The role of vascular endothelial growth factor in heart failure with preserved ejection fraction
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Thesis

THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN HEART FAILURE WITH PRESERVED EJECTION FRACTION

by

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ABSTRACT

To this day heart failure with preserved ejection fraction (HFpEF) remains a poorly understood malady. Half of all heart failure (HF) cases are HFpEF, and the prevalence of HF is on the rise. Unlike HF with reduced ejection fraction, HFpEF has no treatment options and is often times difficult to diagnose because victims of HFpEF often have pre-existing conditions. Vascular endothelial growth factor (VEGF) has been implicated in maintaining myocardial health and is thought to play a role in HFpEF. We sought to test the hypothesis that VEGF-A plays a role in HFpEF in a hypertensive murine model of HFpEF. Using Western blot analysis we found that there was an up regulation of VEGF-A in the homogenized left ventricle (LV) of our HFpEF mice. Unexpectedly, there was a down regulation of VEGF-A in the homogenized tissue from the aorta in those mice. To study the circulating levels of VEGF in our HFpEF mice we used an ELISA. We found that our HFpEF mice had similar levels of circulating VEGF as our control. This suggests that VEGF has paracrine/autocrine role in our HFpEF model rather than endocrine, like our human data suggested. To identify the cells responsible for the expression profile we saw in the homogenized tissue data we looked at the response of adult rat ventricular myocytes (ARVM) and vascular
smooth muscle cells (VSMC) to aldosterone stimulation at short (1hr) and long (24hr) time points at both physiological (50nm) and pathological (1µm) concentrations. To do this analysis we recruited the help of Western blot, ELISA and RT-PCR techniques to construct a consistent VEGF expression profile. The Western blot ARVM data showed statistically significant (P<0.05) increase in VEGF-A to pathological doses of aldosterone, especially at the longer time point. When we tested the VSMC using Western blot analysis, we found that the trend of our n=1 sample suggested a strong response to the physiological dose of aldosterone in the short term. Using the more sensitive ELISA technique to measure the VEGF content of our VCMS we increasing our sample size to n=4 and found no statistically significant (p=NS) response to aldosterone stimulation from the VSMC. However, looking at the trends in the data it is clear that VSMC increases VEGF in response to long-term physiological doses of aldosterone. This is contrary to what we found using Western blot analysis, so we queried the VEGF mRNA from the VSMC to settle the score. Unfortunately, this too proved fruitless. The RT-PCR data was not significant and the trend was that of the ARVM expression profile. We initially turned to VSMC because we hypothesized that they could contribute to the paracrine/autocrine activity similar to what we saw in the LV from the ARVM. It is unclear if VSMC play a role in HFpEF progression, but their lack of consistent response to aldosterone could potential explain the down regulation of VEGF-A we observed in the aorta of our HFpEF mice. We initially sough to test the hypothesis that VEGF-A plays a role in our
HFpEF mouse model, what we found was that ARVM contribute to localized VEGF-A increased production in the LV while in the aorta there is a down regulation of VEGF-A in our HFpEF model, we are unable to make any conclusion about VSMC response to aldosterone because of insufficient sample size. Thus in conclusion, it appears that VEGF-A does play a role in our HFpEF model specifically in a paracrine/autocrine manner in the LV where the ARVM contributes to the increased production of the cytokine.
TABLE OF CONTENTS

TITLE ........................................................................................................................................... i
COPYRIGHT PAGE .................................................................................................................. ii
READER APPROVAL PAGE .................................................................................................... iii
ACKNOWLEDGMENTS .............................................................................................................. iv
ABSTRACT .................................................................................................................................. v
TABLE OF CONTENTS .............................................................................................................. viii
LIST OF TABLES ....................................................................................................................... x
LIST OF FIGURES ..................................................................................................................... xi
LIST OF ABBREVIATIONS ......................................................................................................... xii
INTRODUCTION ......................................................................................................................... 1
Heart Failure With Preserved Ejection Fraction ................................................................. 4
Epidemiology ............................................................................................................................. 7
Vascular Endothelial Growth Factor ......................................................................................... 8
METHODS .................................................................................................................................. 14
Mouse Model of Heart Failure With Preserved Ejection Fraction ........................................ 14
Protein Analysis and Quantification ....................................................................................... 14
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Definitions of HFrEF and HFpEF</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Increased levels of circulating VEGF as seen in 32 heart failure patients</td>
<td>12</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hemodynamic and echocardiographic measurements of left ventricular diastolic function</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Interaction between the five mammalian VEGF molecules and their respective receptors</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>VEGF-A interaction with VEGFR-2 signaling cascade and physiological outcomes</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>Percent of HF patients with acute and stable HF whom have a difference in circulating VEGF levels</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>VEGF-A Expression in Mouse LV</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>VEGF-A Expression in Mouse Aorta</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>VEGF in Human Blood Serum</td>
<td>25</td>
</tr>
<tr>
<td>8</td>
<td>VEGF in Mouse Blood Serum</td>
<td>26</td>
</tr>
<tr>
<td>9</td>
<td>VEGF-A in Aldosterone Treated ARVM</td>
<td>28</td>
</tr>
<tr>
<td>10</td>
<td>VSMC Western Blot Measuring VEGF-A</td>
<td>31</td>
</tr>
<tr>
<td>11</td>
<td>VSMC ELISA Measuring VEGF-A</td>
<td>32</td>
</tr>
<tr>
<td>12</td>
<td>VSMC R-PCR Measuring VEGF-A</td>
<td>33</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

ACE .............................................................. angiotensin converting enzyme
ARVM ............................................................ Adult Rat Ventricular Myocyte
CECs .............................................................. circulating endothelial cells
EF ................................................................. Ejection Fraction
ELISA ........................................................... Enzyme-Linked Immunosorbent Assay
FDA ............................................................... Food and Drug Administration
GAPDH .......................................................... glyceraldehyde 3-phosphate dehydrogenase
HF ................................................................. Heart Failure
HFpEF ......................................................... Heart Failure Preserved Ejection Fraction
HFrEF ........................................................... Heart Failure Reduced Ejection Fraction
HPRT ............................................................ hypoxanthine-guanine phosphoribosyltransferase
LV ................................................................. Left Ventricle
LVEF ............................................................ Left Ventricle Ejection Fraction
RT-PCR ......................................................... Real Time Polymerase Chain Reaction
sEsel .............................................................. soluble E-selectin
TSP-1 ............................................................ Thombospondin-1
VEGF ............................................................ Vascular Endothelial Growth Factor
vWF .............................................................. von Willebrand factor
VSMC ........................................................... Vascular Smooth Muscle Cells
INTRODUCTION

Heart failure (HF) is a devastating disease that places a substantial burden on the healthcare system, the lives of affected individuals, and their families. The clinical manifestations of HF include dyspnea, fatigue with exercise intolerance as well as fluid retention leading to systemic edema (Yancy et al., 2013). HF is often difficult to diagnose because of varied symptoms and multiple causes of the disease (Yancy et al., 2013). Asymptomatic patients can still have HF; because of this, there is no single diagnostic test for HF (Yancy et al., 2013). HF involves impaired heart function and dysfunctions of the heart could be associated with imperfections of the various layers of the heart wall, the heart valves, the great vessels of the heart, or even metabolic conditions such as diabetes (Sharma & Kass, 2014; Yancy et al., 2013). Traditionally, ejection fraction (EF) served as an important metric with which to diagnose HF and also to classify patients with HF into two distinct categories: systolic and diastolic HF (Sharma & Kass, 2014). EF measures the percent of blood volume ejected with each beat and in turn is a benchmark for left ventricular (LV) performance (Sharma & Kass, 2014). LV dysfunction is as variable as the clinical symptoms in HF patients. HF patients exhibit a range of LV sizes and a varied percentage of left ventricular ejection fraction (LVEF): normal LV and preserved LVEF (LVEF≥50%), dilated LV and reduced LVEF (LVEF <40%), as well as a broad range in between (Table 1)(Yancy et al., 2013). HF patients with LVEF of less
than 40 percent are classified as ‘heart failure with reduced ejection fractions’ (HFrEF) patients (Table 1) (Yancy et al., 2013). The HFrEF patient population is significantly easier to diagnose and treat (Yancy et al., 2013). ACE (angiotensin converting enzyme) inhibitors, aldosterone antagonists, and B-adrenergic blockers are three classes of drugs available for HFrEF affected individuals (Braunwald, 2013). On the opposite end of the spectrum, patients with ‘heart failure with preserved ejection fractions’ (HFpEF) have a relatively normal LVEF of greater than or equal to fifty percent. These patients are difficult to diagnose and there are no evidence based therapies available to them (Table 1) (Yancy et al., 2013).
<table>
<thead>
<tr>
<th>Classification</th>
<th>EF (%)</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>I. Heart failure with reduced ejection fraction (HFrEF)</td>
<td>≤40</td>
<td>Also referred to as systolic HF. Randomized controlled trials have mainly enrolled patients with HFrEF, and it is only in these patients that efficacious therapies have been demonstrated to date.</td>
</tr>
<tr>
<td>II. Heart failure with preserved ejection fraction (HFpEF)</td>
<td>≥50</td>
<td>Also referred to as diastolic HF. Several different criteria have been used to further define HFpEF. The diagnosis of HFpEF is challenging because it is largely one of excluding other potential noncardiac causes of symptoms suggestive of HF. To date, efficacious therapies have not been identified.</td>
</tr>
<tr>
<td>a. HFpEF, borderline</td>
<td>41 to 49</td>
<td>These patients fall into a borderline or intermediate group. Their characteristics, treatment patterns, and outcomes appear similar to those of patients with HFpEF.</td>
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<tr>
<td>b. HFpEF, improved</td>
<td>&gt;40</td>
<td>It has been recognized that a subset of patients with HFpEF previously had HFrEF. These patients with improvement or recovery in EF may be clinically distinct from those with persistently preserved or reduced EF. Further research is needed to better characterize these patients.</td>
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Table 1: Definitions of HFrEF and HFpEF (Yancy et al., 2013)
**Heart Failure With Preserved Ejection Fraction**

HFpEF is regularly termed diastolic HF because of the abnormalities observed during LV relaxation and filling (Horgan, Watson, Glezeva, & Baugh, 2014). At the end of contraction (systole), the heart engages in a process of isovolumetric relaxation in which 80% of the LV filling occurs and marks the beginning of diastole. The final stage of diastole is distinguished by atrial contraction which fills the remainder of the LV (Figure 1) (Horgan et al., 2014.). Diastolic abnormalities are often caused by increased fibrosis of the cardiac interstitium and compensatory hypertrophy, leading to reduced compliance of the LV. Compliance is the measure of the tissue’s pressure-volume relationship. In HFpEF the compliance of the LV is diminished meaning the LV has impaired relaxation and greater stiffness, this makes the LV more difficult to fill, drives up diastolic pressure, and as a result exacerbates LV hypertrophy (Horgan et al., 2014). The 2013 ACCF/AHA Guideline for the Management of Heart Failure sites three criteria to make a HFpEF diagnosis: the patient must have clinical signs or symptoms of HF, such as dyspnea, fatigue, or exercise intolerance; a preserved LVEF; and evidence of LV diastolic dysfunction (Yancy et al., 2013). Diastolic dysfunction is a pivotal characteristic in diagnosing HFpEF. Diastolic dysfunction is termed as such because it occurs during diastole phase of the cardiac cycle where at low filling pressure, the ability for the LV to relax is impaired and it is unable to accommodate the necessary blood volume (Horgan et al., n.d.). LV function can be determined either by placing an invasive cardiac
catheter retrograde into the LV using the atria for access, or by performing an non- invasive Doppler echocardiography (Horgan et al., 2014).

Yet another characteristic of HFP EF is an increase stiffening of both the ventricle and the aorta (Borlaug & Kass, 2008). The stiffening of the aorta in particular has both pathological and physiological consequences in the ventricle. In a healthy individual, the vessels are compliant. Via ventricular-vascular coupling, a healthy heart maintains a normal ejection fraction, prevents a large swing in pressure, and as a result avoids both vascular and end-organ damage. HFP EF individuals with ventricular-arterial stiffening are sensitive to blood volume changes and become hypertensive during periods of stress (Borlaug & Kass, 2008).
Figure 1: Hemodynamic and echocardiographic measurements of left ventricular diastolic function (Horgan et al., 2014)
Epidemiology

According to The 2013 ACCF/AHA Guideline for the Management of Heart Failure, Americans over the age of 40 have a 20% chance of developing HF in their lifetime (Yancy et al., 2013). The incidence of HF raises with age and other risk factors such as hypertension, diabetes mellitus, metabolic syndrome, and atherosclerotic disease (Yancy et al., 2013). About half off all HF patients have HFrEF (Braunwald, 2013). The traditional HFrEF population is perceived to be majority white, elderly females with hypertension (Sharma & Kass, 2014). However, as more studies are conducted on global HFrEF populace, the emerging data suggests patient population is far more diverse than previously reported (Horgan et al., 2014). HFrEF is prevalent in a younger, predominantly black population, with elevated rates of hypertension, ventricular hypertrophy, and obesity (Sharma & Kass, 2014). As greater numbers of diverse populations are surveyed, HFrEF seems to disregard not only race, but also sex and ethnicity (Sharma & Kass, 2014).
Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a cytokine that is associated with blood-vessel formation in adults (Olsson, Dimberg, Kreuger, & Claesson-Welsh, 2006). Recently VEGF has been implicated to have an important role in myocardial function, and in turn, HF (Taimeh, Loughran, Birks, & Bolli, 2013). Five known mammalian VEGF subtypes are known (Ferrara, Gerber, & LeCouter, 2003). Their effects are mediated via three distinct receptor tyrosine kinases as seen in Figure 2 (Taimeh et al., 2013). In response to the cytokine stimuli, VEGF receptors have a multitude of effects relating to the vasculature: angiogenesis; new capillary formation from pre-existing vasculature, arteriogenesis; remodeling of pre-existing vessels, and vaculogenesis; and de novo vessel formation (Taimeh et al., 2013). The angiogenic effects are elicited mainly via VEGF-A binding to VEGFR-2. Paradoxically, VEGF-A has a much greater affinity for VEGFR-1 than VEGFR-2; VEGFR-1 is a known negative regulator of VEGF-A. Apart from new vessel formation, VEGF-A interaction with VEGFR-2 has been shown to elicit recruitment and homing of stem cells, increase cell survival, mediate apoptosis, and play a role in vascular permeability as well as vasodilation (Figure 3) (Taimeh et al., 2013). Because of the role VEGF plays in the body it could be of great importance in HFpEF. For example VEGF-A in particular is proven to be of importance in mediating the compensatory cardiac hypertrophy seen in HF (Izumiya et al., 2006). In a cohort of 32 heart failure patients with LV dysfunction, the circulating levels of VEGF were markedly
increased when compared to control (Table 2) (Martínez-Sales et al., 2011). Over 50% of patients with acute HF had a marked increase in circulating VEGF levels, while a diminished percentage of patients with stable HF showed the same increase (Figure 4) (Martínez-Sales et al., 2011). Furthermore, VEGF inhibitors, a new class of anticancer drugs, has shown to most commonly illicit hypertension that can lead to LV dysfunction in cancer patients trying this new therapy (Tocchetti et al., 2013). VEGF inhibitors increase blood pressure in almost 100% of cancer patients that were treated with this type of therapy (Small, Montezano, Rios, Savoia, & Touyz, 2014). In addition, the US Food and Drug Administration (FDA) found that 19%-67% of patients treated with the various FDA approved VEGF inhibitors developed hypertension (Small et al., 2014). Thus, we sought to test the hypothesis that VEGF-A plays a role in HFP EF.
Figure 2: Interaction between the five mammalian VEGF molecules and their respective receptors (Taimeh et al., 2013)
Figure 3: VEGF-A interaction with VEGFR-2 signaling cascade and physiological outcomes (Taimeh et al., 2013)
Table 2: Increased levels of circulating VEGF as seen in 32 heart failure patients with LV dysfunction (Martínez-Sales et al., 2011).

<table>
<thead>
<tr>
<th></th>
<th>Acute HF</th>
<th>Stable HF</th>
<th>Controls</th>
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</thead>
<tbody>
<tr>
<td><strong>Endothelial markers</strong></td>
<td></td>
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<tr>
<td>CECs (cells/ml)</td>
<td>36.9 ± 15.3</td>
<td>21.5 ± 10.0</td>
<td>8.6 ± 5.2</td>
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<tr>
<td>vWF:Ag (%)</td>
<td>325 ± 101</td>
<td>231 ± 82</td>
<td>122 ± 27</td>
</tr>
<tr>
<td>sEsel (ng/ml)</td>
<td>26.3 ± 15.2</td>
<td>21.9 ± 11.9</td>
<td>17.1 ± 6.4</td>
</tr>
<tr>
<td><strong>Angiogenesis markers</strong></td>
<td></td>
<td></td>
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<tr>
<td>VEGF (pg/ml)</td>
<td>411 ± 312</td>
<td>259 ± 226</td>
<td>143 ± 64</td>
</tr>
<tr>
<td>TSP-1 (μg/ml)</td>
<td>57.8 ± 19.1</td>
<td>49.4 ± 15.3</td>
<td>47.1 ± 9.6</td>
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Figure 4: Percent of HF patients with acute and stable HF whom have a difference in circulating VEGF levels. (Martínez-Sales et al., 2011).
METHODS

Mouse Model of Heart Failure With Preserved Ejection Fraction

HFpEF was modeled in wild-type male C57BL/6 mice that underwent uninephrectomy at 10-12 weeks. An ALZET® pumps containing 0.3ug/hr d-aldosterone (HFpEF) or 0.9% saline (control) were implanted. The mice were given 1.0% sodium chloride drinking water for 4-weeks, and had weekly heart rate and blood pressure measurements as well LV structure and function measurements via echocardiography. This is a model of hypertension induced HFpEF. After 4 weeks the mice were sacrificed and their LV tissue, aorta, blood, spleen, and lungs were collected and appropriately preserved.

Protein Analysis and Quantification

After 4 weeks, left ventricular and aortic tissue was collected from wild-type male C57BL/6 mice that underwent uninephrectomy at 10-12 weeks. The tissue was then frozen in liquid nitrogen and stored at -80°C until needed. Tissue samples were homogenized using a Bio-Gen PRO200 Homogenizer machine in 1X lysis buffer containing PMSF. The homogenized tissue was then centrifuged and the supernatant collected and stored at -80°C. After thawing the supernatant on ice, to each cuvette, 2ul was added into 988ul of diluted dye reagent (BioRad Protein Assay Dye Reagent Concentrate, Cat #500-0006, refrigerated). Using a Spectrogenic Genesys 5 instrument the total protein concentration was determined using 1ml of dH₂O as a blank.
Western Blot Analysis

An equal amount of protein was loaded onto Lonza PAGEr Gold Precast 12% Tris-Glycine Gels, and then subjected to electrophoresis. Protein content was determined via spectrophotometry using a Spectronic Genesys 5 instrument as described above. After thawing each sample on ice a calculated amount of dye (Laemmli’s SDS-Sample Buffer (4x, reducing) Boston Bioproducts Cat #BP-110R) was added, samples were then boiled for 5 minutes. Typically, 50-75ug of protein was loaded into each well of the polyacrylamide gel. Invitrogen’s BenchMark Prestained Protein Ladder was also loaded onto the polyacrylamide gel. The BioRad PowerPac Basic power supply was used to perform the protein gel electrophoresis. The protein was then transferred onto polyvinylidene difluoride membrane (GE Healthcare Amersham Hybond) and blocked in a solution of 5% milk in TBST. Membranes were then incubated overnight in 4°C with VEGF-A P-20 (Santa Cruz Biotechnology sc-1836) purified goat polyclonal antibody raised against the N-terminus of VEGF-A of mouse and rat origin. The following day membranes were washed with TBST then incubated with horseradish peroxidase conjugated secondary antibody donkey anti-goat IgG-HRP (Santa Cruz Biotechnology sc-2033). The membrane was then developed using detection reagents (GE Healthcare Amersham ECL Western Blotting Detection Reagent RPN2209), autoradiography film (HyBlot CL autoradiography film E3012), and MACHINE INFO. To determine the amount of GAPDH
(glyceraldehyde 3-phosphate dehydrogenase), as a loading control measure, the membrane was then stripped (ThermoScientific RestoreTM Western Blot Stripping Buffer, Product#21059) and re-probed with primary (abcam Anti-GAPDH antibody-Loading Control ab9484) and secondary (Santa Cruz Biotechnology sc-2005 goat anti-mouse IgG-HRP) antibodies, then developed using the same method as above.

**Isolation and Treatment of Rat Aortic Smooth Muscle Cells**

Rat aortic vascular smooth muscle cells (VSMC) were cultured from adult male Sprague-Dawley rats approximately 200-220g in weight. Using scissors and tongs the aorta was removed and placed in warm M199 media. In a Nuaire biological safety cabinet the fat and connective tissue was removed from the aorta, then a longitudinal cut was made and the endothelium was scraped off. The aorta was then transferred into a collagenase solution and placed in a Fisher Scientific Isotemp CO₂ incubator at 37°C for 30 minutes. Next the adventitial layer was peeled off using tongs. The remaining tissue was minced into 1-2mm size pieces, then digested in a collagenase and elastase solution in a Isotemp CO₂ incubator at 37°C for 1-2 hours. The remaining cells were centrifuged for 10 min at 400 x g and then re-suspended in M199 media with 1% P/S, 10% FBS, and 2mM L-Glutamine. The cells were plated onto P60 plates and incubated for 1 week before being passes for the first time. After the second passaged the cells were treated with aldosterone and collected at various time points.
Isolation and Treatment of Adult Rat Cardiac Myocytes

Adult rat ventricular myocytes (ARVM) were isolated from adult male Sprague-Dawley rats. The heart was removed then attached to a perfusion pump to remove the blood (K-H buffer consisting of 2.36M NaCl, 0.094M KCl, 0.024M MgSO4, 0.024M KH2PO4, and 0.5M NaHCO3) and begin the digestion process (collagenase and hyaluronidase in K-H buffer). After ~25 min of perfusion the heart was transferred to P60 containing trypsin in K-H buffer and digested in a shaking 37°C water bath for 30-40 min. The tissue was then pipette up and down 10 times in a Nuaire biological safety cabinet, then filtered through 80-micron mesh gauze and centrifuged. The supernatant was aspirated and the re-suspended pellet was then ran through BSA. BSA was then aspirated and DMEM was added then the cells were plated on P35 plastic culture dishes that were coated in laminin solution. The ARVM were allowed to adhere and grow for 24hrs in Isotemp CO2 incubator at 37°C before treatment with aldosterone and collection.

Mouse VEGF Immunoassay

The amount of VEGF in mouse blood serum and rat VSMC lysate were determined via VEGF ELISA kit (R&D quantikine ELISA Kit MMV00). After 4 weeks mouse blood was collected from wild-type male C57BL/6 mice that
underwent uninephrectomy at 10-12 weeks then centrifuged for 20 min at 2000 x g. Both mouse blood serum and rat VSMC lysate were stored at -80°C until they were ready to be used in the ELISA assay. The ELISA kit manufacturer protocol was followed. The serum samples were diluted 5-fold as directed, the mouse VEGF standard was diluted and loaded onto the plate as prescribed. The plate was read via a X machine and Y software was used to interpret the results.

RNA Isolation from Adult Rat Vascular Smooth Muscle Cells

The RNeasy® Lipid Tissue Mini Kit by QIAGEN® was used for RNA isolation from adult rat vascular smooth muscle cells. All of manufacturers protocols were followed. Growth media was removed from cells, then 1mL of TRlzol® Reagent was added and cells were scraped off plate then transferred to a clean 1.5mL microfuge tube for storage at -80°C. Samples were thawed then 0.2mL of chloroform was added. After shaking the cells, they were incubated at room temp for 3 min before centrifuging at 12,000g for 15 min at 4°C to remove the TRlzol®. The aqueous phase was carefully removed and placed into a new 2mL collecting tube for RNA isolation. The Qiacube was used according to manufacturer’s instructions. Analysis of RNA yield was quantified using an ND-1000 spectrophotometer and accompanying software.
cDNA Reaction Protocol

The High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems was used for initiating cDNA synthesis. The manufacturers suggested protocol was followed. A 2X Master Mix consisting of 10X RT buffer, 25X dNTP mix, 10X RT random primers, MultiScribe reverse transcriptase and water was made. Samples were prepared by adding 10 uL of RNA sample to 10 uL of the Master Mix and 10 uL of water. Reverse transcription was then performed in a thermal cycler in 4 steps: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, then hold at 4°C. At the end of the cycle 20 uL of cDNA was added to 480 uL of water and stored at -80°C.

Real Time Polymerase Chain Reaction Protocol

The Applied Biosystems ViiA7 Real-Time PCR System was used to measure and quantify RNA isolation from adult rat vascular smooth muscle cells. In a 96 well plate, VEGF and GAPDH contents were measured in duplicate. In each well a 20uL volume consisting of 8uL of sample, 10uL of Sybr® Green, and 2uL of primer was added. Results were analyzed via the Applied Biosystems ViiA7 Real-Time PCR System.
RESULTS

General

VEGF-A expression was examined in the aorta, LV, and serum samples of HFpEF mice. The precise characteristics of the HFpEF mouse model used can be seen in the November 2014 paper published in *Circulation Heart Failure* entitled *Effects of Adiponectin on Calcium Handling Proteins in Heart Failure with Preserved Ejection Fraction* (Tanaka et al., 2014a). Generally, the mice were used to exhibit a hypertension-induced HFpEF model (Tanaka et al., 2014a). HFpEF mice showed signs of LV hypertrophy and diastolic dysfunction, similar to what clinicians observe in the HFpEF patient population (Tanaka et al., 2014b). To gain insight into the molecular mechanism associated with HFpEF, VEGF expression in two distinct rat tissues cultures treated with aldosterone were analyzed. Adult rat ventricular myocyte (ARVM) and vascular smooth muscle cells (VSMC) were isolated, cultured, then treated with physiological and pathological doses of aldosterone at various time points. After collecting and lysing the cells, VEGF content was determined using three distinct methods. Total VEGF protein content was determined via Western blot analysis and enzyme-linked immunosorbent assay (ELISA). The mRNA content in the VSMC was determined via real time polymerase chain reaction (RT-PCR) analysis.
Vascular Endothelial Growth Factor in Tissue

In homogenized HFpEF LV mouse tissue (n=5) there was a statistically significant increase in VEGF-A expression as compared to the LV tissue of the WT mice (n=4) (P<0.05 Figure 5). This confirmed prior studies conducted by Izumiya, who found that VEGF promoted the compensatory cardiac hypertrophy seen in HFpEF (Izumiya et al., 2006). However, when looking at the homogenized aortic tissue of the HFpEF mice, an unfamiliar trend emerged. The homogenized aortic tissue data suggests there is a statistically significant decrease in VEGF levels in the aorta of HFpEF mice (n=15) when compared to the control (n=12) (P<0.05 Figure 6). Sampling a cohort of 15 wild-type male C57BL/6 mice infused with aldosterone for four weeks has not only observable phenotypic consequences, but also unexpected changes in cytokine levels when compared to the 12 wild-type male C57BL/6 mice infused with saline for four weeks.
Figure 5: VEGF-A Expression in Mouse LV. HFrEF homogenized LV mouse tissue (n=5) was compared to control LV mouse tissue (n=4) via Western blot analysis. For the Western blot control Coomassie stain was used. In a two-tailed t-test results were significant P<0.05.
Figure 6: VEGF-A Expression in Mouse Aorta. HFpEF homogenized aortic mouse tissue (n=15) was compared to control aortic mouse tissue (n=12) via Western blot analysis. For the Western blot control GAPDH anti-body was used. In a two-tailed t-test results were significant P<0.05.
Vascular Endothelial Growth Factor in Serum

Along with tissue, the blood from the HFpEF (n=15) and control (n=12) mice was collected. The blood from the mice was collected via the heart using a syringe, and then centrifuged to isolate the serum. Human serum was venously collected from consenting HFpEF patients (n=16) and 3 control patients (n=3) with no history of cardiac disease. The human serum data suggested there would be statistically significant compensatory increase (419.6 ng/mL vs. 68.3 ng/mL) in circulating VEGF in the HFpEF population (P<0.05 Figure 7). However, the results of the mouse blood serum data were quite different. There seems to be no difference between blood serum VEGF level in HFpEF vs. control (P=NS Figure 8). The large cohort of 12 controls and 15 HFpEF mice had almost identical VEGF mean serum levels (53.6 pg/mL vs 53.8 pg/mL).
Figure 7: VEGF in Human Blood Serum. VEGF concentration (pg/mL) is measured in HFrEF human blood serum (n=16) and control human blood serum (n=3) samples via ELISA. In a two-tailed t-test results were significant P<0.05.
Figure 8: VEGF in Mouse Blood Serum. VEGF concentration (pg/mL) is measured in HFpEF mouse blood serum (n=15) and control mouse blood serum (n=12) samples via ELISA. In a two-tailed t-test results were not significant P=NS.
Vascular Endothelial Growth Factor in Adult Rat Ventricular Myocytes

The ARVM’s were isolated from the heart tissue of adult male Sprague-Dawley rats. After culturing the ARVM’s (n=3) in a CO₂ incubator at 37°C, they were treated with physiological (50nm) and pathological (1um) doses of aldosterone. At both doses, long term (24hr) and short term (1hr) responses to aldosterone treatment were measured. Control ARVM not treated with aldosterone were incubated and measured in duplicate n=6. In the physiological range (50nm) only long term exposure to aldosterone elicits a statistically significant increase in VEGF (P<0.05 Figure 9). However, when given a higher does (1um) of aldosterone the ARVM’s had a statistically significant increase in VEGF independent of time (P<0.05 Figure 9). The most significant increase in VEGF expression came from the group of ARVM’s treated with a pathological dose of aldosterone for 24hrs. This group had almost three times the amount of VEGF when compared to control. Thus higher doses of aldosterone are able to stimulate VEGF-A as seen in ARVM.
Figure 9: VEGF-A in Aldosterone Treated ARVM. Cultured ARVM (n=3) were treated with 50nm and 1um aldosterone for 1hr and 24hr. VEGF-A content was measured via Western blot analysis and compared to control (n=6). For the Western blot control GAPDH anti-body was used. In a two-tailed t-test results were significant P<0.05 (*).
Vascular Endothelial Growth Factor in Vascular Smooth Muscle Cells

The VSMC’s were isolated from the aorta of adult male Sprague-Dawley rats. The isolated VSMC’s were placed on a P60 plate, then allowed to adhere and grown for a week in a CO₂ incubator at 37°C. Upon reaching 90% confluence, one plate was passed to four, then allowed to grow for three additional days. After the second passage the VSMC’s were treated with physiological (50nm, 100nm) and pathological (1µm) doses of aldosterone. Both long (24hr, 48hr) and short (1hr) term exposure to aldosterone were measured. In the initial experiment the quantity of VEGF was determined via western blot (P=NS Figure 10). Due to the small sample size (n=1) the experiment did not yield statistically significant results, however, a trend was observed and a more sample was queried via ELISA. The western blot data hinted that at treatment of both physiological (100nm) and pathological (1µm) concentrations of aldosterone, VSMC responded by increasing VEGF production in the short term (P=NS Figure 10). To test this hypothesis the sample size was increased to N=4, a lower physiological dose of aldosterone was tested (50nm), and the 48hr exposure was removed from the protocol. The VEGF protein content (pg/mL) from the four VSMC samples was then tested using an R&D quantikine ELISA Kit (P=NS Figure 11). Unfortunately, no statistically significant trend appeared (P=NS Figure 11). It does seem that in the long term (24hr) at physiological concentrations (50nm and 100nm) the VSMC increase their VEGF production (P=NS Figure 11). This data was contrary to what was observed when
performing the Western blot analysis (P=NS Figure 10). RT-PCR was used to
determine the subtle changes in mRNA quantity of VSMC exposed to
physiological (50nm) and pathological (1µm) aldosterone concentrations at both
long (24hr) and short (1hr) time intervals. VEGF, GAPDH, and HPRT
(hypoxanthine-guanine phosphoribosyltransferase) primers from Integrated
DNA Technologies were used. GAPDH and HPRT served as controls for the RT-
PCR data analysis. The data as presented in Figure 12 suggests that VSMC
respond most to pathological (1µm) long (24hr) exposure to aldosterone (P=NS
Figure 12). The RT-PCR data is of not significant because of the small sample
size and further muddles both the western blot and ELISA VSMC data.
**Figure 10: VSMC Western Blot Measuring VEGF-A.** VEGF-A expression measure in cultured adult rat VSMC (n=1) treated with 100nm and 1um aldosterone for 1hr, 24hr, and 48hr time periods. For the Western blot control GAPDH anti-body was used. In a two-tailed t-test results were not significant P=NS.
**Figure 11: VSMC ELISA Measuring VEGF.** VEGF concentration (pg/mL) was measured in cultured adult rat VSMC (n=4) treated with 50nm, 100nm, and 1um aldosterone at 1hr and 24hr time points. In a two-tailed t-test results were not significant P=NS.
Figure 12: VSMC RT-PCT Measuring VEGF. VEGF mRNA content was measured in cultured adult rat VSMC (n=1) treated with 50nm and 1um aldosterone at 1hr and 24hr time points. In a two-tailed t-test results were not significant P=NS.
DISCUSSION

In this study, we sought to test the hypothesis that VEGF-A plays a role in HFpEF. VEGF-A is essential in maintaining regular myocardial function, the lack of which, causes the heart to transition from hypertrophy to HF as shown by Izumiya et al. (Giordano et al., 2001; Izumiya et al., 2006). In cancer patients treated with VEGF inhibitors, Tocchetti et al. found they developed a multitude of cardiac issues, such as hypertension and diastolic dysfunction (Taimeh et al., 2013). Borlaug and Kass characterized the importance of ventricular-vascular coupling in the progression of HF, which motivated our investigation to query beyond the heart and examine the vasculature (Borlaug & Kass, 2008). We aimed to study the importance of VEGF-A in the heart, aorta, and serum of a hypertension-induced HFpEF mouse model. We tested homogenized tissue samples of aldosterone-infused wild type mice that developed HFpEF, evident by their preserved ejection fraction, hypertension, LV hypertrophy, and diastolic dysfunction. To gain insight into the molecular basis for the phenotypic changes we observed in the ventricle and vasculature of HFpEF mice, we cultured ARVM and VSMC, treated them with aldosterone, then observed changes in VEGF-A at both the protein and mRNA level using various molecular biology techniques.

In a first approach we analyzed VEGF-A in the homogenized ventricle and aorta of our HFpEF mice and compared them with sham mice using Western blot analysis. After 4 weeks of chronic exposure to aldosterone, the mice began to develop HFpEF. HFpEF mice had an increase in VEGF-A expression in the
ventricle, and a decrease in the aorta when compared to control. The former finding was somewhat expected as it is clear VEGF-A plays an essential role in maintaining cardiac function (Giordano et al., 2001; Izumiya et al., 2006). However, the latter finding was both novel and surprising. Other studies suggest that due to the ventricular-vascular relationship, a synergistic increase in VEGF-A in the aorta should appear (Borlaug & Kass, 2008; Taimeh et al., 2013). With the lack of endogenous VEGF-A production in the vasculature we questioned if there was a compensatory increase in systemic VEGF-A production.

A paper published in 2011 by Martínez-Sales et al. led us to look at the serum VEGF-A levels in both the HFpEF patient population as well as our HFpEF mouse model (Martínez-Sales et al., 2011). We expected to confirm Martínez-Sales et al. findings and confirm an increase in VEGF-A serum levels in both the HFpEF patient population and the HFpEF mice. However, in only the former group that finding held true. Of the sixteen HFpEF patients whose serum was analyzed, we found that on average they had a four-fold increase in serum VEGF-A when compared to control. The HFpEF mouse VEGF-A sera were no different from control. In fact, their VEGF-A serum levels were almost identical (53.6 pg/mL vs 53.8 pg/mL). The curious phenotype of our HFpEF mice observed thus far is one of evident paracrine and autocrine effects of VEGF-A in the heart, lack of endocrine VEGF-A secretion, and failure of endogenous production of VEGF-A in the vasculature. A molecular biological explanation to the findings was warranted.
In the next phase of our study, we attempted to narrow down the possible cell types that could be implicated in the VEGF-A expression profiles we observed. Due to their role in the heart’s VEGF paracrine signaling, we isolated and examined ARVM (Giordano et al., 2001). In the aorta we chose to study the VSMC because of their known response to aldosterone (Giordano et al., 2001; Pruthi et al., 2014). After exposing both cell types to physiological and pathological concentrations of aldosterone at long and short intervals, it was interesting to find ARVM responded to both concentrations of aldosterone, and the longer the exposure, the greater the increase in VEGF-A expression. Conversely, the single set of VSMC tested using western blot analysis we observed a short-term response to aldosterone at only the physiological range. The ARVM data corroborated what we observed in the homogenized ventricle data. With the VSMC data we could now explain the reason we saw a decrease in VEGF-A in our homogenized aorta. Potentially what we observed in the vasculature was a temporal relationship between aldosterone stimulation and VEGF-A expression. With VEGF-A elevated for only a short period of time in the VSMC, the cytokine levels then diminished to the miniscule amounts that we detected when western blotting our homogenized aorta. This speculation led us to further explore VSMC expression of VEGF. Although we saw a positive trend in the initial Western blot data, it was not significant due to the small sample size. To remedy this, in a separate experiment, we isolated and treated three more vascular smooth muscle cell lines with aldosterone, then measured their VEGF
expression via ELISA. Unfortunately, the ELISA data did not corroborate what we previously found in our Western blot experiment. The ELISA data suggests VSMC respond most to long-term physiological exposure to aldosterone, not short-term treatment. This trend is not statistically significant; an even larger sample size needs to be queried. The protein expression in the VSMC has led us in two different directions: the Western blot data supports our hypothesis regarding the homogenized tissue data, while the ELISA data calls for a re-analysis of our results. To help settle this discrepancy we turned to RT-PCR to look at the mRNA content in our aldosterone treated VSMC. What we found further complicated our VSMC aldosterone response hypothesis. The RT-PCR data showed a trend that hinted at the possibility that VSMC, at least at the mRNA level, respond most to long-term pathological doses of aldosterone while the physiological doses have little to no effect on VEGF mRNA. The RT-PCR data was not significant and the trend was that of the ARVM expression profile. We initially turned to VSMC because we hypothesized that they could contribute to the paracrine/autocrine activity similar to what we saw in the LV from the ARVM. It is unclear if VSMC play a role in HFpEF progression, but their lack of consistent response to aldosterone could potential explain the down regulation of VEGF-A we observed in the aorta of our HFpEF mice. We initially sough to test the hypothesis that VEGF-A plays a role in our HFpEF mouse model, what we found was that ARVM contribute to localized VEGF-A increased production in the LV while in the aorta there is a down regulation of VEGF-A in our HFpEF model,
we are unable to make any conclusion about VSMC response to aldosterone because of insufficient sample size. Thus in conclusion, it appears that VEGF-A does play a role in our HFpEF model specifically in a paracrine/autocrine manner in the LV where the ARVM contributes to the increased production of the cytokine.
REFERENCES

http://doi.org/10.1016/j.hfc.2007.10.001


http://doi.org/10.1038/nm0603-669

http://doi.org/10.1073/pnas.091415198


Response to Pressure Overload. *Hypertension, 47*(5), 887–893.  
http://doi.org/10.1161/01.HYP.0000215207.54689.31

http://doi.org/10.3233/DMA-2011-0801

http://doi.org/10.1038/nrm1911

http://doi.org/10.1161/ATVBAHA.113.302854

http://doi.org/10.1161/CIRCRESAHA.115.302922

http://doi.org/10.1016/j.cjca.2014.02.011


http://doi.org/10.1161/CIR.0b013e31829e8776
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EDUCATION

Boston University School of Medicine, Boston, MA
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Boston University, College of Art and Science, Boston, MA
May 2012
Bachelor of Arts in Biochemistry and Molecular Biology
Graduated Cum Laude

RESEARCH EXPERIENCE

WHITAKER CARDIOVASCULAR INSTITUTE, Boston, MA July 2014-Present
Masters Thesis Work
• Explored the role of VEGF in heart failure with preserved ejection fraction
• Performed various biochemical assays to quantify VEGF content
• Cultured smooth muscle cells

BETH ISREAL DEACONESS MEDICAL, Boston, MA July 2012-Nov 2012
Research Assistant
• Assay Development and Optimization of Protein Purification in the Hemostasis/Thrombosis Department
• Performed PCR purification and prepared DNA for sequencing.
• Transformed XL-Blue cells, and expressed protein in BL-21 cells.
• Performed protein purification via HPLC.
• Performed immunoassays: ELISA, immunoprecipitation, SDS-PAGE.
• Inventoried and ordered supplies for laboratory exercises.

GENZYME, Waltham, MA Summer 2011
Summer Intern
• Supported 3 separate research projects in the In-Vitro Biology R&D
Department.

• Explored potential oncogenic targets for small molecule inhibition via in-vitro activity assays.
• Maintained HEK293 cell lines and performed ELISA cell-based assays.
• Optimized protein expression in E.coli by varying growth conditions, and ran Western Blot’s.
• Created a 20-minute presentation using excel and power point to showcase the data I collected.

ADDITIONAL EXPERIENCE & LEADERSHIP

INFLUENCERS@, Boston, MA Dec 2012- Aug 2013
Boston General Manager
• Organized events and promote brand awareness on college campuses and via social media.
• Worked closely with CEO and Developers to successfully launch ChatterMob mobile app and website.
• Oversaw day-to-day Boston office operations.

ALBANY MEDICAL CENTER, Albany, NY Summer 2007 & 2010
Summer Volunteer
• Worked with other volunteers to improve patient comfort and care in the Emergency Department

RUGGED CROSSFIT, Boston, MA January 2014-Present
Level 1 Crossfit Trainer

BOSTON UNIVERSITY, Boston MA September 2014-Present
Physiology and Biochemistry Tutor

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Junior Taekwon-Do Instructor
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SKILLS

Lab: Aseptic Technique, Assay Development, Western Blot, Cell Culture, PCR, HPLC, ELISA, Bacterial Transformation, Plasmid Purification, Pour and Run SDS-page and Agarose gels, Gel extraction
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