ROLE OF MECHANICAL VERSUS HUMORAL EFFECTS OF ANGIOTENSIN II ON VASCULAR REMODELING

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Preeti Pandurang Shanbhag

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ROLE OF MECHANICAL VERSUS HUMORAL EFFECTS OF ANGIOTENSIN II ON VASCULAR REMODELING

Approved by:

Dr. W. Robert Taylor, Advisor
Department of Biomedical Engineering/School of Medicine
Georgia Institute of Technology/Emory University

Dr. Samuel C. Dudley, Jr.
Department of Biomedical Engineering/School of Medicine
Georgia Institute of Technology/Emory University

Dr. Hanjoong Jo
Department of Biomedical Engineering/School of Medicine
Georgia Institute of Technology/Emory University

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To my family

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ACE	angiotensin-converting enzyme
ACE-I	angiotensin-converting enzyme inhibitor
Ang II	angiotensin II
ANOVA	Analysis of variance
ARB	angiotensin receptor blocker
AT_1R	angiotensin receptor, type 1
AT_2R	angiotensin receptor, type 2
bFGF	basic fibroblast growth factor
BK	bradykinin
BK B_1	bradykinin B_1 receptor
BK B_2	bradykinin B_2 receptor
cGMP	cyclic guanosine monophosphate
DAG	diacylglycerol
eNOS	endothelial nitric oxide synthase
H_2O_2	hydrogen peroxide
ICAM-1	intercellular adhesion molecule-1
IP_3	inositol triphosphate
MAP kinase	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
O_2^-	superoxide anion
$ONOO^-$	peroxynitrite
PDGF	platelet-derived growth factor

PKC	protein kinase C
PLC	phospholipase C
PTCA	Percutaneous Transluminal Coronary Angioplasty
RAS	renin-angiotensin system
ROS	reactive oxygen species
SD	standard deviation
SEM	standard error mean
SHR	Spontaneously Hypertensive Rat
SOD	superoxide dismutase
TGF- β	transforming growth factor-β
VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cell
WKY	Wistar-Kyoto rat

SUMMARY

Consequences of pathological vascular remodeling include complete arterial occlusions resulting in an ischemic environment, or the development of vulnerable plaques which may rupture and lead to thrombosis. In this study, we investigated the role of Ang II in vascular remodeling. We sought to determine whether the humoral or the mechanical effects of Ang II are the dominant factor driving the remodeling process.

The following experimental groups were used in this study: control group (untreated mice), mice treated with an angiotensin receptor blocker (Candesartan, 0.5 mg/kg/day,SQ), an ACE inhibitor (Captopril, 6 mg/kg/day), and a calcium-channel blocker (Amlodipine, 7.5 mg/kg/day). All mice (n=6 per experimental group) were from the C57Bl/6 background. We implemented the carotid ligation model of vascular injury to study the differences in vascular remodeling. We used multiple time points (7-, 14-, and 21-days post-surgery) to track the progression of the remodeling process as assessed by comparative histomorphometry. At the 7-day time point, we observed that all three treatment groups yielded similar remodeling patterns as evidenced by a significant reduction in neointimal area, medial thickening and hypertrophy compared with the control group. Histomorphometric analysis of carotid sections collected 1mm below the ligation demonstrated that the Amlodipine group had 26% reduction in total vessel area, Candesartan a 36% reduction, and Captopril a 28% reduction ($p<0.05$ in all groups compared with Control), as well as a parallel 38-40% drop in medial thickness. In Day-14 analysis, we did not observe significant differences between the Controls and the treatment groups, although differences were emerging between the treatment groups. Candesartan was found to reduce the extent of negative remodeling observed between the 7- and 14-day Control data, whereas the Captopril group did not exhibit this trend. All three treatment groups exhibited less neointimal formation than Controls, similar to Day-7. By the 21-day time point, the Captopril group underwent positive

remodeling, resembling the Candesartan and Amlodipine groups. Although total vessel area was analogous among all groups, neointimal areas were significantly decreased in the treatment groups.

We report that blood pressure plays a pivotal role in the modulation of vascular remodeling in response to mechanical injury. Although intermediate timepoint analysis suggests that humoral aspects of ACE inhibition or angiotensin-receptor blockade yielded unique effects on the overall vessel caliber, upon reaching the late, 21-day time point, the mechanical factors became predominant. These data support the importance of blood pressure control in the attenuation of pathological vascular remodeling.

CHAPTER 1

INTRODUCTION

1.1 The Importance of Vascular Injury Research

Vascular injury is a ubiquitous term used to generalize the biological response to a broad range of abnormalities afflicting the vessel wall. The type of response is dependent on the short- and long-term nature of the injury, which is apparent based upon the resulting lesion morphology. Examples of the response to injury include neointimal formation, changes to vessel size and caliber, proliferation or apoptosis of cells comprising the vessel wall, and recruitment of inflammatory cells to the site of the lesion. Vascular injury plays a key role in the manifestation of an array of cardiovascular diseases, including hypertension, atherosclerosis, stroke, restenosis, and diabetic vascular complications. Of these, coronary heart disease is the most prevalent cause of death in the developed world, afflicting 35% of the United States and Western European population [1].

There is extensive evidence demonstrating the involvement of the renin-angiotensin system (RAS) in the progression of cardiovascular pathologies. ACE and angiotensin receptors expression are known to become upregulated in areas of vascular injury. Three clinical trials have been published evaluating the benefits of ACE inhibitor treatment in coronary artery disease (CAD), namely: Heart Outcomes Prevention Evaluation (HOPE), European Trial on Reduction of Cardiac Events with Perindopril in Stable Coronary Artery Disease (EUROPA), and Prevention of Events with Angiotensin Converting Enzyme Inhibition (PEACE) [2-4]. Each trial recruited patients that had preserved left ventricular function and risk for coronary or other vascular disease [5]. They used different, yet well studied ACE inhibitors: HOPE administered ramipril,

EUROPA administered perindopril, and PEACE administered trandolapril. The HOPE and EUROPA trials demonstrate a benefit of administration of ACE inhibitors on reducing atherosclerotic complications, whereas the PEACE trial outcomes showed ACE inhibitor therapy ineffective. The main difference in patient populations between these trials was that HOPE and EUROPA trials recruited patients that were at higher risk for adverse cardiovascular events than the stable, low-risk patients comprising the PEACE trial [6].

Atherosclerosis is a very complex, multifactorial disease affecting the arterial wall. Over the years, its origin has been the focus of intense scrutiny, and as a result, several hypotheses have been formulated and published. The results from these clinical trials were inconsistent, supporting the need for further investigation examining the role of Ang II in vascular injury. To date there are no animal models that fully represent the pathogenesis and morphology of human lesions. Because this type of lesion is so complex, several animal models have been developed to differentiate its various underlying biological processes. In this study, we will be utilizing an injury model that was developed to isolate the response of vascular remodeling, particularly neointimal formation.

1.2 Specific Aims

Although the majority of literature regarding vascular injury to date focuses on neointimal hyperplasia and smooth muscle cell proliferation, the emerging notion implicates arterial wall remodeling as being the primary contributor to the underlying mechanisms regulating restenosis [7, 8]. Several models have been developed to study the various aspects of vascular injury, such as medial hypertrophy and thickening, inflammatory cell recruitment, neointimal proliferation, and adventitial fibrosis [7]. In this project, we will employ the flow-cessation vascular injury model to define the role of Ang II in vascular remodeling. We hope to achieve this by evaluating the spatial and

temporal effects of ACE inhibition and angiotensin-receptor blockade via comparative histomorphometry. Our two specific aims are: (1) to characterize the flow-cessation vascular injury model (2) to evaluate the predominance of the humoral versus mechanical effects of Ang II in pathological vascular remodeling.

CHAPTER 2

DEFINING THE ROLE OF ANG II IN VASCULAR REMODELING

2.1 Background and Significance

2.1.1 Vascular Remodeling

Vascular remodeling is a complex, adaptive/maladaptive process by which vessels undergo structural alterations in response to biochemical and/or biomechanical stimuli. It is initially observed in embryonic development during the process of vasculogenesis, when the earliest signs of a developing heart and blood vessels occur, and continues throughout growth and maturation depending on long-term changes in their mechanical and hemodynamic environment [9, 10]. The process of vascular remodeling involves differences in the following cellular processes: cell growth, cell death, cell migration, and production or degradation of extracellular matrix [11].

Multiple factors influence the remodeling process, such as vasoactive substances, growth factors, and hemodynamic changes [12-15]. The endothelium plays a particularly prominent role in remodeling due to its direct exposure to humoral factors, inflammatory mediators, and physical forces [11]. Numerous studies have proven that blood pressure has a pivotal influence on the remodeling of arteries [12, 16, 17]. As supportive evidence for the influence of hemodynamic factors on vessel remodeling, local conditions following balloon angioplasty are characterized by low shear stress and increased wall stress, due to acute lumen area restoration. Constrictive remodeling has been observed as an adaptive mechanism to return to normalized shear stress values; in the case where vascular remodeling is lacking, neointimal growth is replaced [12].

2.1.2 Renin-Angiotensin System

The renin-angiotensin system (RAS) is a central regulator of blood pressure, which is also involved in water and salt balance, tissue growth and perfusion. The RAS is modulated by baroreceptors, whose main function is to detect changes in blood pressure. When the effective circulating volume decreases, followed by a decrease in blood pressure, baroreceptors are activated and send signals via the renal sympathetic nerve, resulting in the granular cells of the juxtaglomerular apparatus releasing renin, a protease that has specificity for angiotensinogen and catalyzes its conversion to angiotensin I (Ang I), a decapeptide. Ang I then transforms due to cleavage of two amino acids into Ang II, an octapeptide, via angiotensin-converting enzyme (ACE). Ang II is a potent vasoconstrictor that mediates its effects upon binding to its receptors, AT_1 and AT_2, although Ang II has greater affinity for the AT_1 receptor [18, 19].

Traditionally, the RAS was believed to have only a systemic influence due to its effects on blood pressure. Once renin is secreted from the kidneys, it is released into the bloodstream where it comes into contact with angiotensinogen produced in the liver, thus producing Ang I. Ang I is then cleaved to form Ang II by ACE predominantly expressed in the lungs. However, recent evidence points to a parallel, tissue-based RAS located in the vascular wall, particularly in areas of atherosclerotic lesions. This was validated by detection of the mRNA of the various components of RAS in macrophages and cell types composing the arterial wall [20]. ACE expression in the arterial wall is predominantly localized in the endothelium, although ACE expression is known to be upregulated in VSMCs upon conversion from contractile to synthetic phenotype [21, 22]. Specifically, Fukuda et al was one of the first groups to demonstrate that VSMCs incorporates all the components necessary to generate Ang II, based on homogeneous cultures of VSMCs derived from SHR [23].

However, the sole component not detected in the vessel wall, and thus not locally synthesized was renin [24], suggesting that the underlying mechanism behind local Ang II production involves the endothelium-mediated cellular uptake of renin from the systemic circulation [20]. Hence, the verification of RAS components in the vessel wall with the additional preconception of the pro-inflammatory effects of Ang II supports the constitutive influence of RAS in vascular remodeling.

2.1.3 Angiotensin-Converting Enzyme

ACE is a zinc carboxypeptidase that is anchored to the plasma membrane [25]. The primary function of ACE in the cardiovascular system is to cleave Ang I to form Ang II and to degrade bradykinin into inactive fragments. ACE is predominantly found in a tissue-bound state and expressed by a variety of other somatic tissues, including renal tubular epithelium, activated macrophages, ciliated gut epithelium, and areas within the central nervous system [26, 27]. Roughly ten percent resides in circulation since it is an ectoenzyme and can slough into the bloodstream, where it is considered plasma ACE [28]. In the vessel wall, ACE is principally expressed in the endothelium, where it produces Ang II adjacent to vascular smooth muscle cells comprising the media [28]. Monocytes and macrophages are also known to express ACE, and more notably, in areas of plaque formation, ACE expression is upregulated in these cell types [29].

In understanding the causal nature of blood pressure control and presence of RAS components, it is known that while renin levels vary in response to changes in blood pressure, ACE levels are far less variable [27]. The use of targeted homologous recombination in mouse embryonic stem (ES) cells has led to the development of a series of transgenic mice with tissue-selective expression of the ACE gene [26, 28, 30]. This was achieved by incorporating a neomycin resistance cassette to disrupt the somatic ACE promoter, followed by the insertion of a tissue-specific promoter to yield the desirable ACE specificity [28]. Therefore, by altering the expression levels of ACE,

insight has and will be gained into the true function and contribution of systemic RAS vs. tissue RAS in various conditions.

2.1.4 Ang II and downstream effects

Ang II, the effector molecule of RAS, is a vasoactive octapeptide (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-COO^-) which plays a role in the etiology of hypertension as well as the pathophysiology of cardiovascular and renal diseases [31, 32]. In these conditions, Ang II generates elevated blood pressure, vasoconstriction, and increased cardiac contractility [31]. Inhibition of ACE and blockade of the AT_1 receptor is a common therapy for treatment of hypertension and atherosclerosis, as current data suggests beneficial effects on endothelial function, vascular smooth muscle cells, and inflammatory vascular processes [33].

In addition to the mechanical effects of Ang II, there are also humoral effects on the cardiovascular system. Ang II contributes to oxidative stress in the vascular wall by activating the NAD(P)H oxidases [34]. The NAD(P)H oxidases are the predominant source of ROS in various cardiovascular pathologies [34-36], including hypertension, atherosclerosis, and post-angioplasty restenosis. Activation of the NAD(P)H oxidases leads to the production of superoxide anion (O_2^-), a short-lived free radical known to react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a strong oxidant. Alternatively, O_2^- can become dismutated by superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2). H_2O_2 is an uncharged molecule that has greater stability than superoxide or peroxynitrite, allowing it to diffuse to neighboring cells where it is involved in the cell signaling of numerous pathophysiological responses depending on the vascular cell type.

The downstream effects of Ang II include the induction of pro-inflammatory genes (ex. ICAM-1, VCAM-1, MCP-1) and activation of matrix-metalloproteases (MMPs). It also plays a role in inducing the phenotypic change of smooth muscle cells from a

contractile to a synthetic, proliferative phenotype [31, 37-41]. Another effect of Ang II is to increase mRNA expression and activity of plasminogen activator inhibitor (PAI-1), implicating its participation in thrombosis, based on in vitro studies using rat aortic smooth muscle cells [42].

2.1.5 Ang II and cell-cycle regulation of VSMCs

A survey of literature regarding the role of Ang II in cell cycle regulation implicates a complex and dual-nature function. Under physiological conditions, Ang II has been found to modulate cell cycle regulatory elements of VSMCs, particularly at the G_1 to S phase, thus confirming its role in cell growth and hypertrophy [43]. This role is dependent on the AT_1 receptor, based on in vitro studies of cultured VSMCs derived from spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) rats using AT_1 and AT_2 selective inhibitors. Signal transduction cascades that are activated upon binding of Ang II to AT_1 receptor include activation of PLC, subsequent generation of IP_3 and DAG, leading to activation of PKC, MAP kinases, and increased intracellular calcium [Ca^{2+}] [44]. This activation of PKC and MAP kinases leads to induction of c-fos and c-myc protooncogenes [45, 46]. Upon comparison of SHR and WKY vascular smooth muscle cells, the SHR cells were characterized by increased DNA synthesis, shorter cell cycle, and accelerated transition from G_1 to S phase compared to WKY cells, presumably due to increased endogenous Ang II production [43, 47-49]. The Ang II participation was validated after treatment with an AT_1R blocker yielded results equivalent to its normotensive counterpart. In addition, the selectivity of the AT_1R was supported as treatment with a selective AT_2R demonstrated no change [50]. An additional finding that supports the role of Ang II in proliferation is Ang II induces intracellular alkalinization correlated with stimulation of DNA synthesis and cellular growth, in addition to increased actin-myosin sensitivity to Ca^{2+} [51].

Although considerable evidence supports the role of Ang II in VSMC hypertrophy, similar studies fail to support its role in VSMC proliferation [52, 53]. Ang II was not found to exert any effects on the G_2 to M phase under physiologic conditions, thereby discounting its mitogenic influence [43]. However, reports have shown that at sites of arterial injury, Ang II does play a mitogenic role on VSMCs [38, 50, 54]. This influence is indirect, as it is thought to be dependent upon the secretion of autocrine growth factors, such as PDGF, bFGF, and TGF-β1 [47, 54]. The mechanisms underlying Ang II-induced VSMC proliferation has not yet been completely defined due to limited data focused on cell cycle regulation.

2.1.6 Angiotensin II receptors

There are four subtypes of Ang II receptors, namely AT_1R (type 1A and 1B), AT_2R (type 2), AT_3R, and AT_4R. The AT_1 receptor is ubiquitously expressed and all the classically-known, proatherogenic effects of Ang II are attributed to this receptor [55, 56]. These downstream effects (anti-apoptotic for SMCs, neointimal hyperplasia, etc.) occur upon the binding of Ang II to its AT_1 receptor. The AT_1 receptor was first cloned by two separate groups in 1991, which has led to further research in the molecular structure and function of this receptor, as well as the development of angiotensin-receptor blockers [57, 58]. Under physiologic conditions, Ang II has much higher specificity for the AT_1 receptor [19]. Both AT_1 and AT_2 receptors are members of the seven transmembrane-spanning, G-protein coupled receptor family [31, 55, 59].

The AT_2 receptor is not well understood for multiple reasons. Firstly, the AT_2 receptor distribution is not homogeneous amongst somatic tissues of the adult animal. Expression is dependent on age, species, vessel type, and pathophysiological state [55, 56, 60, 61]. For example, at sites of vascular injury and wound healing, AT_2 receptor expression occurs most likely as a compensatory mechanism and interestingly sustains levels similar to the total expression level of AT_1 receptor [56, 62]. In this setting, the

main cell types expressing the AT_2 receptor are macrophages and SMCs. The AT_2 receptor response has been observed to be antagonistic to the AT_1 receptor (e.g. anti-proliferative, pro-apoptotic for SMCs, anti-fibrotic, vasodilation, etc.) [56, 62]. Stimulation of the AT_2 receptor has been shown to activate the bradykinin B_2 receptor, resulting in NO synthesis and cGMP upregulation in rabbits and spontaneously hypertensive rats (SHR) [63, 64]. Studies using transgenic mice overexpressing the AT_2 receptor have high cGMP content, but the effects are normalized with NO-synthesis or bradykinin B_2 receptor blockade [55, 65]. Additionally, some studies using the $AT_2R^{-/-}$ mice have shown that AT_2 receptor activation results in decreased ACE [55, 66].

2.1.7 Animal Models

The main animal models used in the study of vascular injury are nonhuman primate, porcine, rabbit, and rodent. Of these, the nonhuman primate and porcine are the most relevant because their lesion distribution, pathogenesis, and morphology mimic that of humans most accurately [7]. However, due to large size, high-cost and difficulty in procurement and handling, their usage is quite limited. The rabbit model is another commonly investigated species in cardiovascular research. The rabbit develops spontaneous and diet-induced atherosclerosis. However, comparative lesion analysis demonstrates a morphology characterized by medial degeneration instead of robust intima-centered lesion development [67, 68]. The development of new strains along with dietary manipulations has circumvented these limitations, although their usage is dependent on the practicality of procurement and cost factors [7, 68].

The major disadvantage of the rodent model is that vessel targeting for reproducible injury is challenging due to size limitations. In addition, there are divergent cellular mechanisms and pathogenesis underlying rat versus human lesions [69]. Although the rodent model does not yield lesions that are most analogous to humans, various injury models to mimic differential aspects of vascular injury have been

implemented to gain further insight into the pathogenesis, morphology, and optimal pharmacologic treatment for lesion development. The most popular and well-characterized rodent model is the balloon injury model, developed by Carmeliet's research group [70]. This model has been extensively used in pre-clinical trials for evaluating the efficacy of ACE-Is and ARBs in vascular injury. In recent years, the mouse model has become particularly favorable because, in addition to their being cost-effective and easily available, advances in gene-manipulating techniques allow for the use of transgenic or knock-out mice to examine the effects of a single gene with the goal of elucidating its function in vascular biology.

2.1.8 Vascular Injury Models

The four major modes of vascular injury in the murine setting are: wire-injury endovascular injury, flow-cessation vascular injury, cuff-mediated perivascular injury, and electric perivascular injury. The size of the mouse vasculature is the main drawback to employing the balloon angioplasty compared to larger animal models. The wire-injury endovascular injury was developed by Lindner's group, involving the insertion of an inert wire into either the carotid or femoral artery resulting in complete endothelial denudation and SMC apoptosis, thereby emulating the effects of PTCA [71]. The major setbacks to this model are surgical variability in yielding a reproducible injury, as well as the lack of a site marker to localize the site of injury for comparative analysis.

The cuff-mediated perivascular injury is unique in that a polyethylene tube is positioned around the femoral artery, thereby mimicking stenosis. This model is particularly useful for studying neointimal proliferation, particularly monocyte and macrophage infiltration [72]. However, it is not ideal for studying vascular remodeling because the polyethylene tube exerts constraints on the overall vessel size, resulting in the inability to evaluate positive or negative remodeling.

The electric perivascular injury model of studying arterial injury involves a single delivery of electric current on the surface of a surgically exposed femoral artery. The induction of this injury model is less biologically relevant, but the wound-healing response is documented to resemble the process of restenosis observed in human arteries. The focal area of injury experienced complete endothelial denudation, ablation of encompassing smooth muscle cells, and transiently induced platelet-rich mural thrombosis followed by infiltration by inflammatory cells [73].

The flow-cessation vascular injury model, our chosen model in this study, involves the ligation of the left common carotid artery distal to the aortic arch, thus inducing permanent changes in shear stress conditions [74]. Ligation provides a marker for analysis, thereby minimizing variability as a result from analysis. This model is favorable for studying vessel remodeling because it does not incorporate any constraints to the overall vessel size. After thorough characterization of the carotid ligation model, the vascular response was found to include smooth muscle cell proliferation, inward remodeling, and neointimal formation, all features of pathological remodeling [74]. Also, the advantage of using the carotid artery in this model is that it eliminates the possibility of remodeling as a result of hypoxia, as it has been shown that blood flow through the brain is conserved via the circle of Willis and flow-mediated vasodilation of the contralateral artery [75]. There are fewer cases of thrombosis in this model compared to the wire endovascular injury or electric perivascular injury because the endothelium is largely intact, thereby functioning as an anti-thrombotic layer protecting the vessel wall. This setting is suitable for studies focused on substances secreted from or targeted to the endothelium [76]. The major disadvantage to this model, is that it results in a complete cessation of blood flow proximal to the ligature and near-stasis conditions distally, which is not necessarily biologically relevant.

2.1.9 ACE Inhibitors and ARBs in Vascular Injury

The seminal paper addressing the prevention of neointimal formation via ACE inhibition employed the rat balloon-injury model [77]. Numerous studies have since been published citing the benefits and vasoprotective effects of ACE inhibition and angiotensin receptor blockade. In support of ACE inhibitors, data demonstrates that reduced neointimal proliferation is caused by decreased bradykinin breakdown, which upon binding to BK B_2 receptors [78, 79] exerts potent vasodilative effects, as well as enhance NO bioavailability through endothelial NO synthase (eNOS) [80, 81]. ACE inhibition causes decreased Ang II generation, leading to an attenuation of cell proliferation [82], expression of pro-inflammatory molecules (ex. MCP-1), and oxidative stress [83, 84].

AT1 receptor blockade, on the other hand, has no effect on ACE activity. In addition to preventing the vasoconstricting, pro-inflammatory, hypertrophic, and hyperplastic effects of Ang II via the AT_1R, its benefits are derived by stimulation of the AT_2 receptor, which has been cited to exert antagonistic effects [56, 85, 86], thus promoting a vasoprotective environment. AT_2R signaling pathways have been found to influence cGMP/NO [64] and BK [64, 65].

2.2 Materials and Methods

2.2.1 Animals

Male mice were used from the C57Bl/6J background (Jackson laboratories, Bar Harbor, Minn). An n=6 was used per experimental group. All mice were 10-12 weeks of age and 25-30g. They were administered standard chow diet (Research Diets) and water ad libitum, and were housed in climate-controlled, pathogen-free conditions with 12/12 hours of light/dark cycle. Mice were used in accordance with the guidelines of the National Institute of Health for the care and use of laboratory animals. All experimental

procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University.

2.2.2 Blood Pressures

Systolic blood pressures were measured using tail-cuff plethysmography (Visitech Systems, USA). All animals were allowed one week to acclimatize to the process by taking daily non-recorded measurements in the effort to avoid anxiety-induced aberrations in blood pressure. After the initial week, blood pressures were recorded from each mouse before the induction of vascular injury. Blood pressures were then recorded post-operatively every 7 days upon approaching the appropriate timepoint.

2.2.3 Flow-cessation vascular injury model

Surgery was performed aseptically as previously described [74, 87]. Mice were anesthetized using ketamine HCl (80 mg/kg, Abbott Laboratories) and xylazine (5 mg/kg, Bayer Corporation) by intraperitoneal injection. Hair was removed from the neck area, followed by a small midline incision of the neck. The left carotid artery was exposed and ligated approximately 1mm below the bifurcation using a 6-0 silk suture (Ethicon). The wound was closed using 4-0 silk sutures. The Captopril and Candesartan treated mice were then implanted with osmotic minipumps (Alzet, Alza Co., Palo Alto, CA, USA) to deliver 6 mg/kg/day of Captopril, and 0.5 mg/kg/day of Candesartan, respectively. Mice were then allowed time for recovery on a warming blanket at 37ºC; then returned to their cages. The mice treated with Amlodipine were administered 7.5 mg/kg/day through diet. The efficacy of the treatments was monitored by measuring systolic blood pressures. Sham mice used in this study did not undergo ligation of the left common carotid artery.

2.2.4 Morphometry

Mice were euthanized at the 7-, 14-, and 21-day timepoints using carbon dioxide asphyxiation, and were pressure perfused at approximately 100 mmHg through the left

ventricle using 0.9% saline for 10 minutes and fixed in 4% paraformaldehyde for an additional 10 minutes. The common carotid arteries were then excised, immersed in 4% paraformaldehyde for 24 hours, tissue processed, and embedded in paraffin wax for morphological evaluation. Ten segments were collected along the length of the carotid with 150-micron spacing between segments, starting proximal to the ligature and proceeding down to the aortic arch. Each segment constitutes ten serial sections of 8 micron thickness, yielding a composite analysis of approximately 2mm in length, as seen in Figure 2-1. Cross sections were stained using hematoxylin and eosin to observe the general morphology of the arteries. Verhoeff's van Gieson staining was used to observe elastin composition of the arteries. Picro-sirius red staining was used to identify collagen composition and orientation in carotid sections, as previously described [88].

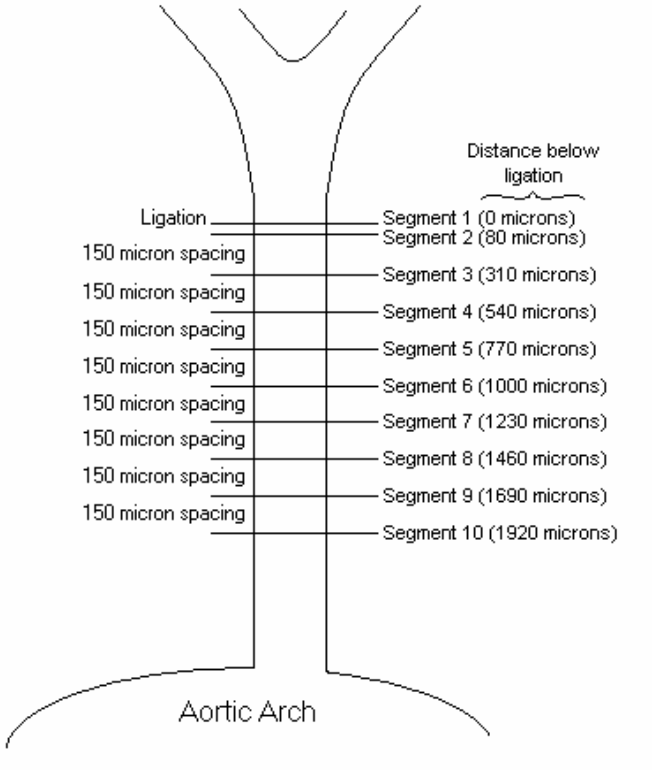

Figure 2-1: Method of Histomorphometric Analysis. Following perfusion, left common carotid arteries were excised at 0-, 7-, 14-, and 21-days post-surgery between the aortic arch and the carotid bifurcation. Arterial cross-sections are collected along 2mm length of the carotid artery, with the ligature as the initial marker for analysis.

Images were obtained using a Zeiss Axioscope equipped with a standard CCD camera (Nikon). Luminal, IEL, and EEL areas were measured by tracing along the surface using ImageJ software. Neointimal areas were calculated by taking the difference between the lumen and IEL areas. Medial areas were calculated by taking the difference between the EEL and IEL areas. Total vessel area is represented by the EEL area.

2.2.5 Analysis

All results are reported as mean ± SEM unless stated otherwise. Statistical analyses were performed using Microsoft Excel (Redmond, WA) and Graphpad Prism (Graphpad Software, Inc., San Diego, CA). Differences between groups were analyzed by two-way ANOVA, followed by Bonferroni's post hoc test. A probability (*P*) value less than 0.05 was considered significant.

2.3 Results

2.3.1 Blood Pressure

After one week of training the mice, pre-surgery systolic blood pressures were measured, and as expected, all basal blood pressures were similar. After blood pressures were recorded, mice underwent ligation of their left common carotid artery, as described in the Materials and Methods section. At the time of the surgery, Candesartan and Captopril osmotic pumps were implanted in select mice in accordance with their treatment category. Mice in the Amlodipine category were administered treatment through diet. The doses of Amlodipine (7.5 mg/kg/day), Candesartan (0.5 mg/kg/day), and Captopril (6 mg/kg/day) were selected to yield equivalent differences in blood pressure compared with the Control group, as seen in Table 1. Results are displayed as mean ± SD.

Table 2-1: Blood Pressures

	Pre-surgery	7-days post-surgery	14-days post-surgery	21-days post-surgery
Control	104.2 ± 3.1 (n=25)	103.3 ± 3.5 (n=25)	104.6 ± 3.85 (n=16)	104.0 ± 4.6 (n=6)
Amlodipine	102.7 ± 3.5 (n=24)	85.3 ± 2.7* (n=24)	84.2 ± 3.4* (n=12)	84.1 ± 1.8* (n=6)
Candesartan	102.1 ± 3.2 (n=20)	85.1 ± 2.5* (n=19)	84.8 ± 2.4* (n=13)	84.8 ± 3.4* (n=5)
Captopril	102.5 ± 3.2 (n=18)	84.3 ± 2.7* (n=18)	85.2 ± 1.6* (n=12)	85.4 ± 2.7* (n=6)

* $P < 0.0001$ vs. Control Pre-surgery

The Control group maintained their systolic blood pressures after surgery. Also, all three treatment groups had an equivalent drop in blood pressures, seen in all mice 7 days after surgery, at the later timepoints, similar reduction in blood pressure were observed. The approximate 15 mmHg drop in blood pressure was found to be statistically significant in all treatment groups compared with the Control group.

2.3.2 Characterization of Flow-Cessation model

Disruption of carotid artery blood flow resulted in early-on outward remodeling (1160.3 ± 24.9 μm^2 compared to 1107.5 ± 11.2 μm^2 in sham mice), followed by significant negative remodeling (949.8 ± 11.3 μm^2) presumably due to permanent changes in shear stress, followed by compensatory vessel enlargement (1092 ± 20.3 μm^2), as seen in Figure 2-2.

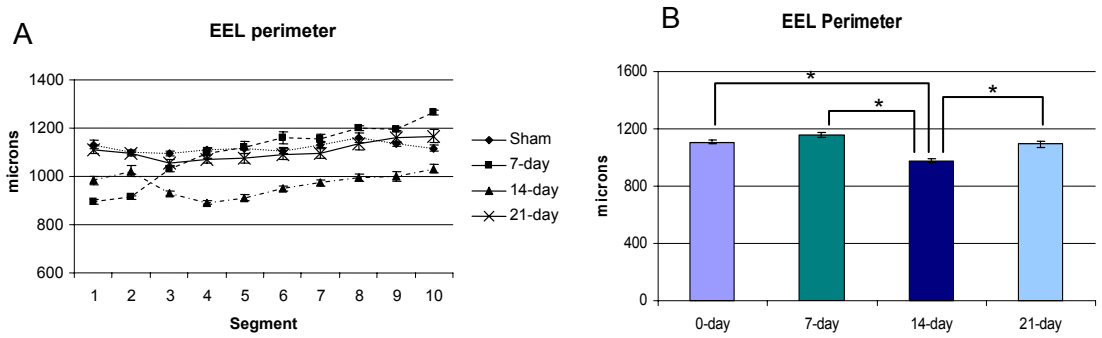

*Figure 2-2: Comparison of EEL perimeters of left carotid artery at 0-, 7-, 14-, and 21-days post-surgery. Panel A: Morphometric analysis of the circumference of blood vessels harvested at 0-, 7-, 14-, and 21-days post-surgery. Cross sectional perimeters of external elastic laminae were measured. Panel B: Temporal analysis of external elastic lamina perimeter of control mice upon ligation of left common carotid artery. Values are obtained from cross-sections collected 1mm (Segment 6) below the ligation. *P < 0.05 versus sham values; all values are depicted as mean ± SEM.*

Figure 2-3 shows a spatial analysis of the typical remodeling response to cessation of blood flow over a time-course span of 7-, 14-, and 21-days post-surgery. The data shown are from 2mm below the left common carotid artery, starting at the ligature (Segment 1) and traversing towards the aortic arch (Segment 10). Outward remodeling is evident in the 7-day analysis due to significant concomitant increase in lumen and total vessel areas. Medial thickening was maximal at the early, 7-day timepoint (as previously observed in [87]), and was observed to a smaller degree in later timepoints following ligation of the carotid artery.

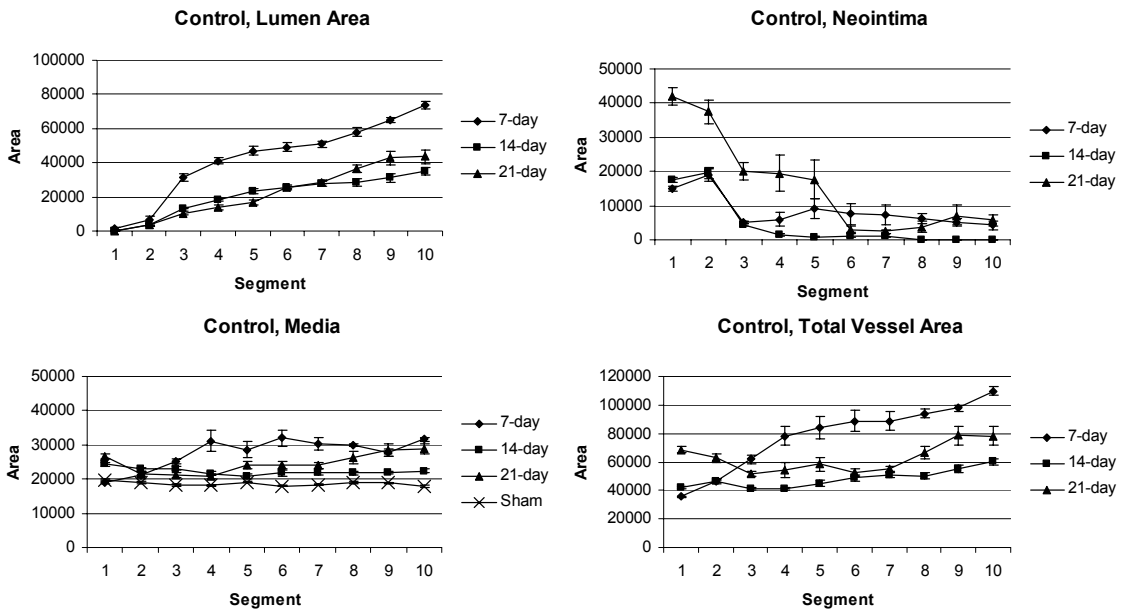

Figure 2-3: Spatial analysis of ligated carotid arteries at 7-, 14-, and 21-days post-surgery. Composite analysis spans 2mm in length down the carotid towards the aortic arch, beginning at the ligation. Cross sectional areas of lumen, neointima, media, and total vessel areas were measured.

Neointimal formation was greatest proximal to the ligature at the 21-day timepoint, where collagen deposition by smooth muscle cells comprising the subendothelial layer is evident, as visualized in Figure 2-4.

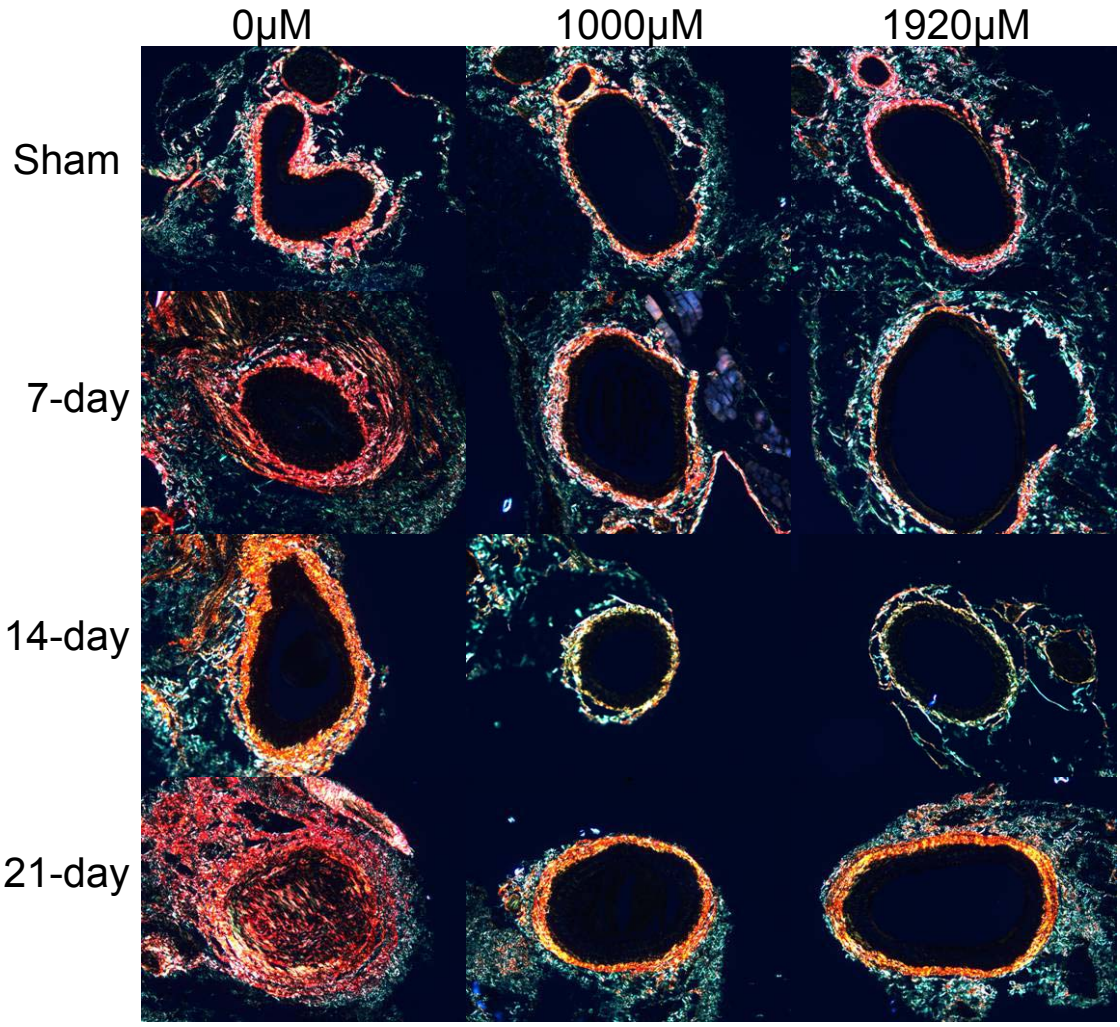

Figure 2-4: Collagen content is greatest proximal to the ligature in the 21-day timepoint. Representative Picro-Sirius stained left common carotid artery sections (8μm thickness) collected from Segments 1, 6, and 10 of sham, 7-day, 14-day, and 21-day experimental groups. Images were viewed under cross-polarized light to view birefringent collagen. In this setting, larger collagen fibers appear bright orange or red, whereas thin-filament collagen fibers appear bright green. Picrosirius staining primarily stains Types 1, 2, and 3 collagen.

2.3.3 Day 7 Analysis

The Captopril, Candesartan, and Amlodipine groups were associated with a significant reduction in medial thickening and neointimal formation upon ligation of their left common carotid arteries compared with the Control group, as seen in Figure 2-5A and 2-5B. In all groups, neointimal formation was greatest proximal to the ligature, and was unvarying from segments 6-10 (1-2mm below the ligation). The difference in neointimal areas among the treated groups and the Control group was found to be statistically significant in cross-sections collected within 100μm to the ligature ($P < 0.001$). Also, medial areas were consistently smaller proximal to the ligature and fairly consistent among the distally collected sections. Medial areas for the Control, $30\pm2\times10^3$ μm^2 at 1mm below the ligation, were roughly 35% greater compared with all three treatment groups, $19\pm1\times10^3$ μm^2 ($P < 0.001$). In addition to the media, total vessel area was also significantly greater in the Control than among the treatment groups. Based on the observation that the Amlodipine, Captopril, and Candesartan data were fairly analogous, we believe that the humoral aspects of ACE inhibition or angiotensin receptor blockade do not play a significant role in these aspects of vascular remodeling at the early 7-day timepoint. Instead, the pressor effect among all treatment groups appears to be the more dominant factor at this stage of remodeling.

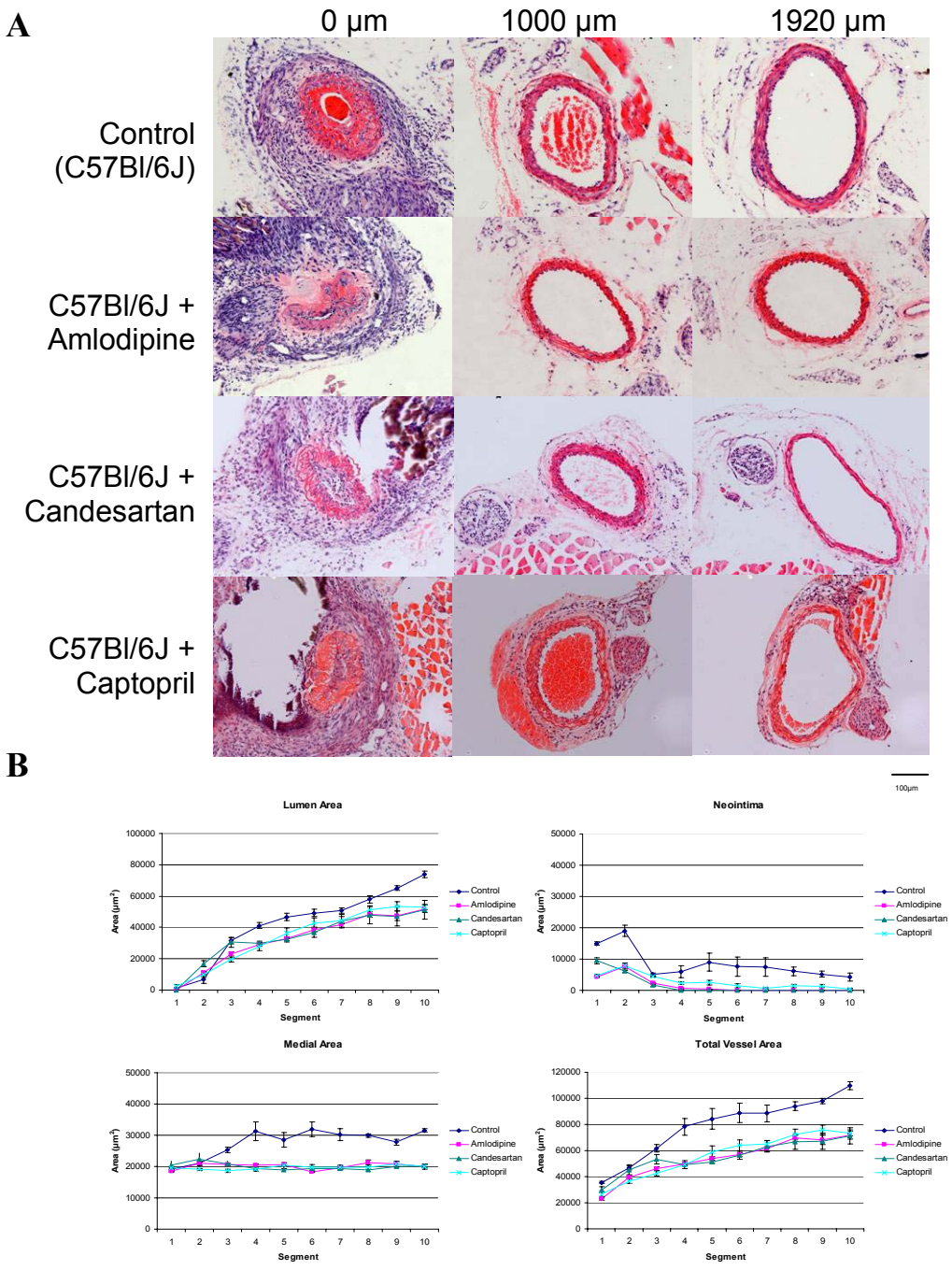

Figure 2-5: 7-Day Morphometric Analysis. Panel A: Representative cross sections of the ligated left common carotid arteries in the Control, Amlodipine, Candesartan, and Captopril groups at varying distances from area of ligation. Ligated arteries were excised after pressure perfusion fixation at 100mm Hg, immersed in 4% paraformaldehyde for an additional 24 hours, then dehydrated and embedded in paraffin. Sections of 8µm were stained with hematoxylin and eosin. Magnification ×20; Scale bar = 100µm. Panel B: Morphometric analysis of ligated carotid arteries. Cross sectional areas of lumen, neointima, media, and EEL were measured.

2.3.4 Day 14 Analysis

Whereas the earlier, 7-day timepoint was the response to acute flow-cessation where blood pressure treatment led to an attenuation of neointimal proliferation and medial thickening, the 14-day timepoint data did not exhibit significant differences between the Control and treatment groups. Candesartan treatment appeared to attenuate the degree of negative remodeling observed in the Control group between the 7- and 14-day timepoints, as seen in the lumen and total vessel areas in Figure 2-6. However, this difference was found to be insignificant ($P > 0.05$). In contrast, the Captopril also did not result in any significant differences in remodeling, although the attenuation was not observed in this timepoint. The only groups exhibiting difference were the lumen and total vessel areas among the Candesartan and Captopril groups (Segments 3-10, $P < 0.05$). The 14-day medial areas among all groups were found to be unvarying. The Control and Amlodipine groups were equivalent in terms of lumen (25646 ± 1482 μm vs. 28760 ± 2143 μm) and EEL areas (48615 ± 1871 μm vs. 46102 ± 2156 μm).

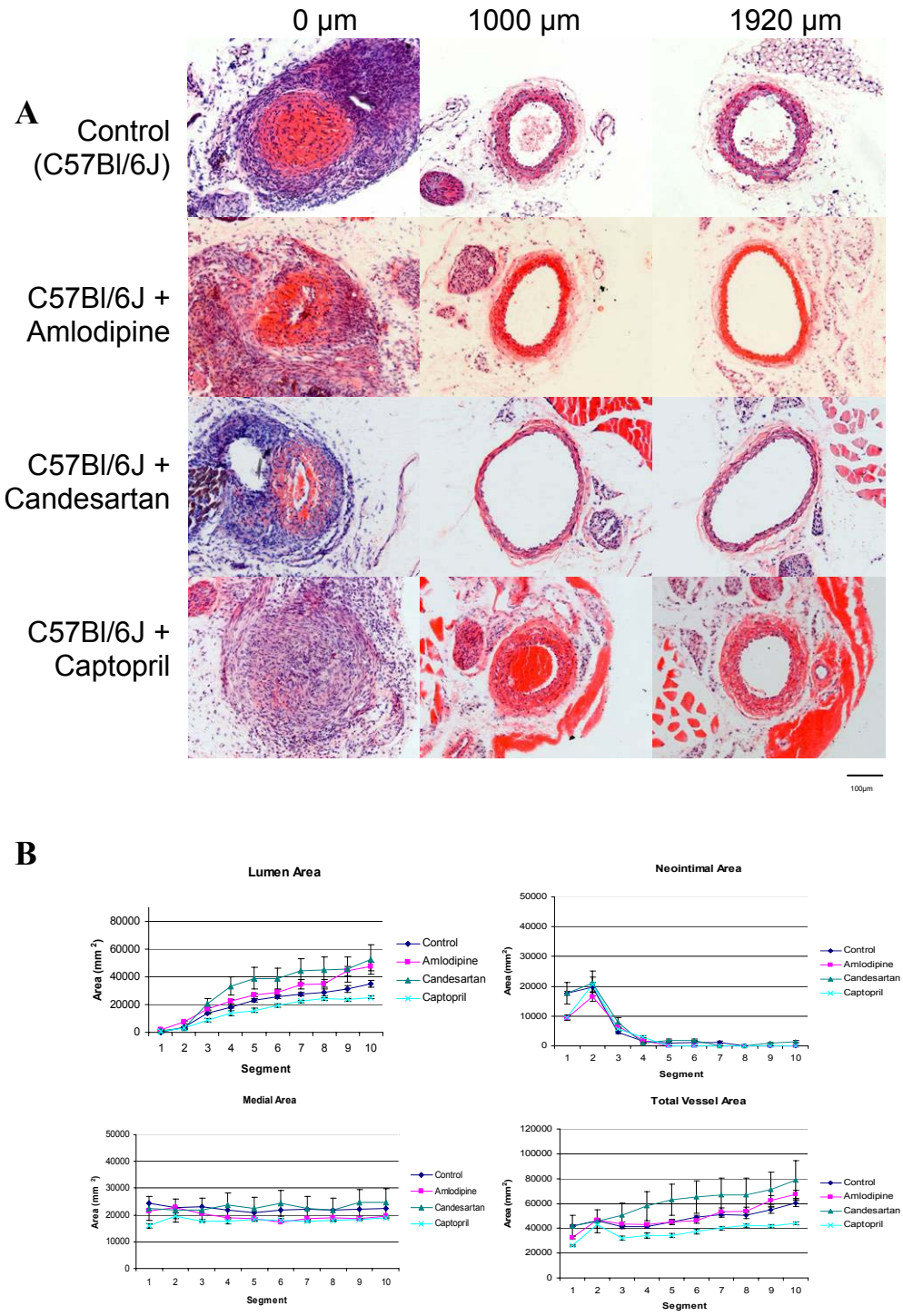

Figure 2-6: 14-Day Morphometric Analysis. Panel A: Representative cross sections of the ligated left common carotid arteries in the Control, Amlodipine, Candesartan, and Captopril groups at varying distances from area of ligation. Magnification ×20; Scale bar = 100µm. Panel B: Morphometric analysis of ligated carotid arteries. Cross sectional areas of lumen, neointima, media, and EEL were measured.

2.3.5 Day 21 Analysis

In this late-stage timepoint, we observed that the neointimal growth was most profound in the Control group. The Control group exhibited consistent values for medial and total vessel areas, yet had smaller lumen areas than the treatment groups, due to the increase in neointimal proliferation. This dramatic change in neointima from the 7- and 14-day to the steady-state timepoint has been previously published by Ivan and colleagues [89]. Also, an interesting new finding was made, which was the pronounced difference in total vessel areas between the 14-day and 21-day Captopril data (36213 ± 1833μm vs. 65770 ± 3406μm). By the 21-day timepoint, the Captopril underwent significant outward remodeling, thereby mimicking vessel characteristics of the Candesartan (59756 ± 3084μm) and Amlodipine (57077 ± 4361μm) groups, as seen in Figure 2-7.

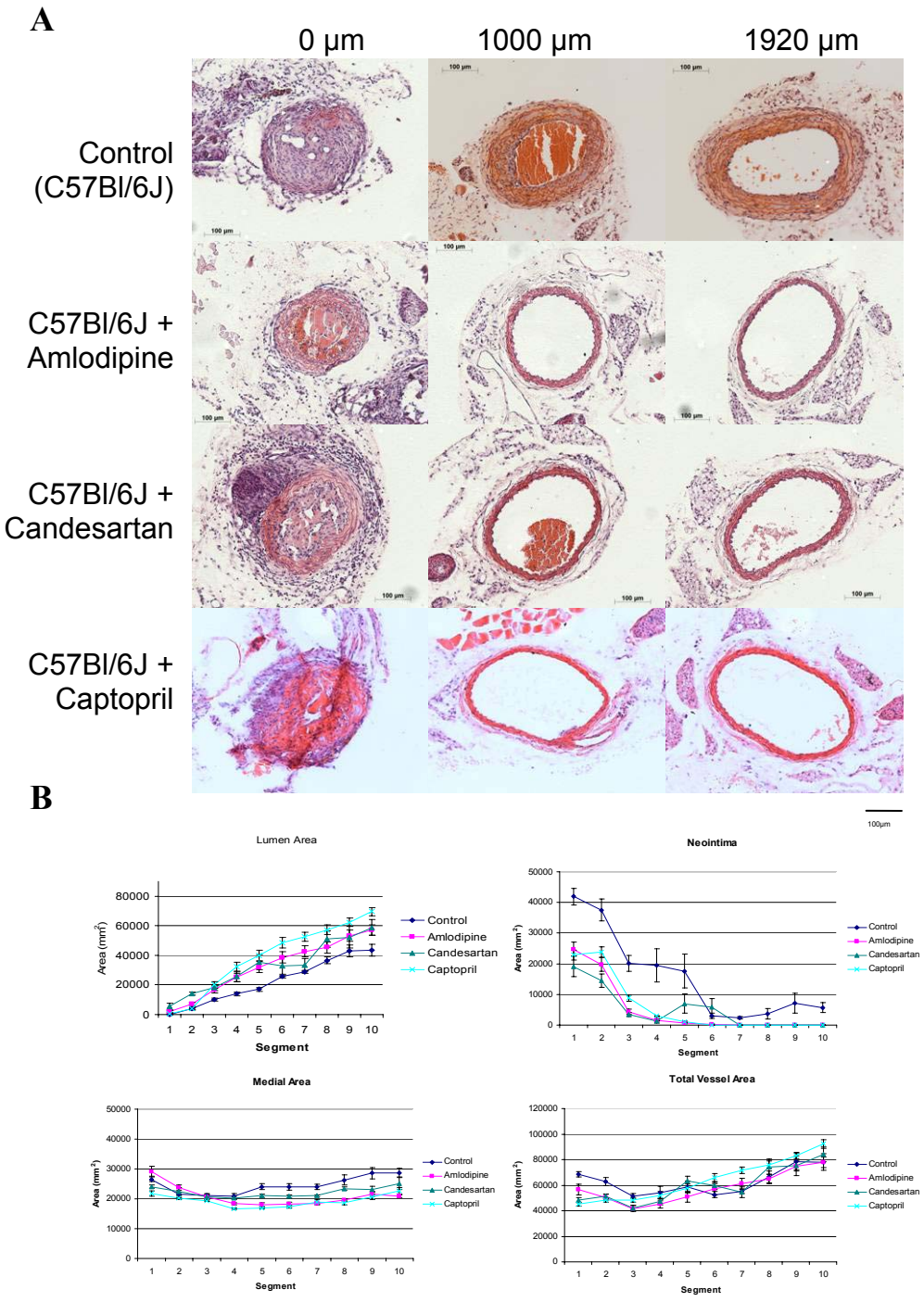

Figure 2-7: 21-Day Morphometric Analysis. Panel A: Representative cross sections of the ligated left common carotid arteries in the Control, Amlodipine, Candesartan, and Captopril groups at varying distances from area of ligation. Magnification ×20; Scale bar = 100µm. Panel B: Morphometric analysis of ligated carotid arteries. Cross sectional areas of lumen, neointima, media, and EEL were measured.

2.3.6 Time Point Analysis

Upon consolidating the 7-, 14-, and 21-day morphometric analyses, we see the characteristic remodeling patterns of each experimental group. As stated earlier during the characterization of the injury model, flow cessation induces an increase in medial thickening, progressive increase in neointimal formation, and reduction in vessel size in the Control group. Treatment with Candesartan, Captopril, and Amlodipine yielded similar remodeling patterns, including significant reduction in neointimal formation and attenuation of medial thickening and constrictive remodeling upon reaching the late, 21-day timepoint. Here, we observe the predominant effect of blood pressure regulation on vascular remodeling, particularly among the 7- and 21-day timepoints.

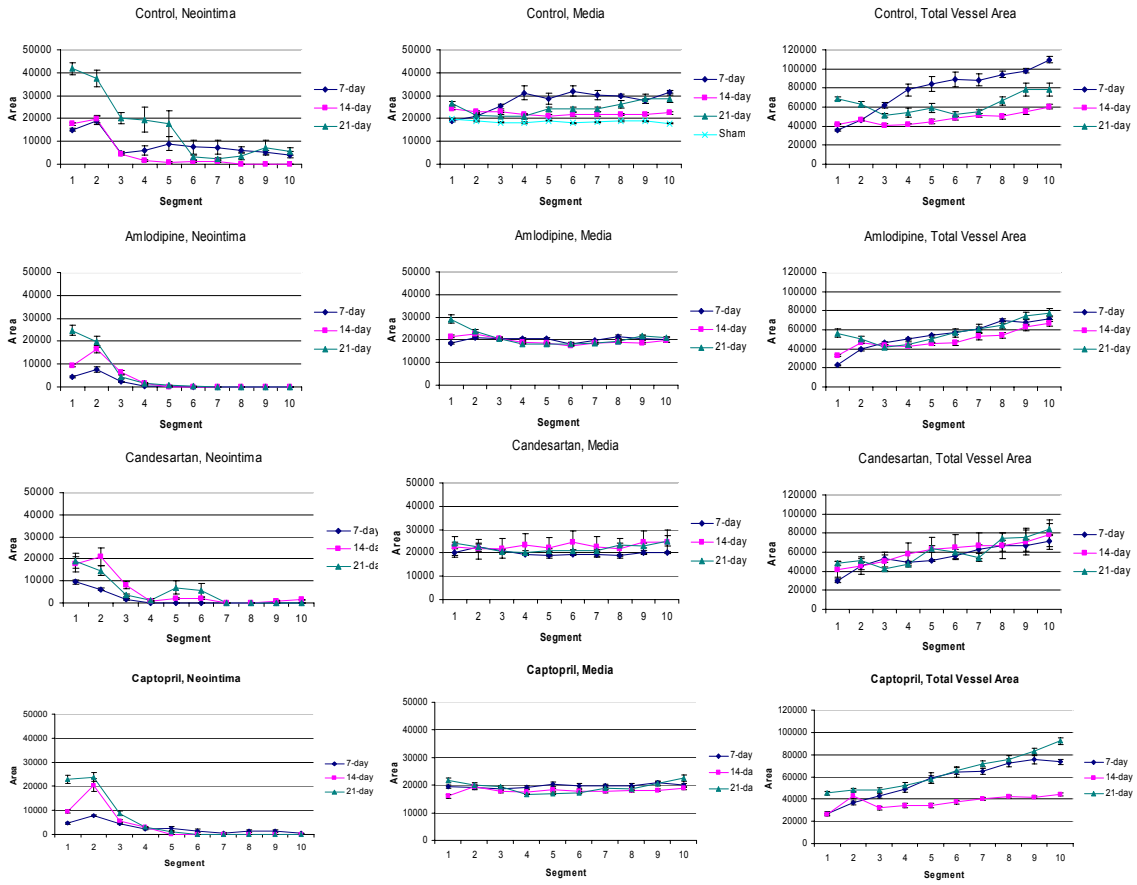

Figure 2-8: Time Point Analysis of Morphometry between Control, Amlodipine, Candesartan, and Captopril groups at 7-, 14-, and 21-day timepoints. Cross sectional areas of neointima, media, and EEL are depicted as a function of time.

2.4 Discussion

Pathological remodeling is one of the primary events that occur in the progression of hypertension and atherosclerosis. Several small animal models have been utilized in order to gain insight into the multitude of factors influencing vascular remodeling [74, 90, 91]. In this study, we sought to determine the predominant role of Ang II in carotid artery remodeling in an acute injury setting, be it humoral or mechanical, and whether this effect is consistently observed over the time course of early- to late-stage remodeling.

Our data strongly supports the preeminent influence of mechanical factors induced by Ang II in long-term vessel remodeling based in a vascular injury setting. Hemodynamic factors, such as blood pressure and wall shear stress have long been known to play a role in adaptive remodeling [82, 92, 93]. The early (7-day) and late-stage (21-day) timepoints both reinforce this belief upon observation of significantly decreased neointimal formation, medial thickening, and preservation of lumen area among all mice that underwent treatment involving lowered blood pressure, despite differences in mode of action (ACE inhibition, angiotensin receptor blockade, or calcium-channel blockade). Previous researchers have demonstrated that transmural pressure stimulates vascular RAS, particularly via endogenous production of Ang II [92, 94]. Therefore, by modulating transmural pressure using ACE inhibitors or calcium-channel blockers, or blocking the effects by AT_1 receptor antagonism, similar outcomes ensue. Because the method of blood pressure regulation appears trivial, it is probable that Ang II does not under these experimental conditions play a significant role in the remodeling process.

In contrast to the 7- and 21-day timepoints, the 14-day timepoint demonstrates insignificant differences compared to the treatment groups. Additionally, characteristic differences emerged between the effects of ACE inhibition and angiotensin receptor

blockade on the structural adaptation of injured vessels among these two experimental groups. Upon comparison of the 7- and 14-day Control results, we observe a transition in the adaptive vessel response to near-stasis conditions, including negative remodeling and neointimal formation. This emphasizes the existence of a hierarchy of factors influencing the response to injury. Because the proliferative index of smooth muscle cells is maximal between the 7- and 14-day timepoints [87], focus is shifted to the influence of Captopril versus Candesartan. In this timepoint, we observe what may be interpreted as the humoral effects of Candesartan and Captopril treatments. Captopril treated mice have been known to manifest low circulating Ang II levels, as well as an increase in circulating BK half-life, due to inhibition of ACE. On the other hand, the Candesartan mice exhibit extraordinarily high levels of circulating Ang II levels due to AT_1R blockade, as well as normal ACE activity. Additionally, AT_2R expression has been upregulated due to the inflammatory setting induced by the ligation of the left common carotid arteries [56, 95, 96]. Studies involving the $AT_2R^{-/-}$ knockout mice have expanded our knowledge on the antagonistic effects this receptor exhibits alongside the AT_1R. $AT_2R^{-/-}$ mice show hypersensitivity to Ang II [97] and have two-fold increase in neointimal formation than $AT_2R^{+/+}$ mice [98], thus emphasizing its vasoprotective, anti-proliferative influence on the vascular wall. Furthermore, Ang II binding to AT_2R is attributed to vasodilation due to increased production of NO [64, 99].

We assumed that Amlodipine was used as a low blood pressure control because its effects are largely pressor-related. Recent lines of evidence point to a vasoprotective role of Amlodipine through its antioxidant effects [100]. The mechanisms of action are different, however, in the attenuation of oxidative stress. Amlodipine, belonging to the dihydropyridine class of Ca^{2+} channel blockers, are found to preserve SOD activity, whereas ACE-Is and ARBs are known to limit Ang II-induced NAD(P)H oxidase stimulation [101, 102]. Therefore, although the calcium-channel blocker treatment was

used as a low blood pressure control, additional antioxidant effects may influence the remodeling process as a result of Amlodipine treatment.

The rapid outward remodeling response observed in Captopril mice between the 14- and 21-day timepoints delineates a particularly interesting observation. Previous studies investigating the effects of ACE inhibition have demonstrated increases in BK leading to activation of the BK B_2 receptor [79, 99, 103], thereby promoting vasodilation. Binding of BK to its B_2 receptor in the endothelium results in an increase in intracellular calcium, leading to eNOS upregulation and subsequent enhanced NO production [78, 80, 103-105].

Based on these findings, we postulate that these phenotypic differences involving differences in AT_2R expression and bradykinin production promote the differences in structural adaptation. The simplest explanation for this unique set of data is that the14-day timepoint is rather an intermediate phase where a “homeostasis” has yet to occur between the various processes of cellular growth, death, migration, and production and degradation of the extracellular matrix. It may be a timepoint where the kinetics underlying the different cellular processes are transitional, thereby allowing the humoral influences of Ang II to become more evident.

The beneficial effects of ACE inhibition and angiotensin receptor blockade on neointimal formation have been reported in several studies involving the balloon angioplasty model of vascular injury in rats [37, 44, 77, 106-110], presumably by inhibiting the mitogenic, hypertrophic, and proinflammatory effects of Ang II. The results of these studies strongly support the humoral aspects of ACE inhibition and angiotensin receptor blockade. Blood pressure control in this setting was demonstrated to only play a minimal role in the attenuation of neointimal proliferation [109]. Because these results are contradictory to the results of our current study, it is important to note the differences in injury settings between these two models. Firstly, with balloon angioplasty, the

resulting arterial injury involves complete endothelial denudation, as well as compression injury to the media [111]. Consequentially, elastic recoil is observed, followed by smooth muscle cell migration and proliferation. There are greater platelet interactions and inflammatory response within the vessel wall observed as a consequence to angioplasty. As a result, the balloon angioplasty injury appears better suited as a model for studying neointimal formation rather than vascular remodeling.

In contrast, the flow-cessation model of vascular injury does not cause endothelial denudation except for the immediate area of ligation. Because the endothelium is largely intact in our model, it allows us to more accurately observe the function of vasoactive molecules secreted from or pharmacologic treatments targeted to the endothelium. It is important to note that although this current study has yielded conclusive results, it was carried out in a very controlled setting. Ligation of the carotid artery does not lead to an ischemic environment due to compensatory flow via the contralateral artery, thereby preventing tonic increases in hypoxia-induced factors. Also, because this study was carried out in a murine setting, alternative Ang II generating pathways are relatively minimal, compared to larger animal models or humans [112].

A survey of literature demonstrates clear differences in lesion development between animal models, presumably due to divergent cellular mechanisms underlying the remodeling process. Even within the murine model, strain differences are known to cause considerable differences in response to vascular injury. Studies comparing several strains of mice have shown significant differences in neointimal formation, degree of inflammatory response, medial area, and vessel diameter characteristics [113-115]. To circumvent these potential sources of variability, we only used mice that were from the same genetic background, C57Bl/6J, instead of using mice from a mixed background.

In summary, modulation of RAS through ACE inhibition or angiotensin receptor blockade did not yield vasoprotective benefits beyond those observed using a calcium-channel blocker. The results of this study convey the influence of hemodynamic factors governing the vessel wall as the predominant regulator of pathological remodeling.

2.5 Conclusion

A hierarchy exists among the factors influencing remodeling of the arterial wall. Upon completion of this project, we believe that the pressor effects of Ang II are the most paramount stimuli for long term vessel remodeling in a pure vascular injury setting. Under normal circumstances, flow cessation yields inward remodeling in addition to neointimal formation. Treatment with ACE inhibitor (Captopril), angiotensin receptor blocker (Candesartan), and calcium-channel blocker (Amlodipine) exhibited similar long-term remodeling characteristics in these experimental settings.

CHAPTER 3

FUTURE DIRECTIONS

3.1 Introduction

Bradykinin, the key effector molecule of the kallikrein-kinin system, has been shown to exert vasodilative effects on the cardiovascular system. The biological functions of BK are dependent upon binding to its constitutive BK B_2 receptor, which is documented to have antiproliferative, antithrombotic, and antioxidant effects [116]. Additionally, at the sites of vascular injury and inflammation, BK B_1 receptor expression is known to become upregulated. The effects of this receptor are not clearly understood although recent evidence in the rat model implicates its role in inflammation and vasoconstriction [117]. Expression of these receptors in the vascular endothelium as well as smooth muscle has previously been published.

Upon completion of our current study, we believe that the differences in remodeling patterns observed among the 14-day treatment groups were partly influenced by increased BK receptor activation due to increased circulating BK levels. Inhibition of ACE has been shown to significantly increase the circulating half-life of BK. Although several lines of evidence suggest that the vasoprotective effects of ACE inhibition are due to the decreased production of Ang II rather than increased bradykinin levels, recent reports have shown that BK upon binding to the BK B_2 receptor leads to an increase in NO bioavailability, due to an upregulation of eNOS [103]. The involvement of BK in the attenuation of oxidative stress has not been fully elucidated.

Recent reports have indicated that angiotensin receptor blockade results in an indirect activation of BK B_2 receptors, due to stimulation of AT_2 receptors, causing an increase in NO bioavailability [118]. Although the activation of the BKB_2 receptor is

evident in both settings of ACE inhibition and angiotensin receptor blockade, the mechanisms underlying the receptor activation are different. Therefore, a potential future direction could involve the investigation of the kinetics of bradykinin receptor activation or possible differential expression of the BK B_1 and BK B_2 receptors causing the constitutive remodeling response. Quantifying the activation of the BK B_1 and BK B_2 receptors under these conditions will allow insight into the function of bradykinin and its receptors in physiologic and pathophysiologic vascular remodeling.

3.2 Potential future studies

3.2.1 Confirming BK receptor presence in remodeling carotid artery

Preliminary evaluation may include immunohistochemistry staining for the BK B_1 and B_2 receptors using the 14-day collected carotid sections from the current study. If the results are found to be similar between the treatment groups, then BK may not be responsible for the observed morphometric variability. However, if BK is responsible for the differences, then we expect to see either differences in the intensity of the stain or possibly differential staining of the BK receptors between treatment groups, thus indicating possible differences in downstream effects of BK.

3.2.2 Evaluation of BK involvement in the differential effects of ACE inhibition and angiotensin receptor blockade

If the results from the preliminary study implicate a difference in levels of BK activity, the next step may involve the use of BK receptor antagonists to observe possible attenuation of the divergent morphological phenotypes between the treatment groups. The experimental design will be similar to that of the current study, and the same flow-cessation vascular injury model will be utilized. The main difference would be the additional treatment of BK receptor antagonists in addition to the ACE-I, ARB, or calcium-channel blocker. Also, the 14-day timepoint appears optimal to conduct this

study since most variability among the treatment groups was observed in this phase of remodeling.

3.2.3 Defining the role of BK receptors in vascular remodeling

In this study, we wish to elucidate the unique roles of the BK receptors in vascular remodeling. To achieve this, we will treat mice with an ACE inhibitor to cause an increase in circulating BK levels. Further treatment with selective BK B_1 or B_2 antagonists should allow us to evaluate the roles of these receptors in remodeling following ligation of their left common carotid arteries.

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