Development of An In Vitro Model to Assess Endothelial Dysfunction from Coarctation of the Aorta

Dylan James Schock

Marquette University

Follow this and additional works at: https://epublications.marquette.edu/theses_open

Part of the Engineering Commons

Recommended Citation
Schock, Dylan James, "Development of An In Vitro Model to Assess Endothelial Dysfunction from Coarctation of the Aorta" (2022). Master’s Theses (2009 -). 733.
https://epublications.marquette.edu/theses_open/733
ABSTRACT
DEVELOPMENT OF AN IN VITRO MODEL TO ASSESS ENDOTHELIAL DYSFUNCTION FROM COARCTATION OF THE AORTA

Dylan J. Schock, B.S.
Marquette University, 2022

Coarctation of the aorta (CoA) is a common congenital heart defect characterized by a stenosis of the descending thoracic aorta. Surgical treatment can alleviate the stenosis and restore physiologic blood flow; however, patients frequently develop cardiovascular morbidity with hypertension (HTN) being most common. Identifying the mechanisms involved with the onset of HTN in CoA patients is difficult due to confounding factors including the severity of coarctation, age of correction, and general lifestyle factors. To account for the variability seen in humans, a rabbit model of CoA and correction was developed previously. Microarray analysis of aortic samples from the model showed natriuretic peptide receptor type c (NPRC) is downregulated in the tissue of CoA rabbits exposed to adverse mechanical stimuli from CoA, which notably persists after correction. RNA-sequencing of samples collected from human CoA patients confirmed NPRC is similarly downregulated, suggesting a role for NPRC in response to CoA. Current efforts to study related mechanisms and function in mechanosensitive aortic endothelial cells (ECs) are limited due to the short-term viability of the tissue once harvested from the rabbit models. The purpose of this investigation is to develop a novel in vitro model using cultured ECs to mimic and complement the results from the rabbit model related to NPRC and endothelial dysfunction. Physiologic (12% elongation) and pathologic (17% elongation) strain conditions derived from Control and CoA rabbit aortic measurements were applied to cultured primary human aortic endothelial cells (HAECs) for 1 hour using the FX-6000T tension bioreactor (FlexCell Int. Corporation). Two-photon imaging of the strain-conditioned HAECs revealed cells that were exposed to pathologic CoA strain levels had significantly less intracellular calcium, $\left[Ca^{2+}\right]_{in}$, mobilization than those exposed to physiologic strain. Collectively, these results show that the in vitro model developed using HAECs generally mimics the in vivo effects that CoA-induced mechanical stimuli have on aortic tissue. Therefore, this model can reasonably be used to help unravel the mechanisms behind coarctation-induced downregulation of NPRC and its involvement in the development of HTN.
ACKNOWLEDGEMENTS

Dylan J. Schock, B.S.

First and foremost, I would like to recognize my graduate advisor, Dr. LaDisa, who generously offered me a position within his lab working on a unique project while serving as a mentor and teaching me the fundamentals of engineering, cardiovascular physiology, and medicine. Additionally, he taught me invaluable skills on how to be a leader and manage a lab, project management and execution, and how to break down and analyze complex problems. These are skills that I will hold onto long into my scientific career and anything else that I may pursue.

I would also like to thank the members of my committee, Dr. Lincoln, Dr. Alli and Dr. Tefft, who were instrumental in helping guide my research and served as mentors. Dr. Alli’s background in biochemistry and molecular biology served as a valuable resource in my understanding of natriuretic peptides and their molecular pathways. I would like to thank Dr. Tefft for not only agreeing to round out my committee with his expertise, but for also providing insight into my experimental design and process. I especially would like to recognize Dr. Lincoln, who not only served on my committee but also generously offered her time, resources, and knowledge in pursuit of my own research.

I would like to recognize Marie Schulte PhD from the Versiti Blood Research Institute, who lent her technical expertise in 2-photon imaging and helped facilitate my experimental design and setup. In addition, Oleg Palygin PhD taught me the fundamentals of 2-photon imaging and methods. I would also like to thank Hilda Jurkiewicz, who taught me the fundamentals of cell culture while helping onboard me into the lab amongst a pandemic.

Last, I would like to express my gratitude to the funding provided to Dr. LaDisa from the National Institutes of Health (NIH) grant No. R01HL142955. This award allowed this research to happen and helped fund me throughout my time at Marquette University and the Medical College of Wisconsin, for which that I am grateful.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS...........................................................................................................i

CHAPTER

I. INTRODUCTION..................................................................................................................1

1.1 Coarctation of the aorta (CoA)..................................................................................1

1.2 CoA-Induced Mechanical Stress & Arterial Remodeling........................................3

1.3 Animal Model of CoA...............................................................................................4

1.4 Natriuretic Peptide Receptor C (NPRC).................................................................6

1.5 Previous Cell Culture & Intact Artery Experiments.................................................8

1.6 Goal of Current Investigation..................................................................................10

II. METHODS.........................................................................................................................12

2.1 Primary Cell Culture...............................................................................................12

2.2 Preparation of Cells for Tension Bioreactor........................................................12

2.3 Two-photon Imaging..............................................................................................15

2.4 NPRC-Specific Inhibitor.........................................................................................17

2.5 CNP Dose Dependence.........................................................................................18

2.6 Strain-time Dependence.......................................................................................18

2.7 Image & Statistical Analysis..................................................................................18

III. RESULTS........................................................................................................................21

3.1 Physiologic Control vs. Pathologic CoA Cyclic Strain........................................21

3.2 CNP Dose Dependence........................................................................................25

3.3 Strain-time Dependence.......................................................................................28

IV. DISCUSSION.....................................................................................................................32
4.1 Review of Motivation for Research……………………………………..32
4.2 Review of Strain-Induced Changes in [Ca$^{2+}$]$_{in}$ Mobilization………..33
4.3 Review of CNP Dose Dependence in HAECs…………………………34
4.4 Review of Cyclic Strain Application Length……………………………35
4.5 Effects of Other Mechanical Stimuli on EC Physiology………………..37
4.6 Limitations & Future Directions……………………………………….39
4.7 Conclusion………………………………………………………………41

BIBLIOGRAPHY………………………………………………………………43
CHAPTER 1: INTRODUCTION

1.1 Coarctation of the aorta (CoA)

Coarctation of the aorta (CoA) is a common congenital heart defect that is typically characterized as a stenosis of the descending thoracic aorta. CoA makes up 5-8% of all congenital heart defects and has an incidence rate of approximately 3 cases per 10,000 births\textsuperscript{1,2}. The pathogenesis of CoA is not fully understood. One leading theory is that during the development of the aortic arch, tissue from the ductus arteriosus infiltrates into the tissue of the descending thoracic aorta\textsuperscript{3}. As the ductus arteriosus begins to close after birth, the ductal tissue in the isthmus area also constricts, ultimately leaving a narrowing in the descending thoracic aorta. The presentation of CoA is heterogenous and can occur in varying severities along the aortic arch or bifurcation. However, CoA most often presents between the left subclavian artery and arterial ligament of the aortic isthmus (Figure 1)\textsuperscript{4,5}. CoA can occur in isolation or in tandem with other congenital heart defects, with the most common being bicuspid aortic valve, hypoplastic left heart syndrome, and ventricular septum defects\textsuperscript{1,6}. In cases of moderate severity, infants with CoA may remain asymptomatic and without clinical diagnosis until early adulthood, when a murmur or hypertension (HTN) is noted\textsuperscript{2}. Other symptoms that are reported usually include difficulty breathing, dizziness, weak pulse, and fatigue\textsuperscript{3}. In more severe cases where CoA has been left untreated, patient prognosis has been poor with 75% mortality by age 43, with the primary causes of mortality including congestive heart failure and aortic rupture\textsuperscript{2}. Treatment exists to alleviate the narrowing, however, there is no known cure.
The most common form of treatment for CoA is surgical intervention, with the first recorded resection and end-to-end anastomosis performed by Crafoord in 1944\textsuperscript{7}. Currently, the clinical guidelines for surgical intervention of CoA are when a peak-to-peak blood pressure gradient (BPG) of $\geq 20$mmHg is measured across the coarctation\textsuperscript{2,8}. While the short-term outcomes of surgical intervention are good, long-term morbidity and mortality remain higher than the general population. Even with surgery to fully restore blood flow, a systematic review reports that post-operative patients have a higher risk of developing chronic arterial HTN, with a mean prevalence of 47.3\% (20-70\%)\textsuperscript{9}. This is of particular importance to clinicians since chronic HTN is often a precursor to various cardiovascular diseases, with the most common being myocardial infarction, stroke, and heart failure.

1.2 CoA-Induced Mechanical Stress & Arterial Remodeling

Experimental and computational models have shown that patients with CoA suffer from altered mechanical stimuli such as wall shear stress (WSS) and strain\textsuperscript{10}. It has long been
understood that HTN and high blood pressure can influence tissue remodeling and is a risk factor for other cardiovascular morbidities such as atherosclerosis and myocardial infarction\textsuperscript{11,12}. On a cellular level, high blood pressure induces changes almost immediately in the stress fibers and cytoskeleton within endothelial and vascular smooth muscle cells (VSMCs), which rearrange themselves to accommodate adverse mechanical conditions\textsuperscript{12}. Although molecular adjustments in the tissue begins to occur within minutes, chronic hypertensive conditions lead to progressive changes in cell-to-cell interactions and extracellular matrix. One current challenge in HTN research involves understanding how the mechanobiological mechanisms that occur at the cellular level translate to pathologic structural and functional alterations at the tissue and organ levels.

The inner luminal layer of the arterial wall consists of a network of mechanosensitive endothelial cells (ECs), which form a barrier between blood and medial VSMCs (\textbf{Figure 2}). ECs are unique in that they are constantly exposed to shear stresses and strain while playing vital roles in cell-to-cell signaling and helping maintain circulatory homeostasis\textsuperscript{13}. One of the most well documented examples of these mechanisms is the role of nitric oxide (NO), a potent vasorelaxant that is produced within ECs in a calcium-dependent manner through a process involving endothelial nitric oxide synthase (eNOS)\textsuperscript{14}. When ECs sense increased mechanical stimuli, they respond by increasing eNOS activity and release NO, which diffuses to VSMCs and induces vasorelaxation. NO initiates a cascade of events in ECs and VSMCs, ultimately inducing vasodilation by lowering intracellular calcium concentrations ([Ca\textsuperscript{2+}]\textsubscript{i}). When pathologic arterial function and structural remodeling occurs due to chronic HTN, the ability of the vessels to vasodilate is reduced due to dysregulation of eNOS signaling factors that inhibit NO production, even when the mechanical stimuli return to physiologic levels\textsuperscript{15}.
Animal Model of CoA

Identifying the mechanisms involved with the onset of HTN in CoA patients is difficult due to confounding factors that include the severity of coarctation, age of correction, co-presenting congenital heart defects, and general lifestyle/environmental factors. There is likely a genetic linkage to the development of CoA, however, adverse changes in local hemodynamics and mechanical stimuli imposed on the tissue due to the stenosis likely have implications in gene expression as well. To mitigate the variability present in humans, a rabbit model of CoA was previously developed by our lab that mimics the hemodynamics and subsequent aortic tissue remodeling and function that occur alongside CoA. Briefly, New Zealand white rabbits underwent thoracic CoA at 10 weeks (~9 human years) using either a permanent silk or dissolvable Vicryl suture. The suture was used to induce a peak-to-peak BPG of 20mmHg across the coarctation, matching the current clinical guidelines for CoA surgical intervention. The use of a dissolvable suture mimics the morphology of CoA following repair (i.e., modest
residual gradient), where the BPG across the coarctation is restored. A comparative control group was also established from littermates who did not undergo surgery in support of replacement, reduction, and refinement consistent with The Principles of Humane Experimental Techniques. Quantification of local hemodynamics, endothelial function, and gene/protein expression was assessed in the aorta and its branches at the conclusion of the study.

**Figure 3.** Left image shows magnetic resonance images showing normal heart, coarctation of the aorta (CoA), and CoA correction (i.e., treatment) along with similarities in humans (left-top) vs an experimental rabbit model (left-bottom). A suture placed in the descending aorta of the rabbit model induces a 20mmHg blood pressure gradient (BPG) that returns to control levels when a dissolvable Vicryl suture is used to replicate CoA repair as compared to a permanent silk suture to mimic untreated CoA. Yellow arrows indicate treatment sites. The middle images show CoA suture (black arrows) and the resulting BPG in control, untreated CoA, and treated CoA rabbits (n = 7/group). Symbols indicate significantly (P < 0.05) different from Control (*) and Treated (§) groups. Right image shows comparison using fluid-structure interaction modeling between Normal and CoA groups from the experimental rabbit models. Normalized peak wall tension is elevated in the region proximal to the coarctation in the CoA groups (Adapted from LaDisa 2019 and Wegter 2017).

The rabbits with induced CoA had elevated wall tension in the tissue proximal to the coarctation (Figure 3, right) as compared to the same region in the control group. This region subsequently underwent remodeling including changes in wall thickness and stiffening, a shift in VSMC phenotype, and decreased endothelial-derived relaxation indicative of endothelial dysfunction. Notably, this pathologic state in aortic tissue persisted even after the stimulus for
remodeling (i.e., CoA) was removed in Corrected rabbits, where the coarctation created with a Vicryl suture dissolved after approximately 1 month.

1.4 Natriuretic Peptide Receptor C (NPRC)

Previous work in our lab used RNA-sequencing performed on aortic tissue collected from CoA patients during surgery to reveal a statistically significant downregulation of natriuretic peptide receptor C (NPRC; aka NPR3) in the tissue proximal to the coarctation as compared to distal. This finding was complemented by microarray analysis of aortic tissue from the experimental rabbit model of CoA, which also showed a significant decrease in NPRC in the proximal region of CoA as compared to control rabbits. Interestingly, this downregulation of NPRC persisted in the corrected rabbits mentioned in the prior section. Taken together, decreased NPRC provides a unique mechanism to further investigate CoA-induced chronic HTN due to the natriuretic peptide systems' involvement with maintaining blood pressure homeostasis.

Natriuretic peptides (NPs) are a family of structurally related circulatory hormones that are involved with fluid retention, blood pressure homeostasis, and cellular proliferation. The primary NPs are termed atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), c-type natriuretic peptide (CNP), and c-type atrial natriuretic factor (cANP; NPRC-specific agonist). ANP is primarily excreted from the atria of the heart while CNP is excreted from vascular cells including ECs. BNP has its named derived from the region that it was originally discovered (brain) and is primarily excreted from the ventricles of the heart. These NPs are further complemented by a family of structurally related receptors, termed natriuretic peptide receptor-A (NPRA), natriuretic peptide receptor-B (NPRB), and NPRC as mentioned above. The NP system creates physiological responses primarily through NPRA, which has preferential binding with ANP and BNP, but not CNP (ANP = BNP >> CNP). NPRB has preferential binding to CNP and less affinity for ANP and BNP (CNP >> ANP = BNP). NPRC has an affinity for all three
NPs, with preferential binding to ANP, followed by a preference for CNP which is slightly preferred over BNP (ANP > CNP ≥ BNP)\textsuperscript{22,25}. The physiologic mechanisms associated with NPRA and NPRB are better understood than NPRC. Activation of either NPRA or NPRB induces guanylyl cyclase activity, which directly increases cyclic GMP (cGMP) formation. This in turn inhibits phospholipase c (PLC) activity, which decreases the production of diacylglycerol (DAG) and inositol triphosphate (IP\textsubscript{3}). The latter of which regulates \([Ca^{2+}]_\text{in}\) levels and signaling\textsuperscript{26}.

NPRC is the most abundant NPR, representing most of the in vivo NPR population and is expressed in the tissues of multiple organ systems\textsuperscript{22,27}. NPRC is particularly abundant in the vascular endothelium and accounts for \(~95\%\) of the total NPR population in ECs\textsuperscript{24}. NPRC was once thought to have only played a metabolic clearance role, acting as a buffer system for more consequential receptors such as NPRA. This theory was bolstered by NPRC having affinity for all NPs while exhibiting no known physiological consequence when activated. However, recent studies have shown that NPRC is coupled to adenylyl cyclase and could play roles in various physiologic processes and signaling (\textbf{Figure 4, left}). When acted upon by an NP, NPRC inhibits adenylyl cyclase and reduces the concentration of cyclic adenosine monophosphate (cAMP). This downregulation of cAMP activates PLC, which in turn modulates \([Ca^{2+}]_\text{in}\) concentration and influences cell proliferation through mitogen-activated protein kinase (MAPK). NPRC binding to both CNP and cANF\textsuperscript{4-23} has been shown to inhibit L-type Ca\textsuperscript{2+} ion channels without influencing other ion membrane channels within mouse myocytes\textsuperscript{28}. Further, it has been shown that stimulation of NPRC with cANF\textsuperscript{4-23} specifically activates \(G_{i-1}, G_{i-2},\) and \(G_{i-3}\), which trigger PLC-\(\beta3\) via the \(\beta\gamma\)-subunits that are linked to the G proteins\textsuperscript{29}. It has been suggested that NPRC has implications with arterial remodeling and may exhibit anti-proliferative effects of medial VSMCs\textsuperscript{28}. Impaired activation of NPRC could lead to downstream phenotypic changes that include vascular remodeling and HTN (\textbf{Figure 4, right}). Total lack of NPRC has been associated
with abnormal skeletal development and greater functional impairment of the left ventricle following ischemia reperfusion injury in NPRC-knockout mice.\textsuperscript{30}

1.5 Previous Cell Culture & Intact Artery Experiments

Prior studies in our lab using cultured primary human aortic endothelial cells (HAECs) showed a rapid decrease in $[\text{Ca}^{2+}]_\text{in}$ when exposed to a single dose of CNP relative to a baseline period.\textsuperscript{30} When exposed to the NPRC-specific agonist cANF, $[\text{Ca}^{2+}]_\text{in}$ increased. Both responses were abolished when the cells were pretreated with a pharmacological NPRC inhibitor (M372049), highlighting the implication of NPRC on calcium signaling within HAECs.\textsuperscript{32} Similar experiments were performed on intact aortic tissue from the rabbit model of CoA described previously. Aortic tissue samples from regions proximal and distal to the coarctation were excised and opened longitudinally to expose the ECs. Measurements made using 2-photon microscopy...
show that interrogation of the vessels with CNP (10μM) caused [Ca^{2+}]_in to decrease rapidly, with the response being attenuated in the proximal region that was exposed to adverse wall strain as compared to distal (Figure 5-right). Taken together, these experiments provide an intriguing framework showing the functional ramifications of altered NPRC expression in the endothelium. Both models showed that normal [Ca^{2+}]_in signaling is activated when treated with a bolus of CNP, however, this behavior was diminished in ECs from aortic regions exposed to elevated mechanical stimuli from CoA^{20}.

![Figure 5. Two-photon imaging of endothelial cells (ECs) taken from excised intact aortic segments of a rabbit model of coarctation of the aorta (CoA). Interrogation of the segments with 10μM of c-type natriuretic peptide (CNP) caused a rapid decrease in intracellular calcium concentration ([Ca^{2+}]_in) within the ECs as indicated by a decrease in Fluo4-AM fluorescence (left). This response was abolished in the ECs of the proximal CoA as compared to distal (right). The region proximal to the coarctation is the same region that was exposed to adverse mechanical strain (Adapted from LaDisa 2019)^{20}

There are numerous prior studies indicating that hemodynamic forces such as strain can be used to modulate NPRC gene expression and the phenotype of ECs in vitro. For example, using a tension-bioreactor, applied cyclic stretch previously induced an autocrine release of Angiotensin II in HAECs^{33}. Angiotensin II has been shown elsewhere to decrease the expression of NPRC messenger RNA (mRNA) levels in cultured VSMCs collected from rat thoracic aorta, whereas NPRA and NPRB expression were not significantly altered^{34}. Cultured bovine aortic ECs exposed to cyclic strain demonstrated a 1.5- to 2.2-fold increase in the amount of adenyllyl cyclase/cAMP activity when compared to unstretched controls^{35}. Increases in intracellular cAMP
and cGMP have also been shown to inhibit NPRC gene expression in rat vascular VSMCs\textsuperscript{36}.

Cyclic strain also induces PKC activity 2- to 3-fold in cultured bovine aortic ECs, which has also been demonstrated to downregulate NPRC expression in cultured Henrietta Lacks (aka HeLa) cells\textsuperscript{37}. This specific mechanism caused downregulation of NPRC in both transcriptional and post-transcriptional pathways, with gene expression being suppressed and immediate receptor loss via enhanced internalization being observed\textsuperscript{37}.

Using basic cell culture and intact artery models to probe for the specific mechanisms linking downregulated NPRC with CoA-induced arterial dysfunction is difficult. Two-photon imaging on ECs is best done on fresh tissue, and the extended duration of surgery to excise the samples combined with the length of the imaging sessions limits the viability of experiments. Additionally, the current protocol used to induce CoA in rabbits takes ~5 months to allow for changes in the tissue to develop, increasing monetary cost and risk of experimental failure.

1.6 Goal of Current Investigation

Considering the current limitations in studying ECs from the intact rabbit aortic segments mentioned above, the objective of the current investigation is to create an in vitro model using cultured HAECs that mimics and compliments the results from the rabbit model of coarctation-induced endothelial dysfunction. Specifically, we used a tension bioreactor to apply physiologic control or pathologic CoA strain conditions measured from the rabbit model to cultured HAECs. Two-photon imaging was used to quantify the strain-conditioned \([\text{Ca}^{2+}]_{\text{in}}\) response when interrogated with CNP, and these results were then compared to outcomes from the rabbit model. Our hypothesis was that applying in vivo pathologic CoA levels of strain on cultured HAECs would cause a less pronounced \([\text{Ca}^{2+}]_{\text{in}}\) response to a dosage of CNP as compared to HAECs that underwent physiologic control levels of strain. Overall, this in vitro model is desirable since it allows study personnel to circumvent some of the current limitations associated with the animal
model recounted above and will permit the investigation of additional mechanisms associated with coarctation-induced downregulation of NPRC and their potential implications on HTN in vivo
CHAPTER 2: METHODS

2.1 Primary Cell Culture

Primary HAECs from Cell Biologics (Chicago, IL) were cultured and expanded using the manufacturers recommended human endothelial cell medium supplemented with 5% fetal bovine serum, 10mL/L of L-Glutamine and Antibiotic-Antimycotic, and 1mL/L of VEGF, HEPARIN, EGF, and FGF. Cultures were maintained in a humidified incubator at 37°C and an atmosphere of 5% CO₂-95% air. Cells were received frozen at passage 3 and initially seeded onto a T25 cell culture flask or stored in liquid nitrogen. Cell media was replenished every 2 days and passaged in a 1:3 ratio every 5-6 days. Cell viability was monitored daily by performing qualitative checks of morphology, pH indicator color, and visual inspection for bacterial/fungal contamination. All culture flasks were coated with a Gelatin-Based Coating Solution (Cell Biologics) for at least 2 minutes and then aspirated prior to seeding. Cells were split in a 1:2 ratio onto T75 culture flasks at passage 7. Once >80% confluent, cells were either frozen and stored at -80°C for later experiments or seeded at 100,000 cells/well onto a six-well Bioflex plate coated with Collagen I from FlexCell International Corporation (Burlington, NC). Experimental cell populations were typically seeded about 2 days before any planned experiment to ensure confluency. Experiments were conducted at passage 8 (P8) to maximize the experimental population and cell viability while also aligning with methods and results from historically published data²⁰.

2.2 Preparation of Cells for Tension Bioreactor

Once the P8 HAECs were confluent and ready for experimentation, the media in each well was aspirated and replaced with fresh media (3mL/well). The Bioflex plates were then transferred to the FX-6000T Tension System (FlexCell International Corporation), a pneumatic bioreactor that induces equibiaxial (i.e., radial and circumferential) strain on cultured cells in monoculture (Figure 6).
Strain was set to be applied cyclically for one hour using a triangular waveform, similar to the shape of the displacement waveform quantified from the preclinical rabbit model mentioned in the introduction. A duty cycle of 33% was similarly set such that 1/3 of the waveform is spent increasing in strain and 2/3 is spent decreasing back to a baseline of 0% elongation. This pattern was chosen to achieve the physiologic strain pattern experienced by the aortic wall over the course of complete cardiac cycle (Figure 7, right)\textsuperscript{38,39}. There was no observable change in strain waveform pattern between the Control and CoA rabbits, thus a similar waveform as described previously was used for both conditions, but with different maximum strain severities (Figure 7, left).
Strain conditions imposed were based on changes in diameter measurements from Control rabbits and CoA rabbits with a ≥20mmHg peak-to-peak BPG imposed. Change in diameter over the course of a cardiac cycle was converted to percent elongation by using ultrasound and MRI measurements made of the proximal aorta from the rabbit models. These derived values were then uploaded to the bioreactor software. An elongation of 12% was subsequently deemed the physiologic Control condition while a 17% elongation was designated as the pathologic CoA condition and representative of that seen in CoA prior to remodeling. To achieve these desired strains, an external air compressor and vacuum system were used in tandem with the FlexCell computerized unit to submit the Bioflex plates to cyclic positive and negative pneumatic pressures, as shown in the experimental setup in Figure 8.
The Bioflex plates used in the study apply uniform radial and circumferential strains to cells grown in monoculture. The percent change in the radial direction is equal to the percent change in the circumferential direction (i.e., product of pi and diameter), thus applying a single value biaxially to cells exposed to Control vs CoA strains was considered sufficient to reasonably replicate the in vivo loading conditions. Cyclic strain was applied for 1 hour in accordance with historically published results on various mechanobiological processes that take place during this time frame\textsuperscript{33,35,40}. Strain-conditioned media was removed and stored at -80°C for potential use in later studies.

### 2.3 Two-Photon Imaging

Average $[\text{Ca}^{2+}]_\text{in}$ mobilization was recorded (n=6 wells/plate) during experimentation that followed cyclic straining of cells. Imaging was performed using an upright Fluoview FV1000 Laser Scanning 2-photon (aka multiphoton) microscope from Olympus (Tokyo, JP) equipped
with Ti:sapphire lasers set to a wavelength of 820nm. Samples were imaged using a 25X (N.A. 1.05 and working distance 2mm) water-immersion objective lens (XLPL25XWMP, Olympus).

Prior to imaging, the strain-conditioned HAECs were loaded with the Ca\(^{2+}\) sensitive dye Fluo-4 AM (3.8μM, Invitrogen) in dimethyl sulfoxide (DMSO) using 0.02% Pluronic acid (Pluronic F-127, Invitrogen) in basal media for one hour. The flexible silicone Bioflex membranes containing the cells were carefully sectioned from the Bioflex plate using a scalpel and pinned down in a silicone elastomer-coated 35mm imaging dish (Figure 9, left). The membranes were immersed in warmed 2mM Ca\(^{2+}\) physiologic salt solution (PSS) consisting of Millipore-filtered water and 8.47g/L NaCl, 2.60g/L HEPES, 0.222g/L CaCl\(_2\), 0.190g/L MgCl\(_2\), and 0.335g/L of KCl.

![Figure 9. Experimental setup for 2-photon imaging. Following cyclic strain application, the rubber membranes containing the strain-conditioned human aortic endothelial cells were segmented from the Bioflex plates using a scalpel and pinned down in a 35mm tissue culture dish coated with a silicone-elastomer (left). The culture dish was then placed within the 2-photon apparatus (right), where 10mL of 2mM Ca\(^{2+}\) physiologic salt solution containing c-type natriuretic peptide was administered across the dish using syringes.](image)

Once the silicone membranes containing the strain-conditioned HAECs were securely pinned down in the tissue culture dish, the dish was transferred into the 2-photon apparatus (Figure 9, right). Brightfield imaging was used to locate the EC layer. The microscope was then switched to multiphoton mode once a desirable population of cells with good morphology was
found. Continuous digital image acquisition settings were set to capture 16-bit images every 1.644s for a maximum duration of 200 frames (328.5s). After starting the experiment, 30 frames were allowed to pass to establish a baseline intensity reading of the cells prior to the addition of any reagents or peptides. At frame 30, 10mL of warmed PSS containing 1.5μM of CNP was gently diluted into the imaging dish via syringe while the same volume of PSS in the dish was simultaneously removed. This dose was chosen to balance cellular response to the agent with toxicity and was further scrutinized in separate dose dependance studies mentioned below\textsuperscript{20,30}. Digital images of each frame during experiments were saved for offline analysis. Changes in intensity reflective of $[Ca^{2+}]_m$ mobilization were imaged with this protocol using HAECs subjected to the Physiologic Control or Pathologic CoA strain conditions mentioned above. Biological replicates (n = 3 Bioflex plates) were obtained using measurements from at least 3 experiments in most cases, with each plate included in the study containing at least one technical replicate (n ≥ 3 wells).

2.4 NPRC-Specific Inhibitor

To further confirm a role for NPRC in the current study, $[Ca^{2+}]_m$ intensity was measured in HAECs exposed to physiologic strain (12% elongation) conditions and then treated with the NPRC-specific pharmacological inhibitor, M372049 (AstraZeneca)\textsuperscript{32}. HAECs at P8 were grown to confluency on Bioflex 6-well plates and then transferred to the FX-6000 bioreactor where physiologic cyclic strain (12% elongation) was applied for 1 hour as described previously. The first two wells were incubated with 10μM of M372049 or equal volume of vehicle (DMSO) in complete media for 1 hour. After incubation with the inhibitor or vehicle, the inhibited media was aspirated and replaced with Fluo4-AM in Basel media for 1 hour. Biological replicates of both the inhibitor and vehicle data were obtained and used for analysis.
2.5 CNP Dose Dependence

The dose-dependent \([\text{Ca}^{2+}]_m\) mobilization in HAECs was measured after interrogation with a range of CNP doses. HAECs at P8 were grown to confluency on Bioflex plates and transferred to the FX-6000 bioreactor where physiologic (12% elongation) or pathologic (17% elongation) strain patterns were applied for 1 hour as described above. The same experimental setup was followed as described. Half the wells (3) in each plate were interrogated with 0.5μM of CNP and the other half treated with a 5μM dose of CNP.

2.6 Strain-time Dependence

To assess the impact of strain duration, \([\text{Ca}^{2+}]_m\) intensity was also measured in response to a dose of 1.5μM CNP after application of 12 hours of cyclic strain using the same rabbit-derived physiologic and pathologic severities described previously. The remainder of the experimental setup was followed as described above.

2.7 Image & Statistical Analysis

Images were quantified using the Loci tool plugin in Fiji (aka ImageJ; National Institute of Health). Regions of interest (ROIs) were identified over five different cells at random, as well as an area of empty space to represent the background fluorescent intensity (Figure 10, left). The Measurement option within the Analyze tool was employed to record the mean intensity, among other features related to fluorescent intensity across all frames. Temporal measurements for each of the 5 recorded ROIs and background were cataloged and saved (Figure 10, right). The raw mean data from each ROI was loaded into Origin6 (OriginLab), where the background intensity values for each frame were subtracted from the 5 ROIs to normalize results.
Figure 10. Example of analysis conducted using 2-photon image stacks of human aortic endothelial cells. Recorded image stacks from 2-photon imaging sessions were imported into Fiji for analysis (left). Five regions of interest (ROIs) were drawn around representative cells of interest for each well. Temporal measurements were then made for each recorded frame to summarize the intensity of Fluo4-AM (right). Each row represents one quantified frame from a single ROI. The mean intensities of each ROI were averaged for each time point to gain a representative value for each well and loaded into Origin6 for further analysis.

The mean Fluo4-AM intensity values (now background corrected) for the first 30 frames before CNP application were averaged for each ROI, creating a baseline value to compare the subsequent change in $[\text{Ca}^{2+}]_\text{in}$. Percent change in the $[\text{Ca}^{2+}]_\text{in}$ relative to the baseline for each recorded time point was calculated.

The resulting changes in percent $[\text{Ca}^{2+}]_\text{in}$ for each ROI were then graphed and a moving average baseline was calculated from the first $\sim$30 frames prior to CNP application using Origin6. The temporal data from each biologic replicates for each condition processed were then loaded into MATLAB for further quantifications and statistical analysis. Average $[\text{Ca}^{2+}]_\text{in}$ mobilization and standard error of the mean were calculated for each well and plate. One-way analysis of variance (ANOVA) was performed between each condition to determine statistical significance. Statistical significance was defined as samples having a p-value $\leq 0.05$. Prism 9 (GraphPad) was used to test for statistical differences and to create associated figures. Quantifications were conducted to best match previously published results from the rabbit model of CoA$^{20}$.

Specifically, the temporal $[\text{Ca}^{2+}]_\text{in}$ mobilization curves and initial absolute decreases in $[\text{Ca}^{2+}]_\text{in}$
after CNP application were quantified and graphed to compare with the in vivo rabbit model to validate the results of the in vitro model presented here. Additionally, the area under the curves (AUC) values for each of the temporal $[\text{Ca}^{2+}]_\text{in}$ graphs were quantified by calculating the definite integral of the curves from 0 to 150 seconds.
CHAPTER 3: RESULTS

3.1 Physiologic Control vs. Pathologic CoA Cyclic Strain

All groups of strain-conditioned HAECs tested generally showed a rapid decrease in 
\([Ca^{2+}]_m\) (\% relative to Baseline) when exposed to a bolus of 1.5\(\mu\)M of CNP. Cells exposed to 
physiologic levels of cyclic strain (i.e., 12%; Physiologic Control group) for 1 hour had a more 
pronounced reduction in \([Ca^{2+}]_m\) than the cells exposed to pathologic cyclic strain (i.e. 17%; 
Pathologic CoA group) for the same duration. This behavior can be seen in Figure 11, where the 
mean temporal \([Ca^{2+}]_m\) was tracked for 100 seconds after administration of CNP, which took 
place approximately 50 seconds after the start of imaging. Both groups of cells had initial 
decreases in \([Ca^{2+}]_m\), but to differing levels, followed by a partial return towards their pre-CNP 
baselines after dilution. The partial recovery of the lost cytosolic \([Ca^{2+}]_m\) peaked between 55 and 
70 seconds after CNP application for both sets of cells.
HAECs that underwent an hour of physiologic cyclic strain followed by incubation with the NPRC-specific inhibitor (i.e., Physiologic + Inhibitor) had an impaired response to CNP as compared to incubation with the vehicle, DMSO (i.e., Physiologic + Vehicle), as shown in Figure 12 (right panel). The Physiologic + Vehicle group had a more pronounced decrease in [Ca^{2+}]_{in} relative to the Physiologic + Inhibitor group. These patterns generally mimic the HAEC responses to CNP after undergoing Physiologic Control vs Pathologic CoA strain levels (Figure 12, left). Following the initial CNP-induced decrease in [Ca^{2+}]_{in}, the Physiologic + Vehicle group did exhibit partial [Ca^{2+}]_{in} recovery, while the Physiologic + Inhibitor cells had increased cytosolic [Ca^{2+}]_{in} levels that went beyond their pre-CNP baselines briefly before steadily decreasing from the initial baseline. This behavior was similar to the results seen from the Pathologic CoA group (Figure 12, left, red plot).
Figure 12. Comparison between the percent change in intracellular calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{in}) shown normalized to baseline intensity for human aortic endothelial cells (HAECs) that underwent 1 hour of physiologic or pathologic strain (left; n=6 Bioflex plates) and those that underwent 1 hour of physiologic strain and were subsequently incubated with an natriuretic peptide receptor type-c (NPRC) inhibitor or vehicle (right, n=3 Bioflex plates). C-type natriuretic peptide (CNP) was diluted into the dish approximately ~50s after the start of imaging, and the subsequent [Ca\textsuperscript{2+}]\textsubscript{in} mobilization was tracked for an additional 100s. Data shown represents mean ± standard error.

The maximum percent decrease in cytosolic [Ca\textsuperscript{2+}]\textsubscript{in} after CNP administration was extracted for all conditions. Figure 13 shows that the Physiologic Control strain group had a significantly more pronounced [Ca\textsuperscript{2+}]\textsubscript{in} decrease in response to CNP (-42.9 ± 2.1%) as compared to the Pathologic CoA (-22.0 ± 2.6%, p ≤ 0.05) and Physiologic + Inhibitor groups (-13.8 ± 2.9%, p ≤ 0.01). The maximum percent decrease of [Ca\textsuperscript{2+}]\textsubscript{in} in the Physiologic + Vehicle group was not significantly different (-47.4 ± 4.2%) from the Physiologic Controls.
The AUC of the absolute change in \([\text{Ca}^{2+}]_{\text{in}}\) from 0 to 150 seconds was quantified using the definite integrals of the \([\text{Ca}^{2+}]_{\text{in}}\) responses (Figure 14-right, shaded regions) and compared across experimental groups. The Physiologic Control group had a higher mean AUC (1490 ± 108 \([\text{Ca}^{2+}]_{\text{in}}\)%) than the Pathologic CoA (662 ± 89 \([\text{Ca}^{2+}]_{\text{in}}\)%, p ≤ 0.01) and Physiologic + Inhibitor groups (510 ± 292 \([\text{Ca}^{2+}]_{\text{in}}\)%, p ≤ 0.01). The AUC of the Physiologic + Vehicle group (1980 ± 263 \([\text{Ca}^{2+}]_{\text{in}}\)%) was not significantly different from the Physiologic Control group.
Figure 14. The area under the curve (AUC) was calculated by performing a definite integral of the change in intracellular calcium concentration ([Ca^{2+}]_{in}) over the course of 150 seconds (right, shaded regions). Bar plots representing the mean AUC values were graphed and compared (left). The Pathologic CoA and Physiologic + Inhibitor groups were significantly different from the Physiologic Control, while the Physiologic + Vehicle group was not significantly different from the Physiologic Control group. Data shown in the bar plot (left) represents the mean AUC ± standard error (SEM). Data shown in the line plot (right) represents mean ± SEM; shaded regions represent area considered when calculating definite integrals.

3.2 CNP Dose Dependence

Three different concentrations of CNP were used to determine dose dependence of the [Ca^{2+}]_{in} mobilization response in strain-conditioned HAECs. Figure 15 demonstrates that as the concentration of the administered CNP increases from 0.5μM to 5μM, the deviation of [Ca^{2+}]_{in} from the pre-CNP baseline generally becomes more pronounced under both physiologic and pathologic strain conditions. Regardless of dose, CNP generally induced an initial rapid decrease in [Ca^{2+}]_{in}, followed by a partial recovery before starting to equalize.
Figure 16 shows that the Physiologic Control group that was treated with 0.5μM of CNP (-25.8 ± 2.3%) elicited a significantly different [Ca$$^{2+}$$]$_{\text{in}}$ response relative to the 1.5μM (-42.9 ± 2.1%, p ≤ 0.05) and 5μM CNP doses (-60.1 ± 3.2%, p ≤ 0.01).

Moreover, the Pathologic CoA group exposed to 0.5μM of CNP (-19.5 ± 2.6%) did not elicit a significantly different response from the HAECs administered with 1.5μM of CNP (-22.0 ± 2.6%). The Pathologic CoA group administered with a dose of 5μM of CNP (-40.9 ± 2.7%) was significantly different from the Pathologic CoA group treated with 0.5μM of CNP (p ≤ 0.05).

When comparing the effects of the various CNP concentrations directly, there were significant differences between the Physiologic and Pathologic strain groups when treated with either 1.5μM (p ≤ 0.05) or 5μM of CNP (p ≤ 0.001). In contrast, 0.5μM of CNP was not potent enough to induce a significantly different [Ca$$^{2+}$$]$_{\text{in}}$ response between the strain conditions.
AUCs were also calculated and compared across all concentrations of administered CNP in Figure 17 using the definite integral method described previously and shown in Figure 14 (right). In cells that underwent physiologic strain, HAECs treated with a dose of 0.5μM of CNP (858 ± 106 [Ca²⁺]ₙ₉%*s) did not have significantly different AUC values from those treated with 1.5μM CNP (1490 ± 108 [Ca²⁺]ₙ₉%*s). There was, however, a significant difference between the AUC values of HAECs dosed with 0.5μM CNP and 5μM CNP (1780 ± 111 [Ca²⁺]ₙ₉%*s, p ≤ 0.01).

Changing the administered CNP dose did not yield a significant difference in mean AUC values of HAECs that underwent pathologic levels of cyclic strain and treated with 0.5μM CNP.
(527 ± 101 [Ca\(^{2+}\)]_{in}\%s). The pathologic mean AUC of HAECs treated with 1.5μM CNP was 662 ± 89 [Ca\(^{2+}\)]_{in}\%s while a 5μM dose of CNP elicited a mean AUC of 340 ± 106 [Ca\(^{2+}\)]_{in}\%s.

A physiologic dose of 1.5μM and industrial 5μM dose of CNP did induce a significant difference in the AUC of HAECs that underwent physiologic strain versus those that underwent pathologic (p ≤ 0.01). A 0.5μM dose of CNP was not potent enough to induce a significantly different AUC between the strain groups, thus making 1.5μM the smallest tested concentration of CNP to elucidate these strain-induced differences in mean AUC.

Figure 17. The area under the curve (AUC) values were calculated by performing a definite integral of the change in intracellular calcium concentration [Ca\(^{2+}\)]_{in} over the course of 150 seconds. In the cells that underwent Physiologic strain (left), there was a significant difference between the AUCs between the cells that were treated with 0.5μM and 5μM of CNP, while the cells that were treated with 1.5μM did not have significantly different AUC values. In the cells that underwent Pathologic strain, the difference in mean AUCs was not significant, regardless of the concentration of CNP applied. Data shown represents the mean AUC ± standard error (n=3 Bioflex plates for 0.5 and 5μM, n=6 Bioflex plates for 1.5μM).

3.3 Strain-time Dependence

HAECs exposed to both 1 and 12 hours of cyclic strain exhibited a rapid decrease in [Ca\(^{2+}\)]_{in} when administered 1.5μM of CNP in PSS. Temporal comparisons of the change in
[Ca\(^{2+}\)]_m (Figure 18) indicate that HAECs exposed to physiologic levels of strain generally had a more pronounced decrease in [Ca\(^{2+}\)]_m mobilization relative to those exposed to pathologic CoA strain, regardless of the length of time that strain was applied. Once CNP was administered, the HAECs from both strain groups exhibited a decrease in [Ca\(^{2+}\)]_m followed by a partial recovery, leading to a leveling of the [Ca\(^{2+}\)]_m transience that generally persisted for the duration of imaging.

Figure 18. Comparison between c-type natriuretic peptide (CNP)-induced changes in intracellular calcium concentration ([Ca\(^{2+}\)]_m) in human aortic endothelial cells (HAECs) that have undergone 1 hour (left; n=6 Bioflex plates) or 12 hours (right; n=6 Bioflex plates) of cyclic strain. For each strain-time, the effects of applying Physiologic (green) and Pathologic (red) levels of strain were compared. Data shown represents mean ± standard error.

As mentioned above, when cyclic strain was applied for 1 hour, there was a significant difference in the maximum [Ca\(^{2+}\)]_m decrease induced by CNP in the cells exposed to Physiologic Control strain as compared to Pathologic CoA (Figure 19; p ≤ 0.05). This difference persisted when the time spent under cyclic strain was extended to 12 hours, where HAECs exposed to physiologic strain had significantly more pronounced [Ca\(^{2+}\)]_m mobilization (-54.9 ± 5.1%) relative to HAECs exposed to pathologic cyclic strain (-26.7 ± 4.3%, p ≤ 0.01). In contrast, the maximum [Ca\(^{2+}\)]_m mobilization in HAECs exposed to physiologic cyclic strain for 1 hour or 12 hours did not differ significantly. HAECs that underwent pathologic cyclic strain for 12 hours had
significantly less \([\text{Ca}^{2+}]_\text{in}\) mobilization than those exposed to pathologic cyclic strain for 1 hour (\(p \leq 0.01\)).

**Figure 19.** Comparison between the maximum decrease in intracellular calcium concentration (\([\text{Ca}^{2+}]_\text{in}\)) in response to c-type natriuretic peptide (CNP) application after human aortic endothelial cells underwent either 1 hour (left; \(n=6\) Bioflex plates) or 12 hours of cyclic strain (right; \(n=6\) Bioflex plates). There was a significant difference between the cells that underwent Physiologic (green) and Pathologic (red) strain levels, regardless of the length of time that cyclic strain was applied. Data within the boxes fall within 1 quartile of the median, while the whiskers extend 2 quartiles from the median. The solid line represents the median. + symbol represents mean. Outliers were determined as all data falling outside of the 90% confidence interval.

The AUC values of HAECs that underwent physiologic cyclic strain (828 ± 108 [\(\text{Ca}^{2+}]_\text{in}\)%) versus pathologic cyclic strain (662 ± 89 [\(\text{Ca}^{2+}]_\text{in}\)%) for 1 hour were significantly different (\(p \leq 0.01\)). This trend continues for the AUC values of cells exposed to 12 hours of physiologic strain (2090 ± 114 [\(\text{Ca}^{2+}]_\text{in}\)%) and pathologic strain (872 ± 130 [\(\text{Ca}^{2+}]_\text{in}\)%), which were also significantly different from each other (\(p \leq 0.001\)).
Figure 20. The area under the curve (AUC) values of human aortic endothelial cells (HAECs) that underwent 1 hour (n=6 Bioflex plates) and 12 hours (n=6 Bioflex plates) were calculated by performing a definite integral of the change in intracellular calcium concentration ([Ca$^{2+}$]$_{in}$) over the course of 150 seconds. Regardless of the length of time that cyclic strain was applied, there was a significant difference between the groups that underwent Physiologic and Pathologic for both 1 hour and 12 hours. Data shown represents the mean AUC ± standard error.
CHAPTER 4: DISCUSSION

4.1 Review of Motivation for Research

Despite there being successful surgical interventions available to alleviate the narrowing associated with CoA, treated patients often experience reduced life expectancy due to long-term morbidities, with the most common being HTN. Previous results from an in vivo rabbit model of CoA found that a 20mmHg peak-to-peak BPG caused by stenosis in the thoracic aorta induces irreversible changes in structure and endothelial function that persists beyond repair\textsuperscript{18}. Microarray analysis of samples from the model showed \textit{NPRC} is downregulated in aortic tissue from the region proximal to the coarctation in rabbits, which notably persists after correction\textsuperscript{20}. Genotyping of samples collected from human CoA patients confirmed that \textit{NPRC} is also downregulated in the tissue exposed to excessive wall tension, suggesting its potential implication with CoA\textsuperscript{20}. Current efforts to study related mechanisms in ECs are limited due to the short-term viability of the tissue once harvested from the rabbit models and the duration of in vivo models lasting up to 4-5 months. The objective of this work was to develop an in vitro model using cultured HAECs to mimic and further complement the results of the rabbit model related to NPRC and endothelial dysfunction. Our hypothesis was that applying in vivo pathologic CoA levels of stain on cultured HAECs would cause a less pronounced $[\text{Ca}^{2+}]_{\text{in}}$ response to a dosage of CNP as compared to HAECs that underwent physiologic control levels of strain. Along with testing this core hypothesis, experiments were completed to determine the dose-dependence of CNP on the cells as well as test how the length of time that HAECs underwent cyclic strain influenced their CNP-induced $[\text{Ca}^{2+}]_{\text{in}}$ mobilization.
4.2 Review of Strain-Induced Changes in $[\text{Ca}^{2+}]_{\text{in}}$ Mobilization

Measurements made of the thoracic aortic diameter during the cardiac cycle revealed a radial strain of 12% in control rabbits over the course of a cardiac cycle. In contrast, a strain of 17% in CoA rabbits exposed to a 20mmHg peak-to-peak BPG was recorded prior to the onset of aortic remodeling. A tension bioreactor was then used to expose these in vivo cyclic strain levels on HAECs grown on a flexible substrate. Physiologic cyclic strain applied on cultured HAECs for 1-hour resulted in a rapid decrease in $[\text{Ca}^{2+}]_{\text{in}}$ when interrogated with 1.5μM of CNP in PSS. This $[\text{Ca}^{2+}]_{\text{in}}$ transient response to CNP was significantly blunted after the HAECs were exposed to pathologic strain for the same duration. This matched the patterns created by administering CNP to the proximal aortic segments of the Control and CoA rabbits, as shown in Figure 21.
Treatment of the strain-conditioned HAECs with the NPRC inhibitor significantly reduced the effect that CNP has on the physiologic Control HAECs, implicating NPRC in this functional \([Ca^{2+}]_{\text{in}}\) signaling response. Due to the rabbit aortic model and cultured HAECs having no known predisposition to downregulated NPRC expression, it can be assumed that the mechanism underlying this functional change is strain-dependent.

### 4.3 Review of CNP Dose Dependence in HAECs

To further scrutinize the impact that the dosage of CNP has on \([Ca^{2+}]_{\text{in}}\) in the current study, strain-conditioned HAECs were interrogated with three different concentrations of CNP: 0.5\(\mu\)M, 1.5\(\mu\)M, and 5\(\mu\)M. Analysis of the temporal \([Ca^{2+}]_{\text{in}}\) mobilizations after CNP
administration indicates that there is a positive correlation between the concentration of administered CNP and the degree of $[\text{Ca}^{2+}]_{\text{in}}$ mobilization that the HAECs undergo in response.

Adjusting the dosage of CNP administered to Pathologic CoA HAECs did not induce as severe a change as was seen in the Physiologic Control cells. Interestingly, the calculated AUC for the larger 5μM dosage of CNP was less than that of smaller 0.5μM dose of CNP. This behavior was somewhat unexpected and contrary to precedence from the Physiologic Control group. However, analysis of the temporal $[\text{Ca}^{2+}]_{\text{in}}$ graphs (Figure 15-right) shows that 5μM applied to the pathologic cells initially induced a larger $[\text{Ca}^{2+}]_{\text{in}}$ response than 1.5μM and 0.5μM, however, its recovery was also much faster had recover closer to its original baseline. This subsequent recovery thus limited the calculated AUC value when comparing the results from other CNP concentrations tested.

In summary, a dosage of 1.5μM of CNP was experimentally determined to be the smallest dose capable of elucidating strain-dependent changes in HAEC function after undergoing either physiologic or pathologic levels of strain. The above results suggests that HAECs that were exposed to physiologic levels of cyclic strain were more sensitive to changes in the dosage of CNP as compared to those that were exposed to pathologic levels of strain. It is known that expression of NPRC is downregulated in the pathologic aortic tissue as shown by the results from human CoA patient genotyping and rabbit model of CoA^20. If the NPRC expression in HAECs are similarly downregulated after pathologic strain, it would be reasonable to surmise that less CNP-sensitive receptors will result in a smaller change in both absolute minimums and AUC values in the pathologic CoA group.

4.4 Review of Cyclic Strain Application Length

The impact of the length of time that cyclic strain was applied in vitro on HAEC function was evaluated by comparing the $[\text{Ca}^{2+}]_{\text{in}}$ responses to CNP after undergoing either 1 or 12 hours
of cyclic strain. Even though pathologic strain has a more pronounced effect on cell function after 12 hours, the difference between the physiologic and pathologic strained HAECs remained significant despite whether strain is applied for 1 or 12 hours. Thus, 1 hour of applied cyclic strain is sufficient to induce a significant difference in the CNP-induced $[\text{Ca}^{2+}]_\text{in}$ mobilization between physiologic and pathologic conditions.

The physiologic changes that occurred after 1 hour of cyclic strain applied to cultured HAECs does not come without precedent. Multiple studies have shown that 1-hour of cyclic stretch on aortic ECs is sufficient to induce physiologic changes in both gene expression and phenotype, some of which can influence the expression of NPRC\textsuperscript{33,35,40}. For example, it has been shown that Angiotensin II, which increases in ECs after 1 hour of cyclic strain, decreases the concentration of $\text{NPRC}$ mRNA in VSMCs\textsuperscript{34}. While the gene expression for $\text{NPRC}$ was decreased in this study, the expression of $\text{NPRA}$ and $\text{NPRB}$ was not significantly altered. Bovine aortic ECs were found to have an increase in adenylyl cyclase activity concentration of cAMP after 1 hour of cyclic strain. Increases in both cytosolic cAMP and cGMP concentration have been shown to inhibit $\text{NPRC}$ gene expression\textsuperscript{36}. Increased cGMP concentrations have been shown to inhibit PLC signaling activity, which in turn decreases the production of DAG and IP; both of which are generated by phosphatidyl inositol bisphosphate and involved with $[\text{Ca}^{2+}]_\text{in}$ signaling and activation of PKC respectively\textsuperscript{22}. PKC activity has been shown to be increased in cultured aortic ECs after 1 hour, as HeLa cells that have had increased PKC activation have also been shown to demonstrate downregulated NPRC expression through both transcriptional and post-transcriptional mechanisms after applying strain for up to 1 hour\textsuperscript{37}. Interestingly, PKC activity in aortic ECs exposed to cyclic strain is significantly elevated after just 5 minutes of cyclic stretch. This upregulated PKC state starts to steadily decrease until 1 hour of strain is applied, where the upregulation then becomes static. This behavior indicates that there are substantial physiologic changes taking place within the first 1 hour of cyclic strain, with diminishing returns for strain that is applied beyond 1 hour. PKC is a primary target of DAG and regulates many intracellular
responses, however the specific molecular mechanisms associated with this interaction are not fully understood\textsuperscript{41}. Recent studies have focused on the impact of protein kinase D (PKD), which has known implications in cell growth, gene expression, and protein trafficking in ECs. DAG regulates both the localization of PKD and phosphorylates PKD by using PKC\textsuperscript{41}. The 1-hour study duration applied in the current investigation is also favorable as it limits the potential for failed experiments while allowing for the further assessment of NPRC-induced mechanisms associated with elevated levels of strain.

### 4.5 Effects of Other Mechanical Stimuli on EC Physiology

The FX6000 tension bioreactor is specifically designed to induce cyclic biaxial strain on cells in vitro. ECs, however, are constantly exposed to multiple kinds of concurrent mechanical stimuli created when interfacing with circulating blood. The three main types of mechanical stimuli experienced by ECs in vivo are WSS from blood flow, hydrostatic pressure from blood pressure, and cyclic stretch (i.e., strain) from vessel deformation. Normalized wall tension (the product of pressure and radius) was previously found to be substantially elevated in the tissue proximal to the coarctation based on computational modelling, as opposed to proximal WSS which generally remained consistent\textsuperscript{21}. Based on this observation, indices associated with adverse deformation in the proximal aorta were the focus of this investigation when evaluating CoA-induced endothelial dysfunction and NPRC regulation. ECs in vivo are also consistently exposed to both shear stresses and hydrostatic pressure. However, it is debated whether hydrostatic pressure meaningfully influences aortic EC physiology\textsuperscript{42,43}. The in FX6000-T tension bioreactor presented above can manipulate one of these variables (cyclic stretch); however, all three types of mechanical stimuli would likely need be applied concurrently to fully recapitulate the in vivo hemodynamic consequences on aortic EC growth and physiology in the presence of CoA.
Along with wall tension, it is well documented that ECs will change their morphology and gene expression based on the WSS acted on them from blood flow. The exact magnitudes and combinations of these individual stresses and how they affect EC physiology are not fully understood. For example, in vitro exposure of cultured HAECs to steady flow has been shown to increase mRNA levels of ICAM-1 as compared to exposure of hydrostatic pressure alone; while pulsatile flow decreased mRNA levels of ICAM-1 in HAECs as compared to steady flow\textsuperscript{42,44}. Along with the morphological changes that occur, shear stress from blood flow can also alter and increase the transients of secondary messengers such as $[\text{Ca}^{2+}]_{\text{in}}$ and IP\textsubscript{3}\textsuperscript{45,46}. This influence on intracellular signaling can have implications on the development of cardiovascular disease such as arteriosclerosis. Computational modeling of fluid-structure interactions of the rabbit model of CoA revealed that WSS in the region proximal to the stenosis generally remains constant even as CoA severity increases\textsuperscript{21}. So, despite the influence that WSS has on endothelial physiology, it likely plays a minimal role in the development of the endothelial dysfunction and downregulation of NPRC as seen in the experimental rabbit model of CoA.

As blood is pressurized by the heart and pumped through the vasculature, dynamic changes in pressure occur between the systolic and diastole phases of the cardiac cycle. This constant rhythm of pressurization and depressurization induces hydrostatic pressure on the endothelium that lines the inner wall of the vasculature, primarily the aorta which is located distal to the heart. Computational modeling and measurements of the rabbit experimental model have found elevated blood pressure in the region proximal to the coarctation. Little is known about how NPRC expression in ECs is influenced and what mechanisms are related to this dynamic hydrostatic pressure. Prior investigations on the affects that hydrostatic pressure has on cell viability are mixed\textsuperscript{42,43,47}. Biologic tissues and cells have been found to be nearly incompressible, thus it is unknown what effect hydrostatic pressure would have on cellular physiology\textsuperscript{47}. It has, however, been proposed previously that the pressure transients during the initial pressurization
step and final depressurization step can induce physiologic regulation and stimulation in ECs\textsuperscript{43}. For example, cultured bovine aortic ECs experienced changes in orientation and elongation in random directions and less VE-cadherin expression in response to hydrostatic pressure\textsuperscript{43}. However, other investigations involving cyclic pressurization and depressurization cyclic on cultured bovine aortic ECs found no significant changes in EC physiology\textsuperscript{48}.

4.6 Limitations & Future Directions

The current results should be interpreted within the constraint of several potential limitations. The aortic tissue that led to results from the rabbit model were contained within intact segments. This is in juxtaposition to the ECs in the current study that are of human origin and were grown in culture. HAECs represent a single cell type growing in a 2-dimensional monoculture environment free of the systemic changes that are found within the animal during homeostasis and experimentation, along with the influence of mechanical stimuli mentioned before. The flexible membranes in the Bioflex plates that the HAECs were cultured on are capable of applying uniform radial and circumferential cyclic strain. While the circumferential strain matched the values derived from the rabbit model, radial strain is difficult to measure in vivo. Thus, it remains to be determined if the radial strain imposed biaxially from the bioreactor matches the same strain conditions that are experienced in vivo.

Due to limitations with the imaging protocol, 2-photon imaging had to be conducted in a separate building from where tissue culture took place. Care was taken to insulate the cultures from temperature fluxes and mechanical disturbances during the transfer from bioreactor to microscope, however future work will aim to use an imaging system that is situated closer to the location of the bioreactor and tissue culture hood. The cultured HAECs were much more sensitive to environmental conditions as compared to the intact arterial segments. Attempts were made to keep the cultures at a stable temperature of 37°C while imaging, however heat was inevitably lost
during the process of loading and removing cells from the imaging baseplate. The cultures also
thrive in a humid atmosphere consisting of 5% CO₂, conditions that were unable to be met with
the current imaging setup.

Immunohistochemical staining for NPRC protein expression has been evaluated
previously in the aortic tissue collected from the CoA rabbit models. Commercially available
primary NPRC antibodies have been previously found to be non-specific and not reliable in
accurately evaluating NPRC expression (A. Alli, personal communication). An in-house NPRC
primary antibody was developed and used with our previous work, however, limitations on
availability prevented the use of this antibody for the current study. Once available, future work
will aim to compare the NPRC protein expression between the Physiologic Control and
Pathologic CoA groups. In addition, RT-PCR can be applied to gain a holistic understanding of
how cyclic strain regulates NPRC expression in both transcriptional and post-transcription
pathways.

While the rabbit model of CoA suggests that decreased NPRC expression is a secondary
consequence of CoA, it is possible that there is a casual genetic basis for decreased NPRC
expression that is also linked to the presentation of CoA. Prior genotyping of DNA samples from
human CoA patients was conducted to determine if HTN may be associated with the single
nucleotide polymorphism (SNP) NPRC variants rs2270915 and rs146301345. Both SNPs were
predicted to be highly intolerant and damaging, with a significant majority of the CoA patients in
the cohort having a rs2270915 genotype. Further studies of these named SNPs are needed, as
rs2270915 and rs146301345, either appearing alone or together, were not found to reliably
determine HTN status.

Additional work was attempted to recover the lost [Ca²⁺]ᵢ in response to CNP by treating
the Pathologic CoA HAECs with the NPRC-specific agonist, cANF. There is published evidence
to suggest that treatment of arterial tissue with cANF may induce relaxation of the vessels and
potentially restore normal EC function that was altered from hypertensive levels of strain. Past
work conducted within our lab indicates that cANF directly influences the HAECs in culture. It was found that 0.75μM of cANF increased the $[\text{Ca}^{2+}]_\text{in}$ of cultured HAECs, with the response blocked by the NPR-C inhibitor$^{20}$. In mice models, the right ventricular systolic pressure (RVSP) was significantly elevated in NPRC-knockout mice versus the NPRC-positive wildtype$^{31}$. This is indicative of NPRC’s role in the presence of HTN and RVSP function$^{31,49}$. Additionally, cANF may induce vascular smooth muscle relaxation in human small arteries, with the vasodilation effect being more pronounced in the vessels that were experiencing endothelial dysfunction and decreased NO bioavailability$^{50}$. Compiled together, results from these studies creates a framework for the implication of cANF in the recovery of normal function in diseased arterial endothelium and tissue$^{50}$. Initial attempts to apply cANF’s ability to recover lost endothelial function in our model was unsuccessful. Pathologic CoA cells incubated with 0.75μM of cANF did not exhibit any more $[\text{Ca}^{2+}]_\text{in}$ mobilization than those treated with a vehicle. The mechanism behind this behavior is currently unknown, however, the presented evidence suggests that cANF provides an intriguing paradigm for future studies to potentially recover lost endothelial function from CoA.

4.7 Conclusion

Collectively, the results of this investigation show that the in vitro model developed using HAECs in a tension bioreactor successfully mimicked the in vivo effects that CoA-induced mechanical stimuli have on the aortic tissue from the rabbit model. The $[\text{Ca}^{2+}]_\text{in}$ response to CNP in strain-conditioned HAECs was generally consistent with the data observed from interrogating ECs from the intact aortic segments. Therefore, this model can reasonably be used to help unravel the mechanisms behind coarctation-induced downregulation of NPRC and its involvement in the development of chronic HTN. Future investigations into these underlying mechanisms can potentially provide the framework to discover underlying new therapies for CoA-induced endothelial dysfunction and HTN; with the ultimate goal being to translate these results to the
clinic where life-changing treatments can then be applied to children and adults suffering from CoA.
BIBLIOGRAPHY


