. 7582-7.**
26. **Leventhal, C., et al., *Endothelial trophic support of neuronal production and recruitment from the adult mammalian subependyma.* Mol Cell Neurosci, 1999. 13(6): p. 450-64.**
27. **Sun, C., et al., *The effect of brain-derived neurotrophic factor on angiogenesis.* J Huazhong Univ Sci Technolog Med Sci, 2009. 29(2): p. 139-43.**
28. **Iwai, M., et al., *Possible inhibition of focal cerebral ischemia by angiotensin II type 2 receptor stimulation.* Circulation, 2004. 110(7): p. 843-8.**

29. **McCarthy, C.A., et al., *Angiotensin AT2 receptor stimulation causes neuroprotection in a conscious rat model of stroke.* Stroke, 2009. 40(4): p. 1482-9.**
30. **Namsolleck, P., et al., *AT2-receptor stimulation enhances axonal plasticity after spinal cord injury by upregulating BDNF expression.* Neurobiol Dis, 2013. 51: p. 177-91.**
31. **Steckelings, U.M., et al., *AT2 receptor agonists: hypertension and beyond.* Curr Opin Nephrol Hypertens, 2012. 21(2): p. 142-6.**
32. **Steckelings, U.M., et al., *Non-peptide AT2-receptor agonists.* Curr Opin Pharmacol, 2011. 11(2): p. 187-92.**
33. **Jehle, A.B., et al., *A nonpeptide angiotensin II type 2 receptor agonist does not attenuate postmyocardial infarction left ventricular remodeling in mice.* J Cardiovasc Pharmacol, 2012. 59(4): p. 363-8.**
34. **Bosnyak, S., et al., *Stimulation of angiotensin AT2 receptors by the non-peptide agonist, Compound 21, evokes vasodepressor effects in conscious spontaneously hypertensive rats.* Br J Pharmacol, 2010. 159(3): p. 709-16.**
35. **Gelosa, P., et al., *Stimulation of AT2 receptor exerts beneficial effects in stroke-prone rats: focus on renal damage.* J Hypertens, 2009. 27(12): p. 2444-51.**
36. **Chen, J., et al., *Endothelial nitric oxide synthase regulates brain-derived neurotrophic factor expression and neurogenesis after stroke in mice.* J Neurosci, 2005. 25(9): p. 2366-75.**

Chapter 7

SUMMARY

Our findings demonstrate that lowering blood pressure using angiotensin receptor blockers (ARBs) is not essential to activate BDNF/TrkB. In addition the ability of candesartan to activate BDNF/TrkB is both eNOS and reperfusion dependent.

APPENDIX

Includes 1 unpublished manuscript

1. **Overriding the Hypotensive Effect of Candesartan Aggravates Stroke Outcome.**

   Ahmed Alhusban, Anna Kozak, Adviye Ergul, and Susan C Fagan

APPENDIX 1

OVERRIDING THE HYPOTENSIVE EFFECT OF CANDESARTAN AGGRAVATES STROKE OUTCOME

Ahmed Alhusban, Anna Kozak, Adviye Ergul, Susan C Fagan

To be submitted to pharmacotherapy

Abstract:

Background: Altering blood pressure during the acute stage after stroke is generally avoided over concerns to expand the area of low cerebral perfusion. Agents that alters RAAS system has been shown to positively affect stroke outcome. Assessing whether their reported beneficial effect is related to blood pressure lowering is still to be mechanistically proven.

Method: Male normotensive rats were implanted with blood pressure transmitters and pumps containing angiotensin II, α-phenylephrine, or vasopressin solutions. One week after pumps implantation, animals were subjected to 3 hours of middle cerebral artery occlusion followed by reperfusion. At the time of reperfusion, candesartan (1mg/kg) was administered intravenously and the blood pressure and stroke outcome were assessed.

Results: Infusion with the different agents elevated mean arterial blood pressure. Reducing blood pressure acutely in these animals was associated with mortality in the different models used.

Conclusion: Altering blood pressure in hemodynamically unstable animals might aggravate stroke outcome and worsen outcome.

Introduction:

Hypertension has been identified as a major risk factor for stroke [1]. Interventions intended to reduce blood pressure has been demonstrated to reduce the risk of stroke recurrence and incidence [2-5]. Both clinical [3, 4, 6] and experimental data [7-12] suggested a more beneficial effect of agents that alter renin-angiotensin-aldosterone system (RAAS) on stroke. Critical analysis of available data suggests that the beneficial effect of these agents might not be due to blood pressure reduction [2, 4, 11]. Verifying whether the pro-recovery effect of these agents is blood pressure lowering independent might help adopting the use of these agents in doses that does not affect blood pressure during the acute stage to improve stroke outcome.

Materials and methods:

Animals: All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Charlie Norwood Veterans Affairs Medical Center (09-04-008). Male wistar rats (160-180g) were implanted with blood pressure transmitters as previously described [7]. Animals were allowed two weeks for recovery, after which their baseline blood pressure was followed up for two days. In all procedures anesthesia was induced using 5% isoflurane and maintained using 2% isoflurane delivered through inhalation throughout the procedure.

Dose response studies: animals were anesthetized and the dorsal aspect of the neck and inter-scapular area was shaved and scrubbed for surgery using 70% ethanol followed by iodine. A midline incision was introduced and a pocket was made in the subcutaneous compartment. Programmable pumps (iPRECIO™; Primetech, Tokyo,

Japan) filled with a solution α-phenylephrine (49.1mM) were implanted inserted in the subcutaneous pocket and the incision was closed using clips. The pumps were programmed to deliver solutions with increasing concentrations of the dissolved agent. Each particular concentration was infused for 36 hours and blood pressure and heart rate were followed up throughout the whole experiment time.

Blood pressure and stroke: all infused agents were purchased from Sigma-Aldrich (St. Louis, MO). Animals implanted with blood pressure transmitters were prepared as mentioned above and were implanted with Alzet™ mini osmotic pumps (model 2M3, 7 day; Alza, Palo Alto, CA). Implanted pumps were filled with solutions of angiotensin II (AngII; 1.4mM), vasopressin (2.9mM) or α-phenylephrine (0.3mM). Blood pressure response to the infused agent was followed up for one week after which cerebral ischemia was induced through middle cerebral artery occlusion (MCAO) for three hours. At the time of reperfusion animals received a single I.V. injection of candesartan 1mg/kg. Blood pressure and heart rate were continuously followed up.

Middle cerebral artery occlusion: Cerebral ischemia was induced through MCAO as has been reported previously. Briefly, the ventral aspect of the neck was shaved and scrubbed using 70% ethanol. A midline incision was made and the common carotid artery was isolated. The bifurcation of the common carotid artery was then identified and the external and internal carotid artery was isolated. The external artery was then cauterized and a small incision was made in its wall. A silicone coated suture was inserted through the incision and pushed into the internal carotid artery all the way till the origin of the middle cerebral artery. Three hours after the occlusion the suture was removed and the wound was closed.

Results:

Phenylephrine induced a dose dependent mild increase in blood pressure: To assess the effect of phenylephrine on blood pressure, iPRECIO pumps were used to deliver a solution of phenylephrine with 100ug/kg/hr increment dose increase every 36 hours. Phenylephrine induced a modest dose dependent increase in mean arterial blood pressure (Figure 1A and B). Interestingly, the hypertensive effect of phenylephrine was lost shortly after the infusion ceased.

Angiotensin II and vasopressin infusions increased mean arterial blood pressure: In agreement with previous reports continuous infusion of AngII (0.4ug/kg/min) induced a rapid 20-25mmHg increase in the mean arterial blood pressure (Figure 2A) [13]. Four days after infusion initiation MAP decreased by about 10mmHg and was maintained throughout the duration of the experiment. On the other hand, vasopressin infusion (20ug/kg/hr) induced about 30mmHg increase in MAP (Figure 2B). This increase lasted for 4 days then MAP decreased to about 120mmHg and was maintained throughout the duration of the experiment.

Candesartan treatment induced a robust short term reduction in MAP in vasopressin infused animals: Similar to our previous reports, MCAO induced a sharp increase in MAP [7]. Candesartan treatment at the time of reperfusion induced an acute short term reduction in blood pressure (Figure 3A). This reduction was then followed by a rebound in MAP which led to the animal death.

Candesartan treatment at the time of reperfusion reduced MAP to less than baseline values: Candesartan treatment at the time of reperfusion reduced blood

pressure to less than baseline values. This reduction was consistent in both phenylephrine and AngII infused animals (Figure 3B and C). Similar to what was observed in vasopressin infused animals, candesartan induced reduction in MAP was associated with animal mortality. In contrast to vasopressin infused animals, AngII and phenylephrine infused animals did not have the blood pressure rebound.

Discussion: Our results demonstrated the deleterious effect of acute candesartan induced blood pressure reduction in animals with induced increase in blood pressure. Surprisingly, despite the ability of candesartan to reduce blood pressure after MCAO, this decrease was not translated into a beneficial effect with regard to mortality. Unfortunately, this increased incidence of mortality deterred us from further pursuing whether candesartan induced blood pressure is a prerequisite for the improved neurobehavioral outcome previously reported by our lab and others.

Hypertension development is associated with an increased oxidative stress with development of endothelial dysfunction and vascular remodeling [14-18]. These complex and inter-related effect of hypertension makes it impossible to know whether the observed beneficial effects of blood pressure reduction are related to the reduced mechanical stress imposed on blood vessels walls or to other blood pressure independent effect of hypotensive agents. In this investigation we managed to establish a dose response relationship between phenylephrine infusion and blood pressure levels. Accordingly, current findings allow for studying the pure mechanical effects of increased blood pressure without the interference imposed by oxidative stress and vascular remodeling.

In a shocking contrast to our expectations, early candesartan treatment resulted in high mortality. This effect was consistent among all experimental groups. Considering this observation we considered it to be unacceptable to proceed further with this experiment at this level. This unexpected finding can be explained by a number of causes. One simple explanation is an operator induced development of intracranial hematoma. This hematoma can develop as a result of cerebral artery rupture as a result pushing the suture more into the cerebral arteries. In our experiments we were unable to detect any appreciable intracranial hematomas when the cranial cavity was opened postmortem. Another explanation would be related to the duration of time blood pressure was increased before induction of ischemia. Usually blood pressure increase is induced for two weeks before experiments are initiated [19, 20]. This duration allows for vascular remodeling to occur [19, 21]. The duration of time we used is sufficient to induce a pre-hypertensive state in animals as been previously reported [22]. In addition, the levels of blood pressure increase were comparable to what was previously reported for the same duration of treatment [19, 22]. Despite the good argument in favor of extending the duration of hypertension induction, the goal of this investigation was to assess whether the pure hemodynamic effect of blood pressure reduction is essential for improving stroke outcome. If the duration of induction is extended, vascular remodeling will ensue [21]. This effect will interfere with understanding the results and will make it difficult to conclude with confidence whether blood pressure reduction is essential for improving stroke outcome. Accordingly, it would be a reasonable approach to review the design of the experiment and balance the pros and cons for extending the duration of vasoactive substance infusion.

In light of the previous discussion, we were unable to draw a conclusion about whether blood pressure reduction is essential to improve stroke outcome. But it's noteworthy to take our findings into consideration when stroke victims present with unstable hemodynamic state. In this case blood pressure reduction must be cautiously considered as an intervention.

References:

1. *Go, A.S., et al., Heart disease and stroke statistics--2013 update: a report from the American Heart Association. Circulation, 2013. 127(1): p. e6-e245.*
2. *Schrader, J., et al., The ACCESS Study: evaluation of Acute Candesartan Cilexetil Therapy in Stroke Survivors. Stroke, 2003. 34(7): p. 1699-703.*
3. *Schrader, J., et al., Morbidity and Mortality After Stroke, Eprosartan Compared with Nitrendipine for Secondary Prevention: principal results of a prospective randomized controlled study (MOSES). Stroke, 2005. 36(6): p. 1218-26.*
4. *Yusuf, S., et al., Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. N Engl J Med, 2000. 342(3): p. 145-53.*
5. *Rodgers, A., et al., Blood pressure and risk of stroke in patients with cerebrovascular disease. The United Kingdom Transient Ischaemic Attack Collaborative Group. BMJ, 1996. 313(7050): p. 147.*
6. *Randomised trial of a perindopril-based blood-pressure-lowering regimen among 6,105 individuals with previous stroke or transient ischaemic attack. Lancet, 2001. 358(9287): p. 1033-41.*
7. *Fagan, S.C., et al., Hypertension after experimental cerebral ischemia: candesartan provides neurovascular protection. J Hypertens, 2006. 24(3): p. 535-9.*
8. *Kozak, W., et al., Vascular protection with candesartan after experimental acute stroke in hypertensive rats: a dose-response study. J Pharmacol Exp Ther, 2008. 326(3): p. 773-82.*
9. *Guan, W., et al., Vascular protection by angiotensin receptor antagonism involves differential VEGF expression in both hemispheres after experimental stroke. PLoS One, 2011. 6(9): p. e24551.*
10. *Dai, W.J., et al., Blockade of central angiotensin AT(1) receptors improves neurological outcome and reduces expression of AP-1 transcription factors after focal brain ischemia in rats. Stroke, 1999. 30(11): p. 2391-8; discussion 2398-9.*
11. *Ito, T., et al., Protection against ischemia and improvement of cerebral blood flow in genetically hypertensive rats by chronic pretreatment with an angiotensin II AT1 antagonist. Stroke, 2002. 33(9): p. 2297-303.*
12. *Engelhorn, T., et al., The angiotensin II type 1-receptor blocker candesartan increases cerebral blood flow, reduces infarct size, and improves neurologic outcome after transient cerebral ischemia in rats. J Cereb Blood Flow Metab, 2004. 24(4): p. 467-74.*

13. *Muthalif, M.M., et al., Angiotensin II-induced hypertension: contribution of Ras GTPase/Mitogen-activated protein kinase and cytochrome P450 metabolites. Hypertension, 2000. 36(4): p. 604-9.*
14. *Park, J.B. and E.L. Schiffrin, Small artery remodeling is the most prevalent (earliest?) form of target organ damage in mild essential hypertension. J Hypertens, 2001. 19(5): p. 921-30.*
15. *Schiffrin, E.L., Remodeling of resistance arteries in essential hypertension and effects of antihypertensive treatment. Am J Hypertens, 2004. 17(12 Pt 1): p. 1192-200.*
16. *Schiffrin, E.L., L.Y. Deng, and P. Larochelle, Morphology of resistance arteries and comparison of effects of vasoconstrictors in mild essential hypertensive patients. Clin Invest Med, 1993. 16(3): p. 177-86.*
17. *Briones, A.M. and R.M. Touyz, Oxidative stress and hypertension: current concepts. Curr Hypertens Rep, 2010. 12(2): p. 135-42.*
18. *Touyz, R.M., Molecular and cellular mechanisms in vascular injury in hypertension: role of angiotensin II. Curr Opin Nephrol Hypertens, 2005. 14(2): p. 125-31.*
19. *Simon, G., G. Abraham, and G. Cserep, Pressor and subpressor angiotensin II administration. Two experimental models of hypertension. Am J Hypertens, 1995. 8(6): p. 645-50.*
20. *Diz, D.I., P.G. Baer, and A. Nasjletti, Angiotensin II-induced hypertension in the rat. Effects on the plasma concentration, renal excretion, and tissue release of prostaglandins. J Clin Invest, 1983. 72(2): p. 466-77.*
21. *Simon, G., G. Cserep, and C. Limas, Development of structural vascular changes with subpressor angiotensin II administration in rats. Am J Hypertens, 1995. 8(1): p. 67-73.*
22. *Young, C.N., et al., ER stress in the brain subfornical organ mediates angiotensin-dependent hypertension. J Clin Invest, 2012. 122(11): p. 3960-4.*

Figure 1: Phenylephrine increases mean arterial pressure in a dose dependent manner. Animals were implanted with iPRECIO pumps that infused phenylephrine at increasing concentrations. Doses from 0-800ug/kg/hr resulted in a dose dependent manner (A). Extending the infusion range did not result in appreciable change in MAP (B). n=1-2.

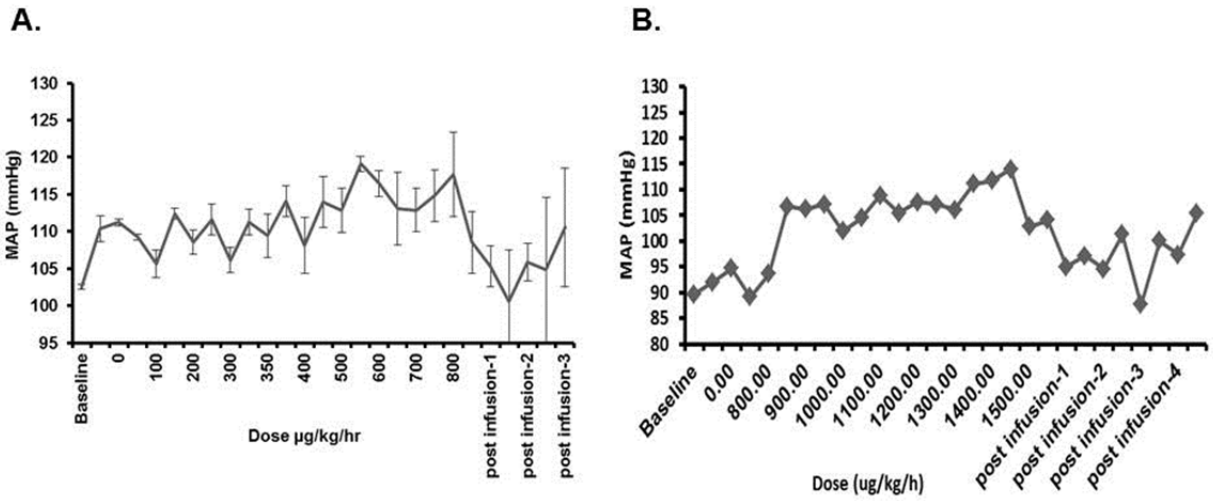

A.
MAP (mmHg)
130
125
120
115
110
105
100
95
Baseline
0
100
200
300
350
400
500
600
700
800
post infusion-1
post infusion-2
post infusion-3
Dose µg/kg/hr
B.
MAP (mmHg)
130
125
120
115
110
105
100
95
90
85
80
Baseline
0.00
800.00
900.00
1000.00
1100.00
1200.00
1300.00
1400.00
1500.00
post infusion-1
post infusion-2
post infusion-3
post infusion-4
Dose (ug/kg/h)

Figure 2: Angiotensin II and vasopressin infusion increased MAP. Constant rate infusion of angiotensin II (A) and vasopressin (B) increased MAP. n=1-2.

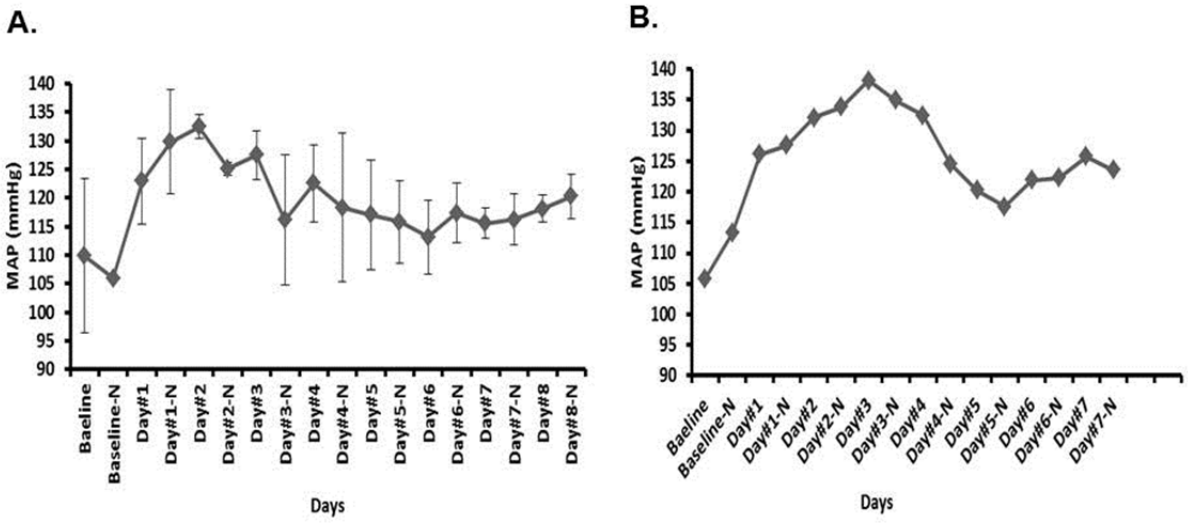
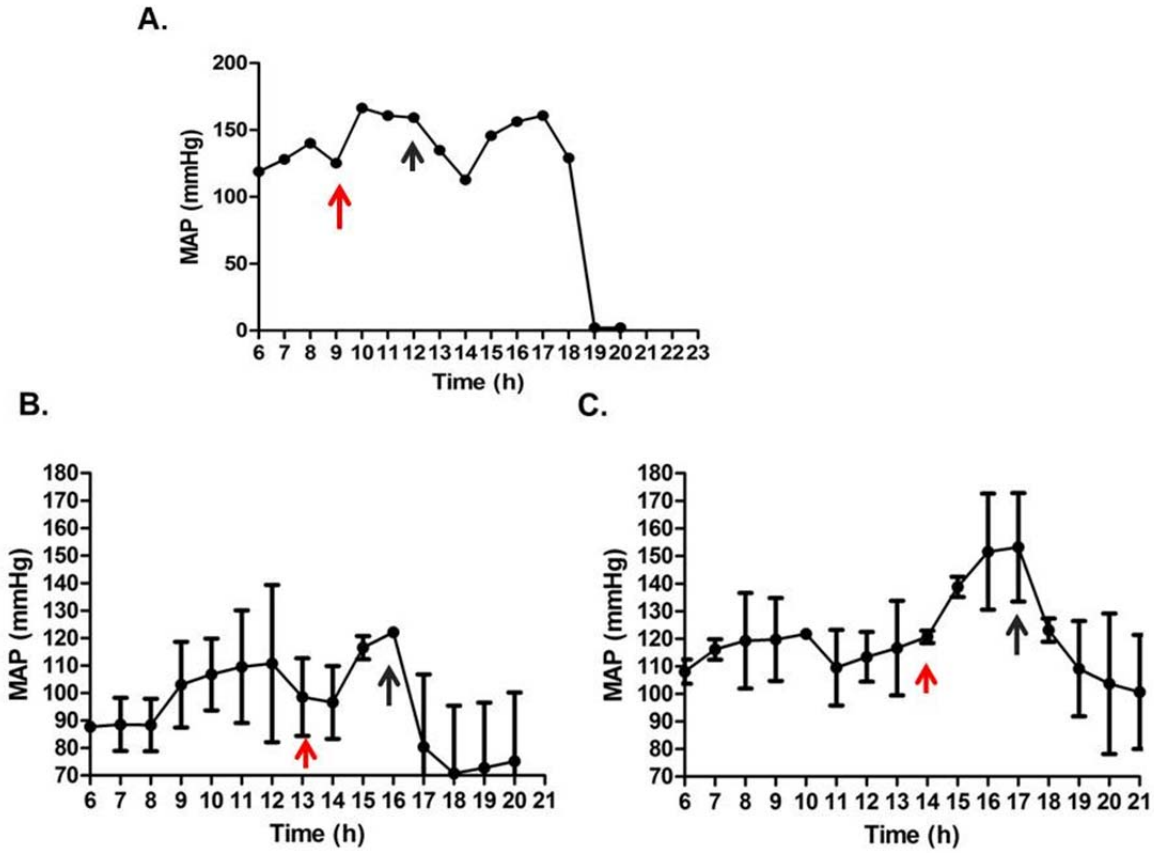

A.
MAP (mmHg)
140
135
130
125
120
115
110
105
100
95
90
Baeline
Baseline-N
Day#1
Day#1-N
Day#2
Day#2-N
Day#3
Day#3-N
Day#4
Day#4-N
Day#5
Day#5-N
Day#6
Day#6-N
Day#7
Day#7-N
Day#8
Day#8-N
Days
B.
MAP (mmHg)
140
135
130
125
120
115
110
105
100
95
90
Baeline
Baseline-N
Day#1
Day#1-N
Day#2
Day#2-N
Day#3
Day#3-N
Day#4
Day#4-N
Day#5
Day#5-N
Day#6
Day#6-N
Day#7
Day#7-N
Days

Figure 3: Candesartan administration induces an acute reduction in MAP followed by a rebound. Administering candesartan at the time of reperfusion in vasopressin infused animals acutely reduced MAP (A). MAP abruptly rebounded which led to animals death (A). This observation was further replicated in animals infused with both phenylephrine (B), and angiotensin II (C) infused animals. Red arrows indicate the time of ischemia induction; black arrows indicate the time of candesartan administration. n=1-2.


A.
MAP (mmHg)
200
150
100
50
0
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (h)
B.
MAP (mmHg)
180
170
160
150
140
130
120
110
100
90
80
70
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Time (h)
C.
MAP (mmHg)
180
170
160
150
140
130
120
110
100
90
80
70
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Time (h)