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THE MECHANICAL PROPERTIES OF NON-FAILING AND FAILING HUMAN MYOCARDIUM

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THE MECHANICAL PROPERTIES OF NON-FAILING
AND FAILING HUMAN MYOCARDIUM

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine at the University of Kentucky

By

Cheavar Anthony Blair

Lexington, Kentucky

Director: Dr. Kenneth S. Campbell, Professor of Physiology

Lexington, Kentucky

2017

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ABSTRACT

THE MECHANICAL PROPERTIES OF NON-FAILING AND FAILING HUMAN MYOCARDIUM

Heart failure is a clinical syndrome that manifests when there are structural and functional impairments to the heart that reduces the ability of the ventricles to fill or eject blood. The syndrome affects ~6 million Americans and is responsible for nearly 300,000 deaths annually. At the core of the syndrome are dysfunctional sarcomeres, the machinery that drives cardiac contraction and relaxation. By assessing the mechanical properties of human cardiac tissue, the information provided in this dissertation will provide data that demonstrates how sarcomeric dysfunction contributes to heart failure in the left and right ventricles. Additionally, these data will supply information on how probable therapeutics impact the mechanical properties of the heart and the clinical implications. Thus, the overall objective of this project is to assess the mechanical properties of failing and non-failing human myocardium while concomitantly studying the molecular mechanisms contributing to heart failure and work towards therapy.

Mechanical experiments were performed with human cardiac samples obtained from patients who were receiving heart transplants and from organ donors who

did not have a history of heart failure. Cardiac samples were homogenized and chemically permeabilized (pores in the membrane). Multicellular preparations from failing and non-failing hearts were attached between a force transducer and a motor to determine the mechanical properties.

In the first study, we compared the mechanical properties of cardiac samples from the right and left ventricles of non-failing and failing hearts, as well as determined the relative phosphorylation levels of specific sarcomeric proteins. The results show that in non-failing hearts, calcium sensitivity was higher in the left ventricle, and in failing hearts, calcium sensitivity was higher in the right ventricle. The shift in the pattern of the calcium sensitivity data from non-failing samples to failing samples underpin a statistical interaction between heart failure status and the ventricles of the heart for calcium sensitivity. This interaction suggests that heart failure is altering the sensitivity of the myofilament to Ca^{2+} differently in the right ventricle. The mechanical data also demonstrated that heart failure significantly reduced isometric force and maximum power in both ventricles. Biochemical assays suggest that the cause of the interaction observed in the calcium sensitivity data is driven by the phosphorylation profile of sarcomeric proteins.

We then determined the effects of two small molecules (omecantiv mecarbil and para-Nitroblebbistatin) on the mechanical properties of human myocardium. The results of those studies demonstrate that omechantiv mecarbil increases calcium

sensitivity and slows the rate of force development in a dose-dependent manner without altering maximum isometric force. Conversely, para-Nitroblebbistatin reduced isometric force, power, and calcium sensitivity without changing shortening velocity or the rate of force development.

Lastly, we measured the effects of engineered troponins on the mechanical function of failing tissue. The results show that troponin C and troponin I designed to either increase or decrease calcium sensitivity can significantly increase or decrease calcium sensitivity without altering maximum force, shortening velocity or the rate of tension development.

The findings reported in this dissertation have revealed novel mechanical data from non-failing and failing human cardiac tissue. These data present three significant results. First, the right vs. left ventricular comparison data shows that heart failure in humans reduces maximum force and power in both ventricles equally while altering myofilament calcium sensitivity of the left and right ventricles in different ways. The change in calcium sensitivity may reflect ventricle specific post-translational modifications of sarcomeric proteins. Second, the use of myosin modulators revealed that molecules like omecamtiv mecarbil and para-Nitroblebbistatin that directly target myosin function can modify calcium sensitivity and the rate of force development in human cardiac tissue. Third, the engineered troponin study showed that engineered troponins C and I can alter myofilament calcium sensitivity without affecting myosin kinetics. Clinically, the

results of the small molecules and engineered protein studies suggest that small molecules and engineered proteins could potentially serve as therapy for patients suffering from heart disease.

KEYWORDS: Right and left ventricles, human hearts, cardiac specific small molecules, calcium sensitivity, sarcomere, engineered proteins

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October 3, 2017

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This dissertation is dedicated to my lovely mother, Donna Grandison

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CHAPTER 1

Introduction

1.1. The heart as two pumps

The normal mammalian heart is a muscular pump that is divided into four chambers, two atria, and two ventricles. The heart can be viewed as two separate pumps, the right pump consisting of the right atrium and right ventricle (RV) and the left pump composed of the left atrium and left ventricle (LV). The atriums sit atop the ventricles where they receive blood from the low-pressure venous systems which then makes its way into the ventricles. The RV and LV which are separated by the interventricular septum then ejects the blood into the low pressure pulmonary and high-pressure systemic systems respectively. As a result, the RV is a low-pressure pump, while the LV is a high-pressure pump.

The ability of the ventricles to generate adequate pressures to pump blood into the pulmonary and systemic vasculature is derived from the capacity of individual cardiomyocytes to produce force. There are a series of events that must take place for a cardiomyocyte to contract and produce force (electrical stimuli, Ca^{2+} induced Ca^{2+} release, etc.). However, at the fundamental level, it is the shortening of sarcomeres that produces the pressure in the ventricles. Cardiac muscle can enhance the amount of force/pressure each ventricle generates by increasing sarcomere length. Sarcomere length in the heart increases proportionally with preload (stretching of the ventricles because of increased end-diastolic volume). Higher preload not only stretch sarcomeres but also increase

stroke volume (volume of blood pumped per beat). The mechanism that describes the heart's ability to increase stroke volume and contractility (intrinsic ability of the heart to contract independently of preload or afterload) in response to an increase in preload is the Frank-Starling mechanism ¹ (Fig 1.1).

The right and left pumps must eject similar stroke volumes (volume of blood pumped per beat) while working against different afterloads (load/pressure imposed on ventricles during ejection). Therefore, each ventricle must generate different pressures to eject blood. It is assumed that the thinner RV produces lower pressures in comparison to the thicker LV during systole solely because of the differences in structure. While it is not debatable that the structure of the ventricles contributes to the amount of pressure they can produce, variations in the mechanical properties of individual cardiomyocytes could augment the geometrical effects.

1.2. Right and left ventricular mechanics

Echocardiography and magnetic resonance imaging (MRI) are two techniques used to measure the global mechanical function of the RV and LV. The mechanical parameters commonly assessed by both methods are strain (deformation of an object) and strain rate (strain per unit time) ². Strain is produced when stress (force per unit cross-sectional area) is applied to the walls of the ventricles ³. Measuring strain and strain rate can be used to determine the velocity of contraction and relaxation, and estimate the filling pressures of the

ventricles⁴. MRI and echocardiographic measurements are important for understanding global pump function. However, neither technique can be used to measure mechanical properties at the cellular level.

To measure the mechanical properties of the RV and LV at the cellular level, researchers have used either intact isolated cells, trabeculae, or papillary muscle or permeabilized (single or multicellular preparations with holes in the membrane) samples. With intact isolated single cells, an investigator uses a high-speed camera to record the mechanical properties when the cell contracts and relaxes. Whereas experiments using either intact trabeculae, papillary muscle, or permeabilized samples utilize a force transducer and a motor. The mechanical parameters that have been reported from isolated intact single cells include time to peak contraction and relaxation, the rate of shortening and re-lengthening, and time to 50% or 90% relaxation. Studies that have used intact trabeculae or papillary muscles have also reported mechanical parameters similar to that reported in intact single cells, but have also recorded force/tension.

In permeabilized samples, cells are not living, as the membrane has been skinned (perforated). However, the contractile structure is still in place, which will contract when exposed to Ca^{2+} . Permeabilized samples allow researchers to measure contractile properties such as sensitivity of the myofilaments to Ca^{2+} and the rate of force development which cannot be assessed using intact

samples. Also, permeabilized samples can also be used to measure maximum force, maximum shortening velocity, and power.

In animals, both intact and permeabilized tissue from the RV and LV have been used to compare the mechanical properties of the ventricles⁵⁻¹². In human¹³, only intact cells have been measured. Data from intact cells have conflicted, whereas in permeabilized samples, the results have been more consistent. However, the reports from permeabilized samples in animals have raised questions as to whether mechanical differences exist in permeabilized samples from humans, as the animal data from permeabilized tissue have revealed inter-ventricular differences that are not measurable in intact cells. Thus, an assessment of the mechanical properties of the RV and LV in intact and permeabilized cells is needed to determine if mechanical differences exist between the ventricles.

1.3. Right and left ventricular differences

The RV and LV of healthy mammalian hearts have different embryologic, metabolic, structural, functional, and electrophysiological characteristics. However, it is unclear if mechanical differences exist between the cells of the ventricles of healthy hearts, and the subsequent effect of heart failure on the contractile properties of both ventricles, as previous studies have reported inconsistent findings.

1.3.1. Embryological origins

The embryological origins of RV and LV are different (Table 1.1 ¹⁴). During gastrulation, cardiac progenitor cells emerge from the mesodermal layer ¹⁵. The cardiac progenitors that differentiate into the cardiomyocytes forming the LV originate from the primary heart field (first population of cells to migrate to heart-forming region), whereas progenitor cells that develop into the RV arise from the secondary heart field (second set of cardiac progenitors to migrate into the heart-forming region) ¹⁴⁻¹⁶. Consequently, there are differences in the genes that regulate ventricular formation, such as the basic helix-loop-helix transcription factors *HAND1* & *HAND2*, which specifically regulate LV & RV formation respectively ¹⁴⁻¹⁹.

1.3.2. Functional and structural differences

Under normal physiological conditions, the RV and LV generate different pressures to pump the same volume of blood during each cardiac cycle (Table 1.1 ¹⁴). The RV creates ~25 mm Hg to pump blood into the low resistance pulmonary vasculature, while the LV produces ~120 mm Hg as it ejects blood into the high resistance systemic circulation ¹⁴. Consequently, by virtue of Laplace's law, the low-pressure RV is thin walled and the high-pressure LV is thick walled ²⁰.

The shapes of the ventricles also vary (Table 1.1 ¹⁴). The LV has a prolate ellipsoid shape, a contrast to the elaborate RV that is triangular when viewed from the side and crescent shaped when viewed in cross-section ^{21,22}.

Additionally, the ventricles show clear anatomical differences in myocardial architecture. In the LV, cells in the mid-myocardium (middle layer) are arranged circumferentially in the short-axis plane of the equator with fibers angles of 20° to -20° , whereas the sub-epicardial (outermost layer) and sub-endocardial (innermost layer) cells are layered obliquely and helically with fiber angles ranging from 30° to 80° ^{14,21}. As a result, the contraction of the LV is circumferential and radial with added rotational and twisting motions ¹⁴. Conversely, myocytes in the RV are arranged predominantly longitudinal, creating the peristaltic contraction seen in the RV ²².

1.3.3. Metabolic and electrophysiological differences

The RV and LV pump the same stroke volume. However, because of the low resistance of the pulmonary circulation, the stroke work of the RV is ~75% less than that of the LV. As a result, the RV is not as metabolically active ²⁰.

Electrophysiological differences between the RV and LV has been reported in healthy mice ^{5,23}, rats ²⁴, dogs ^{25,26}, and guinea pigs ²⁷. In humans, differences have been reported between the ventricles of diseased hearts²⁸. However, the nature of the electrophysiological difference varies amongst species. In rodents (mice and rats), the action potential duration of the RV is shorter than the action potential duration in the LV ^{5,29}, while in larger mammals (dogs), the notch during phase 1 of the action potential is smaller in LV compared to the RV ²⁶. In small and larger animals, functional differences in the electrophysiological properties of

the RV and LV have mainly been the result of differential expression and activity of K⁺ channels in the ventricles ³⁰. In rats, Clark et al. ²⁹ demonstrated that the heterogeneity seen in the action potential waveforms between the RV and LV is the result of the variance in the distribution of transient outward K⁺ currents. In larger dogs, Di Diego and colleagues ²⁶ also showed that the primary reason for the differences in the phase 1 notch between the RV and LV is because of higher transient outward K⁺ currents in the RV. Kondo et al. ⁵ in mice went one step further in that they not only showed that interventricular differences in K⁺ currents exist, but they also demonstrated that mechanically cells from the RV and LV differ as a result of electrophysiological variances.

1.3.4. Mechanical properties in the RV and LV

Studies that have compared the mechanical properties of non-failing RV and LV have used intact and permeabilized cells from several different models (Table 1.2). In intact isolated single cells, the data are inconclusive. Reports from healthy cats ¹², and human ¹³ have shown that cells from both chambers behave similarly. Conversely, studies using intact single cells from pigs ⁸ and mice ⁵ have shown differences. In pigs, the RV shortened more than the LV, whereas, in mice, cellular shortening was greater in the LV.

Mechanical data comparing the RV and LV using intact papillary muscles, trabeculae, and permeabilized samples have been more consistent. For example, in intact papillary muscles from dogs ⁹ and rats ¹¹, force was similar in

both ventricles. Force was also similar between the RV and LV of intact trabeculae ⁶ and permeabilized ^{7,10} tissues from rats. In addition to the mechanical force data, the two studies that have compared the sensitivity of the myofilaments to Ca²⁺ in rats have also been consistent in that they both showed that the LV was more sensitive to Ca²⁺ in non-failing preparations. Furthermore, studies using permeabilized samples demonstrated that heart failure had a significant effect on the mechanical properties of the ventricles, which studies in intact samples have also shown (Table 1.3). Thus, experiments using permeabilized human myocardium from the RV and LV are needed to (1) determine if the inter-ventricular differences observed in rats are also present in human hearts, and (2) assess the impact of heart failure on the mechanical properties of both chambers.

1.3.5. Right-sided and left-sided heart failure

Heart failure is a clinical syndrome that manifests when there are structural and functional impairments to the heart that reduce the ability of the ventricles to fill or eject blood ³¹. There are more than 23 million people worldwide who are affected by the disease, of which, ~6 million are Americans ^{32,33}. The mortality rate for heart failure is high, as ~50% of patients die within the first five years of diagnosis ³⁴⁻³⁶.

Patients who suffer from heart failure can have right-sided dysfunction, left-sided dysfunction, or dysfunction in both ventricles. Clinically, RV dysfunction has been

under-appreciated, as it was believed that RV failure was primarily a result of impaired LV function ^{37,38}. However, several studies have shown that left-sided and right-sided heart failure can develop independently of each other ³⁹⁻⁴³. These are important findings because they imply that it might be useful to develop chamber-specific therapies to help patients who have predominantly left or mostly right-sided dysfunction. Data quantifying the mechanical properties of cells from the RV and LV could be used to identify potential therapeutic targets.

1.4. Systolic dysfunction

Diastolic and systolic heart failure are the two most common forms of the syndrome encountered in clinical practice ⁴⁴. These forms of heart failure are the result of either diastolic (impaired ventricular relaxation, compliance or filling) or systolic (impaired contractile or pump function) dysfunction. In comparison to a normal heart, patients suffering from diastolic dysfunction had similar stroke volume but reduced left ventricular relaxation and increased passive stiffness ⁴⁵. Consequently, these patients have increased pressures during diastole, which can be represented by the pressure-volume loop as an upward shift during filling (Fig 1.2 ⁴⁶). The hallmark of systolic dysfunction is a reduction in stroke volume/ejection fraction (Fig 1.2 ⁴⁶), which leads to the inability of the heart to supply enough blood to meet basal metabolic demands ⁴⁷.

Studies have shown that patients who suffer from diastolic heart failure may not experience systolic heart failure. For example, Baicu et al. ⁴⁸ demonstrated that

left ventricular systolic performance, function and contractility, in general, remains normal in patients who have diastolic heart failure. However, patients who have systolic heart failure almost certainly have diastolic heart failure as well⁴⁹. As a result, patients suffering from systolic heart failure not only display reduced stroke volume/ejection fraction, but they also exhibit increased filling pressures (Fig. 1.2⁴⁶). The principal mechanism responsible for systolic heart failure is impaired contractile function^{45,46}. Thus, a lot of attention has been given to understanding contractile proteins and ways to improve contractile function.

1.5. The sarcomere

The sarcomere is the basic unit of muscle. Each sarcomere is bound by two Z-discs which enclose the thin (actin-containing) and thick (myosin-containing) filaments (Fig. 1.3A⁵⁰). There are hundreds of proteins found in the sarcomere. However, it is the proteins comprising the thick and thin filaments that drive muscle contraction. The proteins directly involved in carrying out a muscle contraction in the thin filament are, actin, the polymerized backbone, tropomyosin, a filamentous protein that wraps around actin monomers inhibiting the interaction between actin and myosin, and the troponin complex (Fig. 1.3B⁵⁰). The trimeric troponin complex consists of troponin C (cTnC), the Ca²⁺ binding subunit, troponin T (cTnT), the subunit that binds the troponin complex to tropomyosin, and troponin I (cTnI), the inhibitory subunit⁵¹.

The thick filaments consist of the molecular motor myosin II and associated proteins, including the cardiac modulatory protein myosin binding protein C (cMyBP-C) ⁵² (Fig. 1.3B ⁵⁰). Myosin has two heavy chains and four light chains. The myosin heavy chains contain three regions, a head (also referred to as either the motor domain, cross-bridge or subfragment 1 (S1)), a neck, and a tail ⁵³. The motor domain has the ATPase and the actin binding sites which respond to ATP binding. Attached to the motor domain are essential (ELC) and regulatory (RLC) light chains, which can alter the function of the cross-bridges ⁵⁴.

1.5.1. Cross-bridge cycle

Muscle contracts when thick and thin filaments slide past each other ⁵⁵. The process is initiated by an action potential which causes an increase in intracellular Ca^{2+} through a process termed Ca^{2+} -induced Ca^{2+} -release ⁵⁶. High amounts of Ca^{2+} then binds to cTnC resulting in the conformational change of the troponin complex. The structural alteration in the troponin complex then leads to a shift in tropomyosin exposing binding sites on actin for myosin cross-bridges to bind ⁵⁷. The attachment of actin and myosin because of increased Ca^{2+} sets the stage for the thick and thin filaments to slide past each other and generate force through the actions of the cross-bridges.

Lymn and Taylor first proposed the cross-bridge cycle in 1971 ⁵⁸. It explains the kinetic properties of the actin-myosin complex during a contraction ⁵⁹⁻⁶¹ (Fig. 1.4 ⁶²). The binding of myosin to actin in the presence of ATP initiates the cross-

bridge cycle, which stimulates the ATPase in the cross-bridges⁵³. In the absence of ATP, the actin-myosin interaction forms a strong (rigor) complex. Upon the binding of ATP to the ATPase, cross-bridges briefly dissociate from actin and then re-attaches through weak hydrostatic bonds (step 1). The hydrolysis of ATP to ADP + P_i then causes a conformational shift in the heads of the cross-bridges leaving the myosin heads in a pre-stroke position (step 2). This reaction (ATP to ADP + P_i) primes the cross-bridges for re-attachment to actin in a more strongly bound force-producing state (step 3). During the transition period where the myosin-actin interaction goes from a weakly to a strongly bound state, inorganic phosphate is released leaving ADP (weakly bound to strongly bound are represented in figure 1.4⁶² as 3a for weakly bound and 3b for strongly bound). The next step in the cycle is the force producing state, as the release of inorganic phosphate causes a conformational shift of the heads of cross-bridges (~10 nm), pulling actin filaments towards the center of the sarcomere, this is referred to as the “power stroke” (step 4). The final step in the cycle (step 5 Fig. 1.4⁶²) is the release of ADP which leaves cross-bridges and actin in a rigor state until the next ATP molecule binds starting the cycle again.

The weakly to the strongly bound transition period of the cross-bridge cycle (steps 3a and 3b in Fig. 1.4⁶²) is of great importance, as it serves as a molecular target for therapy for patients suffering from heart conditions. Studies have shown that this transition period can be altered with drugs. For examples, reports in dogs⁶³ and rats⁶⁴ show that the cardiac myosin activator omecamtiv mecarbil

increased the rate of inorganic phosphate release which forces cross-bridges into the strongly bound force-producing state faster. Conversely, Blebbistatin a small molecule that inhibits myosin II function has been shown to slow down the release of inorganic phosphate during the weakly to strongly bound transition state, trapping cross-bridges in weakly bound state longer⁶⁵⁻⁶⁸. The use of these compounds or similar molecules may serve as therapy for patients suffering from systolic heart failure, or hypertrophic (hypercontractility) cardiomyopathy respectively.

The use of small molecules to modulate the cross-bridge cycle to improve cardiac function may serve as a plausible option for patients suffering from cardiac diseases. However, some cardiomyopathies are the result of mutations in contractile proteins. Therefore, small molecules may not be beneficial to these patients. Another area of research that has proved a therapeutic option for patients suffering from cardiac disease is gene therapy. Thus, there is ongoing research trying to devise ways to alter the contractile function of the heart by modifying sarcomeric proteins. The modifications that are made to these proteins are then used to replace dysfunctional proteins or change the phosphorylation status of a particular sarcomeric protein to improve cardiac function.

1.5.2. Phosphorylation of myofilament proteins

The heart must continually adapt to changes in hemodynamic load as well as respond to neurohumoral stresses on a beat-to-beat basis. To handle the

constant changes, dynamic regulation of contractile apparatus is needed. Post-translational modification of sarcomeric proteins is a primary mechanism for altering cardiac function on a beat-to-beat basis ^{69,70}. One of the most studied post-translational modifications is phosphorylation ⁷¹. In the sarcomere, studies have shown that phosphorylation of thin and thick filament proteins can promote or depress cross-bridge kinetics ^{72,73}. Additionally, alteration in contractile protein structure and function by post-translational modifications have been implicated in heart failure ^{74,75}.

There are several known kinases (ex. Serine/Threonine kinases such as protein kinases A, C, D, G, & Ca²⁺/calmodulin protein kinase II) and phosphatases (ex. protein phosphatase I) that modulate contractile protein function ⁷⁶. The known targets of these kinases and phosphatases are cTnI, cTnT, and tropomyosin in the thin filament, ⁷⁷⁻⁷⁹ and MyBP-C and RLC in the thick filament ⁸⁰⁻⁸³. Stimulation of the α and β adrenergic receptors on cardiomyocytes can enhance or reduce contractile protein phosphorylation by augmenting or decreasing kinase or phosphatase activity ⁸⁴. One of the most studied pathways that are used to alter kinase activity in the heart is the β -adrenergic pathway. At the contractile protein level, β -adrenergic stimulation has been shown to enhance PKA levels which phosphorylate several contractile proteins including cTnI.

The phosphorylation of cTnI is of importance in the forthcoming chapters, as phosphorylation of this protein is known to alter myofilament Ca²⁺ sensitivity ⁸⁵, a

mechanical parameter measured in these studies. In chapter 5, the effects of engineered cTnI and cTnC on Ca^{2+} sensitivity are discussed.

1.6. Overall objective

The overall aim of this project is to assess the mechanical properties of failing and non-failing human myocardium while concomitantly studying the molecular mechanisms contributing to heart failure and work towards therapy.

1.6.1. Compare the mechanical properties of the RV and LV of non-failing and failing human hearts. (Chapter 2)

Hypothesis: Myocardium from the RV and LV of human hearts has different mechanical properties.

Rationale: The RV and LV of human hearts have different embryologic, metabolic, structural, functional, and electrophysiological characteristics.

However, it is unclear if mechanical differences exist between the chambers in non-failing and failing hearts.

1.6.2. Determine the effects of omecamtiv mecarbil on the mechanical properties of failing human hearts. (Chapter 3)

Hypothesis: The cardiac myosin activator omecamtiv mecarbil will augment mechanical force.

Rationale: Omecamtiv mecarbil is a drug design to enhance the rate of inorganic phosphate release in the actin-myosin cycle. By increasing the rate of phosphate release, the molecule increases the number of myosin heads strongly bound to actin at any given time during the cross-bridge cycle. Thus, cardiac tissue incubated with the drug should produce higher mechanical forces.

1.6.3. Assess the effects of para-Nitroblebbistatin on the mechanical properties of failing human hearts. (Chapter 4)

Hypothesis: Para-Nitroblebbistatin will decrease the maximum force and calcium sensitivity in human cardiac samples incubated with the molecule.

Rationale: para-Nitroblebbistatin is a molecule designed to keep cross-bridges in the weakly bound state with actin. Therefore, failing tissue incubated with the molecule should produce less force and have lower calcium sensitivity as a result of the molecule reducing the duty ratio (number of myosin heads in strong force generating state).

1.6.4. Examine the effects of engineered proteins on the mechanical properties of failing human myocardium. (Chapter 5)

Hypothesis: Cardiac troponins C and I engineered to increase or decrease Ca^{2+} sensitivity will alter pCa_{50} .

Rationale: The effects of engineered troponins C and I have been demonstrated *in vivo* in rodent myocardium. However, how these proteins change the mechanical properties of human samples is not known. By showing similar effects in human hearts, engineered troponins C & I may serve as therapy for patients suffering heart failure.

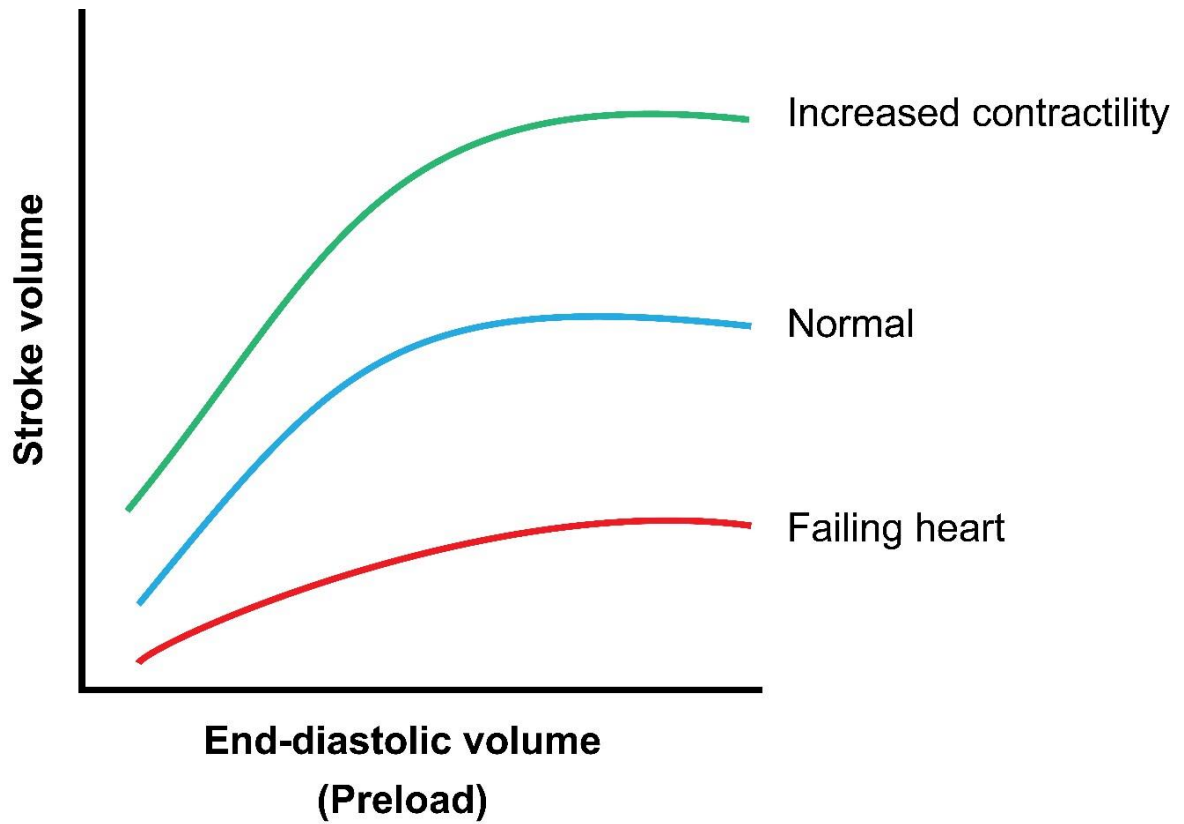


Figure 1.1. The Frank-Starling mechanism

Curves represent a change in stroke volume in response to increasing preload.

Red line (heart failure), blue (normal healthy hearts), green (when contractility is increased in the heart).

Table 1.1. Differences between the left and right ventricles under normal conditions

	Left ventricle	Right ventricle
Embryological origin	Primary heart field	Secondary heart field
Morphological characteristics	Bullet shape; prolate ellipsoid	Complex, crescentic
Myocardial characteristics	Thick smooth walls; fine trabeculations	Thin, heavily trabeculated walls
Myocardial architecture	Predominant radial myocyte orientation in the mid layers; subendocardial myocytes follow right-hand helix configuration; subepicardial myocytes form left-hand helix	Predominant longitudinal myocyte orientation; angulated intrusion of superficial myocytes toward the endocardium
Physiological pump conditions	High-resistance, high-pressure pump; dominant radial thickening and contraction during ejection	Low-resistance, low-capacitance pump; peristaltic-like motion from inflow to outflow during ejection
Flow characteristics	Well-defined isovolumic contraction and relaxation; no hangout period	No or minimal isovolumic periods; hangout period

Table modified from Friedberg & Redington, 2014¹⁴

Table 1.2. Non-failing mechanical properties in the RV and LV reported in the literature

Model (Intact cells/trabeculae/papillary muscle/permeabilized)	Mechanical property	Non-failing RV	Non-failing LV	RV vs. LV comparison
Cats (intact cells)¹²	Rate of shortening (resting lengths/s)	0.67 ± 0.19	0.67 ± 0.12	Not significant
	Rate of relaxation (resting lengths/s)	0.69 ± 0.22	0.69 ± 0.18	Not significant
	Extent of contraction (% of resting length)	10.6 ± 2.6	10.6 ± 1.5	Not significant
Dogs (papillary muscle)⁹	Total tension (g/mm ²)	7.2 ± 1.6	7.9 ± 1.7	Not significant
	Maximum rate of tension development (mm ² /g/s)	31.8 ± 8	28 ± 4	Not significant
	Time to peak tension (ms)	336 ± 26	401 ± 42	Significant difference (p<0.005) RV reached peak tension faster
Pigs (intact cells)⁸	Percent shortening (%)	5.7 ± 0.2	4.6 ± 0.1	Significant difference (p<0.05) RV shortened more
	Velocity of shortening (µm/s)	87 ± 4	50 ± 1	Significant difference (p<0.05) RV shortened faster
	Velocity of re-lengthening (µm/s)	80 ± 4	50 ± 1	Significant difference (p<0.05) RV re-lengthened faster
	Time to peak contraction (ms)	200 ± 4	247 ± 4	Significant difference (p<0.05) RV contracted faster
	Time to 50% relaxation (ms)	98 ± 3	101 ± 2	Not significant

Rats (papillary muscle)¹¹	Time to peak force (ms)	130 ± 3	151 ± 4	Significant difference (p<0.01) RV reached peak force faster
	Active force (g/mm ²)	6.0 ± 0.4	6.2 ± 04	Not significant
	Maximum rate of force development (g/mm ² /s)	74 ± 6	70 ± 4	Not significant
	Maximum shortening (%)	18 ± 1	13 ± 0.5	Significant difference (p<0.05) RV shortened more
	Maximum shortening velocity (muscle lengths/s)	1.98 ± 0.14	1.13 ± 0.07	Significant difference (p<0.05) RV shortened faster
Rats (trabeculae)⁶	Force (mN/mm ²)	Values not presented (figure)	Values not presented (figure)	Not significant
Mice (intact cells)⁵	Sarcomere length shortening (µm)	0.033 ± 0.003	0.041 ± 0.003	Significant difference (p<0.01) LV shortened more
	Rise time (50% to peak, ms)	54.7 ± 1	51 ± 1	Not significant
	Decay time (50% to baseline, ms)	117 ± 5	98 ± 3	Significant difference (p<0.01) LV decay faster
Human (intact cells)¹³	Time to peak shortening (s)	Values not presented (figure)	Values not presented (figure)	Not significant
	Time to relaxation (s)	Values not presented (figure)	Values not Presented (figure)	Not significant
Rats (Permeabilized cells)⁷	Maximum force (mN/mm ²)	28.1 ± 1.7	26.7 ± 1.3	Not significant

	EC ₅₀ (μmol/L) (1.77 ± 0.01	1.36 ± 0.06	Significant difference (p<0.05) LV more sensitive to Ca ²⁺
Rats (Permeabilized strips)¹⁰	Maximum force (mN/mm ²)	0.85 ± 0.15	1.5 ± 0.25	Not significant
	pCa ₅₀	5.7 ± 0.09	6.0 ± 0.05	Significant difference (p<0.05) LV more sensitive to Ca ²⁺

*EC₅₀ & pCa₅₀ indicates the Ca²⁺ concentration at 50% of maximum force, and is the index for myofilament Ca²⁺ sensitivity.

Table 1.3. Comparison of non-failing and failing RV and LV mechanical properties reported in the literature

Model (Intact cells/trabeculae /permeabilized)	Mechanical property	Non-failing RV	Non-failing LV	Failing RV	Failing LV	Non-failing RV & LV vs. Failing RV & LV
Pigs (intact cells)⁸	Percent shortening (%)	5.7 ± 0.2	4.6 ± 0.1	3.1 ± 0.1	2.3 ± 0.1	Significant difference (p<0.05) Failing RV & LV shortened less than non-failing RV & LV. Failing LV shortened less than failing RV
	Velocity of shortening (µm/s)	87 ± 4	50 ± 1	48 ± 2	33 ± 1	Significant difference (p<0.05) Failing RV & LV velocity of shortening was slower than Non-failing RV & LV. Failing LV velocity of shortening was slower than failing RV
	Velocity of re-lengthening (µm/s)	80 ± 4	50 ± 1	50 ± 3	32 ± 1	Significant difference (p<0.05) Failing RV & LV re-lengthened slower. Failing LV re-lengthened slower than failing RV
	Time to peak contraction (ms)	200 ± 4	247 ± 4	242 ± 4	234 ± 4	Significant difference (p<0.05) Failing RV took longer to get to peak contraction, and failing LV got to peak contraction faster than non-failing LV
	Time to 50% relaxation (ms)	98 ± 3	101 ± 2	102 ± 3	105 ± 3	Not significant
Rats (trabeculae)⁶	Force (mN/mm ²)	Values not present	Values not present	Values not present	Values not present	Not significant

		ed (figure)	ed (figure)	ed (figure)	ed (figure)	
Human (intact cells)¹³	Time to peak shortening (s)	Values not presented (figure)	Values not presented (figure)	Values not presented (figure)	Values not presented (figure)	Not significant
	Time to relaxation (s)	Values not presented (figure)	Values not presented (figure)	Values not presented (figure)	Values not presented (figure)	Not significant
Rats (Permeabilized cells)⁷	Maximum force (mN/mm ²)	28.1 ± 1.7	26.7 ± 1.3	14.9 ± 1.0	13.3 ± 0.8	Significant difference (p<0.05) Failing RV & LV produced less force
	EC ₅₀ (μmol/L)	1.77 ± 0.01	1.36 ± 0.06	1.59 ± 0.08	2.42 ± 0.18	Significant difference (p<0.05) Non-failing LV is more sensitive to Ca ²⁺ than failing LV
Rats (Permeabilized strips)¹⁰	Maximum force (mN/mm ²)	0.85 ± 0.15	1.5 ± 0.25	1.7 ± 0.35	1.7 ± 0.27	Significant difference (p<0.05) Failing RV produce more force than non-failing RV
	pCa ₅₀	5.7 ± 0.09	6.0 ± 0.05	6.0 ± 0.09	6.0 ± 0.09	Significant difference (p<0.05) Failing RV is more sensitive to Ca ²⁺ than non-failing RV.

*EC₅₀ & pCa₅₀ indicates the Ca²⁺ concentration at 50% of the maximum force and is the index for myofilament Ca²⁺ sensitivity.

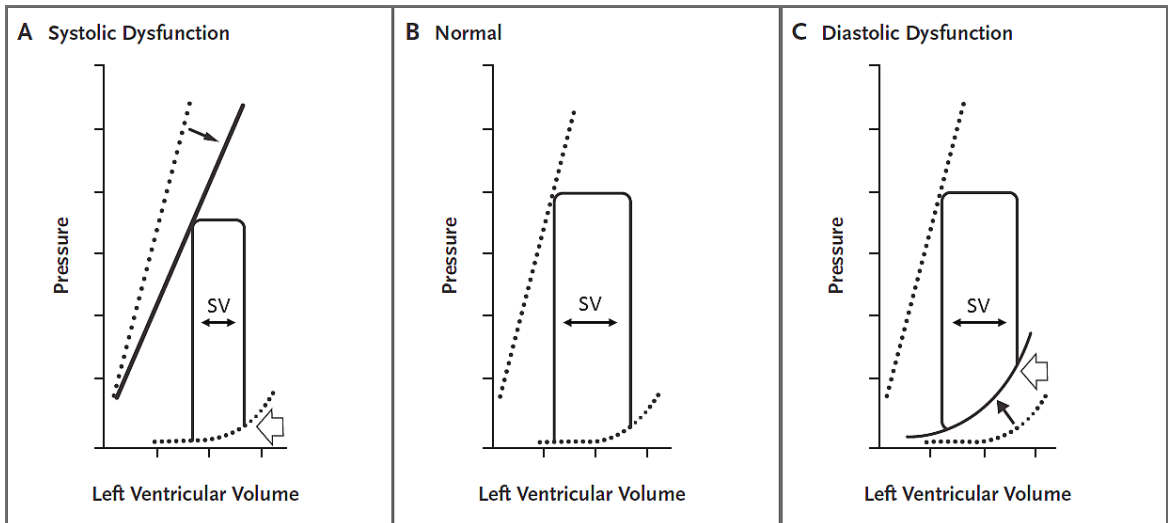


Figure 1.2. Left Ventricular Pressure-Volume Loops in Systolic and Diastolic Dysfunction

A) Systolic dysfunction with decreased stroke volume, black line displaced downward. B) Normal pressure-volume loop. C) Diastolic dysfunction, the diastolic pressure has shifted up (solid curved black line). (Modified from Aurigemma and Gaasch, 2004 ⁴⁶)

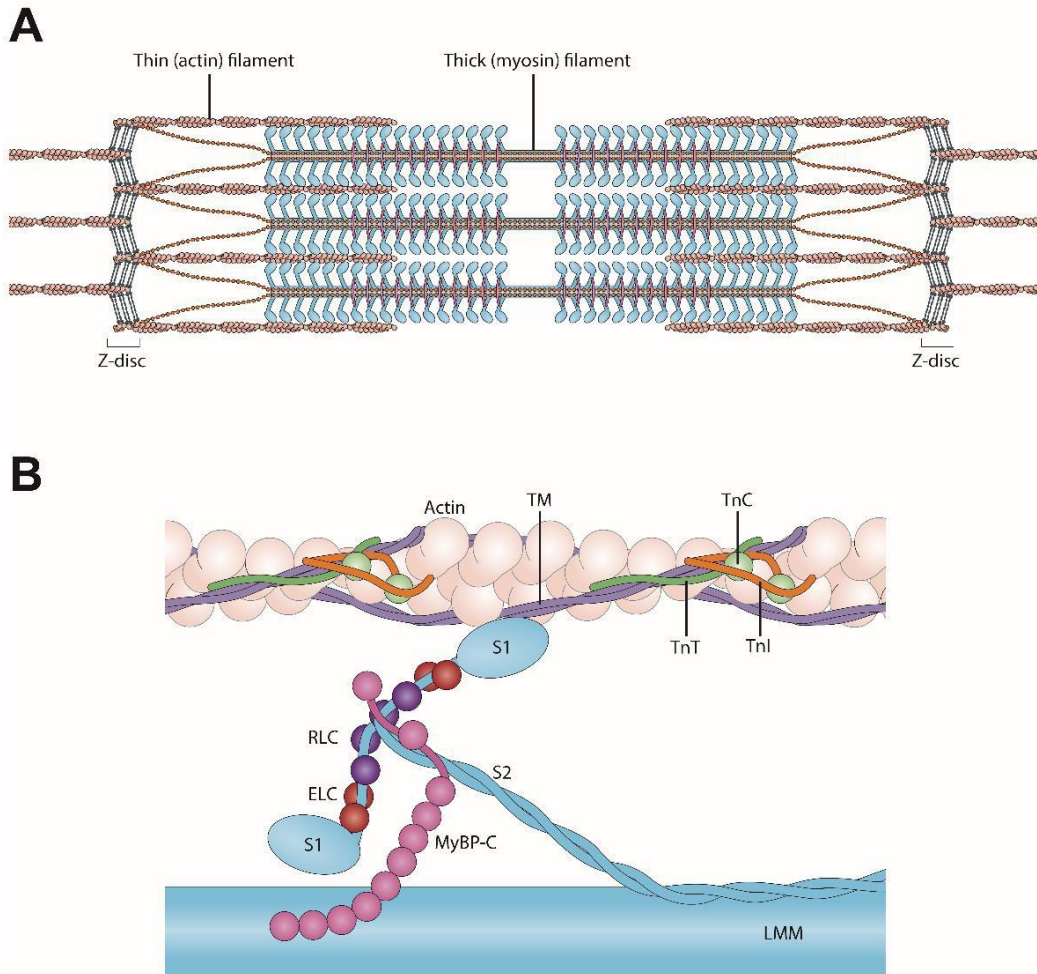


Figure 1.3. Sarcomere

A) Schematic of sarcomere bounded by two Z-disc with thick and thin filaments.

B) Close up of selected thin and thick filament proteins. Thin filament proteins: actin, tropomyosin (TM), troponin C (TnC), troponin T (TnT), troponin I (TnI); thick filament proteins: S1 subfragment/cross-bridges (S1), myosin binding protein C (MyBP-C), regulatory light chain (RLC), essential light chain (ELC), lever arm (S2), light meromyosin (LMM). (Modified from Hwang and Sykes, 2015

50)

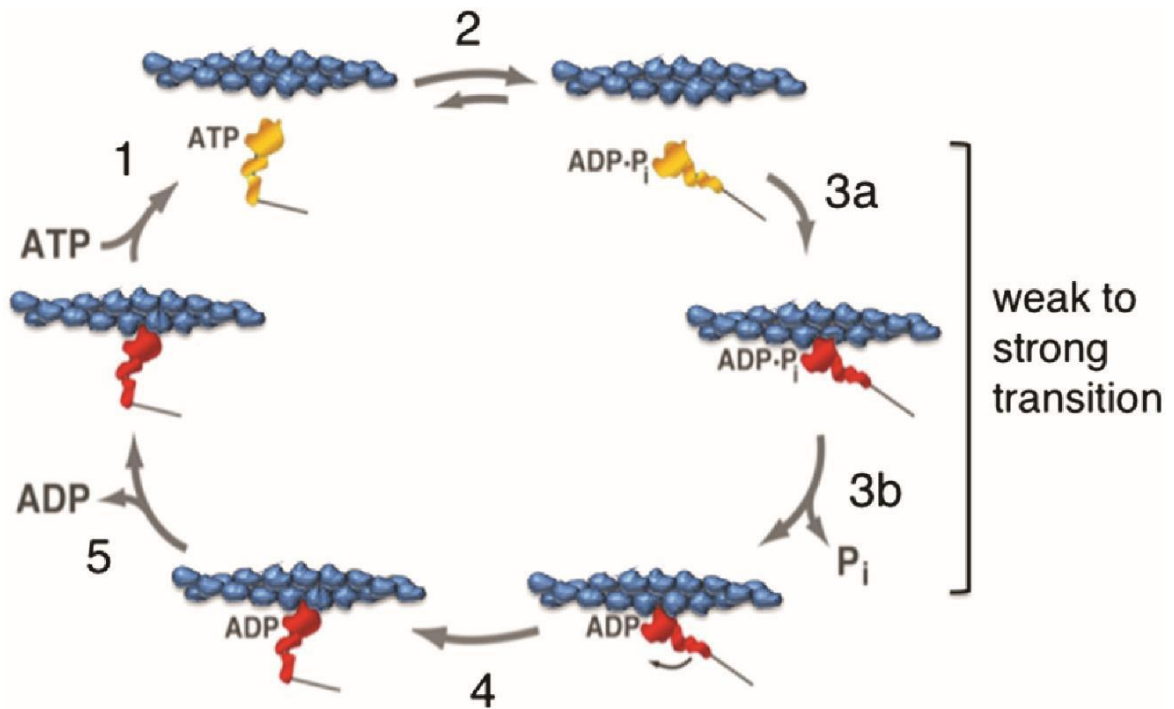


Figure 1.4. Actin-myosin cross-bridge cycle

Yellow cross-bridges represent actin and myosin in the weakly bound state; red cross-bridges represent actin and myosin in the strongly bound state. Step 1: Binding of ATP to cross-bridge dissociates myosin heads from actin. Step 2: Hydrolysis of ATP to ADP + P_i locking myosin heads in pre-stroke configuration. Step 3 (a and b): Transition of actin-myosin interaction from weakly bounded to strong binding, which results from the release of inorganic phosphate. Step 4: Powerstroke, cross-bridges shift ~10-nm pulling actin to the center of the sarcomere. Step 5: ADP released, actin and myosin remain in rigor until new ATP binds. (Adapted from James Spudich, 2014 ⁶²)

Chapter 2

The Ca^{2+} sensitivity of right ventricular myocardium increases more than the Ca^{2+} sensitivity of left ventricular myocardium in human heart failure

2.1. Introduction

In healthy human hearts, the left ventricle (LV) is a thick-walled bullet shaped chamber that generates high pressure (~120 mm Hg during systole) to pump blood around the systemic circulation. Conversely, the right ventricle (RV) is a thinner crescent shaped low-pressure pump that produces ~25 mm Hg as it ejects blood into the pulmonary vasculature^{5,14}. While it is clear that the distinct chamber structures contribute to the inter-ventricular difference in systolic pressure, it is not known if variation in myocardial contractile properties augments the geometrical effects. Neither is it known whether heart failure affects cells from the LV and the RV in the same way.

The impact of heart failure on LV myocardial function has been reported extensively. Studies using skinned cardiac samples from patients with end-stage heart failure have shown that the failing heart is more sensitive to Ca^{2+} and develops less contractile force than samples obtained from organ donors who did not have heart failure⁸⁶⁻⁹³. However, it is less clear how heart failure alters the contractile properties of tissue from the RV.

Previous reports that have looked at the effects of heart failure on maximal force (F_{\max}) and Ca^{2+} sensitivity (pCa_{50}) in the RV and LV have used animal models.

Belin et al. ⁷ demonstrated that heart failure in rats reduced F_{\max} in permeabilized myocytes from both ventricles. Conversely, Perreault and colleagues ¹⁰ showed that heart failure increased F_{\max} in the RV and had no effect on the LV. Both studies revealed that non-failing cells from the LV are more sensitive to Ca^{2+} than non-failing cells from the RV. In failing tissue, Perreault et al. ¹⁰ found that heart failure increased pCa_{50} in the RV but not in the LV, while Belin et al. ⁷ reported that heart failure reduced pCa_{50} in the LV without altering pCa_{50} in the RV.

The data reported in this manuscript extend these studies to human myocardium. Our results show that pCa_{50} values measured for multicellular preparations from non-failing LV were greater than pCa_{50} values measured for non-failing RV specimens. In failing hearts, the opposite was true. Failing RV samples had higher pCa_{50} values than failing LV samples. Data quantifying the relative phosphorylation levels of myofilament proteins suggested that the shifts in Ca^{2+} sensitivity reflected changes in the relative phosphorylation of cardiac troponin I (cTnI). There were no inter-ventricular differences in maximum force, maximum power output or maximum shortening velocity.

2.2. Materials and Methods

2.2.1. Procurement of human samples

The human cardiac samples used in this study were acquired using the collection protocol described by Blair et al. ⁹⁴. Briefly, hearts procured from patients undergoing cardiac transplants at the University of Kentucky and from organ

donors who did not have heart failure were given to a researcher immediately after being removed from the patient. The samples were obtained from through-wall sections cut from the distal region of the LV and RV. The LV samples were then further dissected transmurally, forming sub-epicardial, mid-myocardial, and sub-endocardial specimens. RV samples were not separated transmurally, as the RV wall is typically thinner and the dissection is harder to perform consistently. The specimens were snap-frozen in liquid nitrogen within 30 minutes of being removed from the patient and stored in the vapor phase of liquid nitrogen at -150°C until use. The University of Kentucky Institutional Review Board approved all procedures, and subjects gave informed consent.

Previous data from our laboratory⁹⁵ showed that heart failure has a greater impact on the contractile properties of samples from the middle transmural region of the LV than on samples from the LV sub-epicardium or LV sub-endocardium. This study compared samples from the RV and the mid-myocardium of the LV to optimize the probability of detecting statistically significant effects.

2.2.2. Clinical characteristics from patients with heart failure and donors

The samples from patients undergoing heart transplants came from 7 males (classification of heart failure: 5 ischemic and 2 non-ischemic) and 5 females (classification of heart failure: all non-ischemic). The donor samples came from 3 males (cause of death: 2 head trauma and 1 unknown) and 2 females (cause of death: stroke). The mean age of the patients with heart failure was 48 (range 19

to 68), while that of donors was 41 (range 22 to 48). Patients' characteristics are summarized in table 2.1.

2.2.3. Multicellular preparations

Multicellular preparations (Fig. 2.1) were obtained by mechanically disrupting ~100 mg of tissue from the LV mid-myocardium or the RV. The preparations were then permeabilized using 1% v/v Triton detergent (Fig. 2.1.1). A total of 79 multicellular preparations from 17 patients (5 non-failing and 12 failing hearts) were analyzed. The average length of the preparations used in this study was $860 \pm 248 \mu\text{m}$, and the average cross-sectional area was $4.05 \pm 2.08 \times 10^{-8} \text{ m}^2$. Cross-sectional area was estimated assuming a circular profile.

2.2.4. Experimental set-up

Preparations were attached to a force transducer (resonant frequency, 600 Hz; Model 403, Aurora Scientific, Aurora, Ontario, Canada) and a motor (step time 0.6 ms; model 312B, Aurora Scientific) (Fig. 2.1.2) and stretched to a sarcomere length of $2.24 \mu\text{m}$ in a solution with a pCa ($= -\log_{10}[\text{Ca}^{2+}]$) of 9.0. All experimental measurements were conducted at 15°C using SLControl software⁹⁶.

2.2.5. Solutions

Two set of solutions were used in this study. A relaxing solution to isolate and permeabilize preparations, and experimental pCa ($= -\log_{10} [\text{Ca}^{2+}]$) solutions with varying concentrations of Ca^{2+} . The relaxing solution contained (in mmol L^{-1}): 4

ATP, 2 EGTA, 20 imidazole, 100 KCl and 7 MgCl₂. The final pH of the relaxing solution was adjusted to 7.0 with 4M KOH. To permeabilize the multicellular preparations, double strength relax, water and two protease inhibitors (phenylmethanesulfonyl fluoride 500 μmol L⁻¹ and leupeptin 40 μmol L⁻¹) were added to three beakers, the fourth beaker contained the aforementioned and triton. To make a range of activating solutions (pCa solutions), two pCa (4.5 & 9.0) solutions were initially made. Intermediate pCa solutions ranging from pCa 5.0 to 6.4 were made by combining different volumes of pCa 4.5 and 9.0. pCa 4.5 contained (in mmol L⁻¹): 7 EGTA, 20 Imidazole, 52 KCL, 14.5 Creatine phosphate, 4.9 ATP, 7 CaCl₂, 5.23 mgCl₂, sufficient amounts of KOH to get pH to 7.0. The composition of pCa 9.0 (in mmol L⁻¹) was: 7 EGTA, 20 Imidazole, 68 KCL, 14.5 Creatine phosphate, 4.83 ATP, 5.41 MgCl₂, and 13.7 μM CaCl₂ plus ample amount of KOH to reach pH 7.0. The ionic strength was held constant at a 180 mM in all solutions.

2.2.6. Force, rate of tension development, Ca²⁺ sensitivity measurements

Preparations were initially immersed in pCa 4.5 (saturating Ca²⁺ concentration). Maximum isometric force (F_{max}) was calculated as the force developed in this solution at steady-state (Fig. 2.1.3). Once force reached steady state, the rate of tension development, k_{tr} , was measured by rapidly shortening the preparation by 20%, holding it at the short length for 20 ms, and then re-stretching the preparation to its original length. The k_{tr} was subsequently calculated by fitting the portion of the force record immediately after the re-stretch with a single

exponential function of the form $F(t) = A + B(1 - \exp(-k_{tr}t))$. In this equation, $F(t)$ is the force at time t , and A and B are constants (Fig. 2.1.3). Calcium sensitivity (pCa_{50}) was determined by repeating the force measurements with different solutions that had pCa values in the range 9.0 to 5.0. pCa_{50} values were calculated by fitting the resulting data to a modified Hill equation of the form $F = F_{pas} + F_{Ca} ([Ca^{2+}]^n / ([Ca^{2+}]^n + [Ca^{2+}_{50}]^n))$, where F_{pas} is the force measured in pCa 9.0 solution; F_{Ca} is Ca^{2+} activated force; n is the Hill coefficient, and $[Ca^{2+}_{50}]^n$ is the free Ca^{2+} concentration required to develop half the maximum Ca^{2+} dependent force.

2.2.7. Shortening velocity and power measurements

To measure the shortening velocity and power, the multicellular preparations were allowed to shorten for 80 ms against pre-set loads that ranged from 0 to 100% of the maximum tension measured in pCa 4.5 solution. The shortening velocity in each trial was determined by fitting a regression line to a plot of fiber length against time during the final 50 ms of the force clamp. The mean force was also determined during this time. The resulting data were then fitted using a hyperbolic equation of the form $(F+a)(V+b) = (F_0+a)b$, where F is the force developed at a shortening velocity of V , F_0 is the isometric force, and a and b are constants with dimensions of force and velocity respectively. Power values were calculated as the product of force and velocity. Fig. 2.1.4 illustrates a representative force-velocity and power curves.

2.2.8. Phosphorylation of myofilament proteins

The phosphorylation status of selected sarcomeric proteins was determined using the protocol described in the supplemental materials of Haynes et al. ⁹⁵. Chemically permeabilized multicellular preparations utilized in the mechanical measurements were initially homogenized in urea-thiourea sample buffer (in mol L⁻¹, 8 Urea, 2 Thiourea, 0.075 M DTT, and 0.05 Tris-HCl, with 3% SDS w/v, pH 6.8). 1 µg of protein was then loaded onto each lane of precast polyacrylamide gels (12% 26 well Tris-HCl, Bio-Rad, Hercules, CA). Gels were first stained with Pro-Q Diamond phosphoprotein stain (Invitrogen, Carlsbad, CA) and scanned with a Typhoon 9410 scanner (GE Healthcare, Piscataway, NJ) to detect phosphorylated proteins. The gels were then de-stained and subsequently stained again with Sypro Ruby (Invitrogen, Carlsbad, CA) to assess total protein content. The amounts of phosphorylated or total protein represented by each band were determined by integrating the densitometry profile followed by appropriate background correction. These calculations were performed using GelBox software developed by our laboratory. Values from multiple gels were normalized using data from a single homogenate that was run on each gel. Relative phosphorylation was calculated as the ratio of the integrated densities calculated for the Pro-Q Diamond and Sypro Ruby stains.

2.2.8.1. Western blot analysis of cTnI Ser-23/24 phosphorylation

TnI Ser23/24 specific phosphorylation was determined by Western blot similar to that previously described Nixon et al. ⁹⁷ and Salhi et al. ⁹⁸. Briefly, whole cell

homogenates from above were separated on a 12% (29:1) Laemmli gel and transferred to Immobilon FL PVDF membrane (Millipore Sigma, Billerica, MA). Following blocking with 1% BSA in TBS, TnI Ser-23 and Ser-24 phosphorylation (pTnI S23/24) was detected by incubation with the rabbit anti-phosphorylated TnI Ser-23/24 antibody (Cell Signaling Technology, Inc.). Following washes, membranes were incubated with an anti-rabbit Cy5 labeled fluorescent secondary antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA), washed and visualized on a Typhoon 9410 imager (GE Healthcare, Piscataway, NJ). Following TnI Ser-23/24 phosphorylation imaging, total TnI was determined in the same membrane by re-incubation with the mouse anti-cardiac TnI antibody (Fitzgerald; clone C5). Following washes, the membrane was incubated with an anti-mouse Cy2 labeled fluorescent secondary antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA), washed again and visualized as above. Sequential development using different primary/secondary combinations allows for quantification of phosphorylated and total TnI species in the same membrane. Phosphorylation at Ser-23/24 and expression of TnI was then quantified from the individual images with ImageQuant TL v8.0 (GE Healthcare, Piscataway, NJ) and Ser-23/24 phosphorylation defined in arbitrary units as the TnI Ser-23/24 phosphorylation signal divided by the total TnI signal.

2.2.9. Statistical analysis

The experimental data were analyzed in SAS 9.4 (SAS Institute, Cary, NC) using linear mixed effects models incorporating two main effects (heart failure status

and ventricular region) and their interaction. The linear mixed models were chosen for the analysis for a few reasons. First, they accounted for the unbalanced design (5 non-failing and 12 failing hearts). Second, the linear mixed models took into consideration that certain regions (LV and RV) came from the same person (repeated measures) and that from that LV or RV sample, there were 2 to 3 preparations (hierarchical nesting). Thus, the linear mixed models provided more statistical power than a standard two-way ANOVA tests when multiple samples from each heart are analyzed. Post-hoc analysis was performed using Tukey-Kramer corrections. P values less than 0.05 were considered significant. Data are reported as mean \pm SEM.

Table 2.1. Patients' characteristics

Age	Gender	Diagnosis	Cardiomyopathy/cause of death
22	Male	Non-failing	Head trauma
60	Female	Non-failing	Stroke
28	Male	Non-failing	Head trauma
47	Female	Non-failing	Stroke
48	Male	Non-failing	Unknown
68	Male	Failing	Ischemic
42	Male	Failing	Ischemic
53	Male	Failing	Ischemic
54	Male	Failing	Ischemic
64	Male	Failing	Ischemic
20	Male	Failing	Non-ischemic
35	Male	Failing	Non-ischemic
56	Female	Failing	Non-ischemic
66	Female	Failing	Non-ischemic
19	Female	Failing	Non-ischemic
60	Female	Failing	Non-ischemic
36	Female	Failing	Non-ischemic

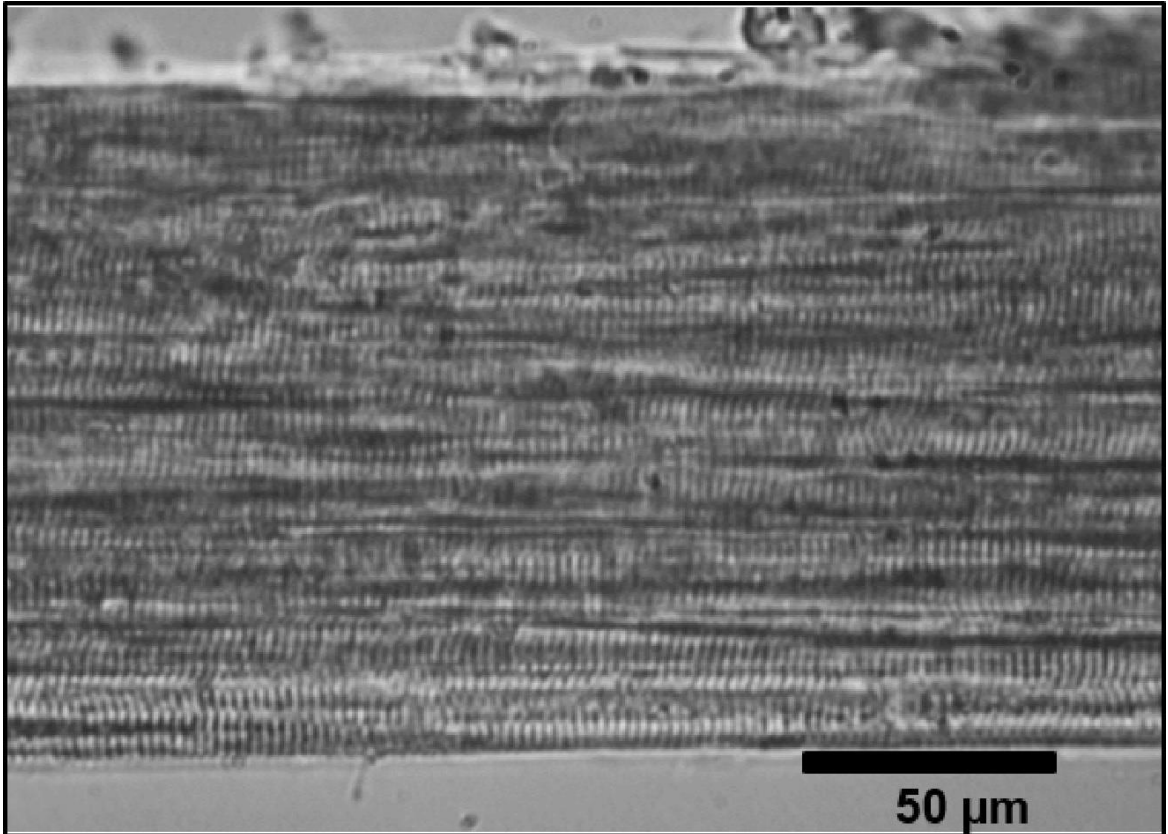


Figure 2.1. Multicellular preparation

4X magnification of permeabilized multicellular preparation. Each preparation used in this and forthcoming studies contained between 10-20 single cardiomyocytes.

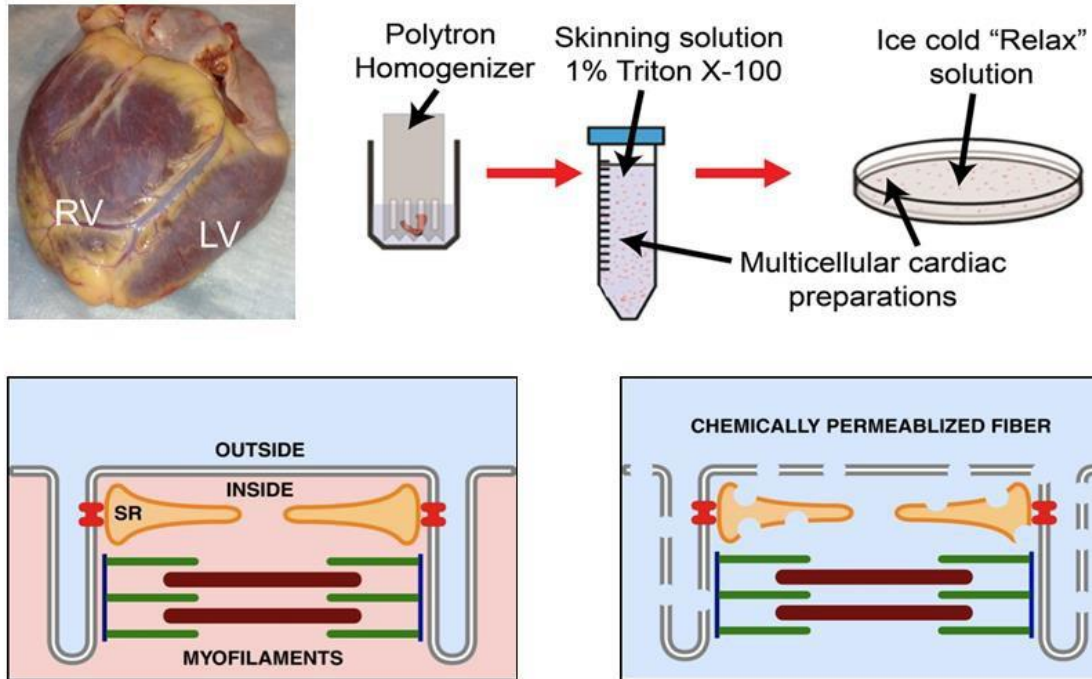


Figure 2.1.1. Permeabilization of preparations

Top left, human heart displaying right and left ventricles. Top excluding human heart, homogenization, and permeabilization (skinning) process. Bottom left, intact membrane. Bottom right, permeabilized membrane.

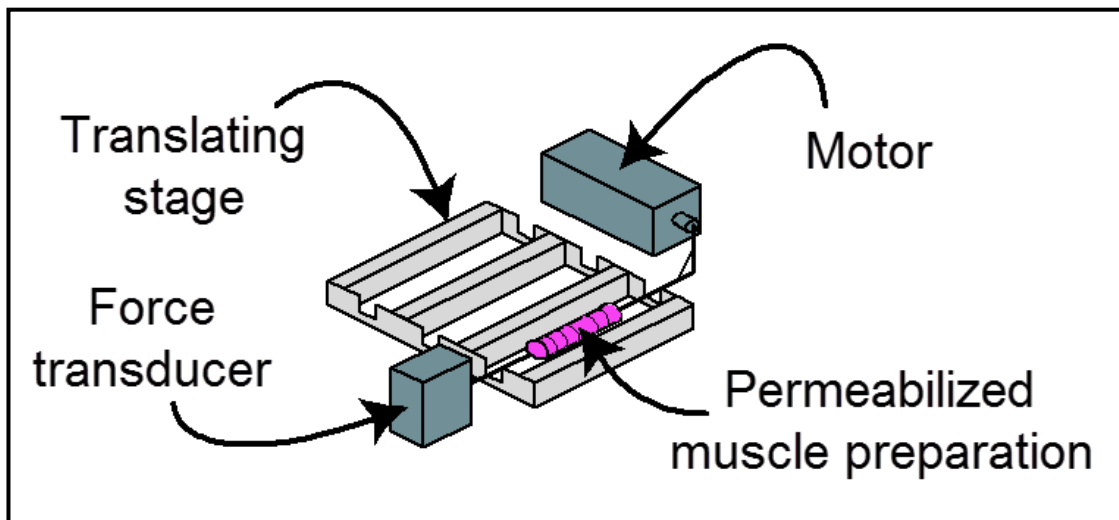


Figure 2.1.2. Experimental setup

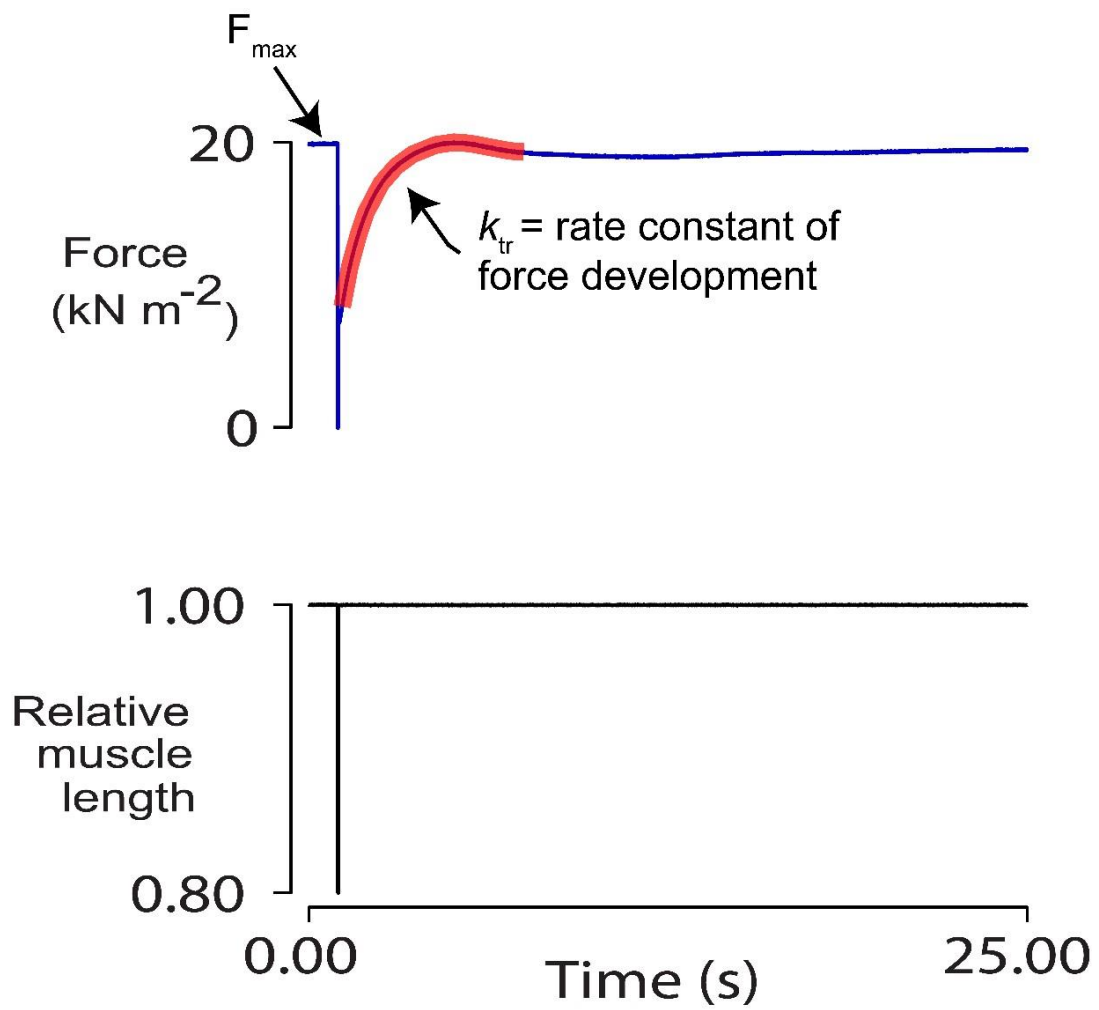


Figure 2.1.3. Experimental record

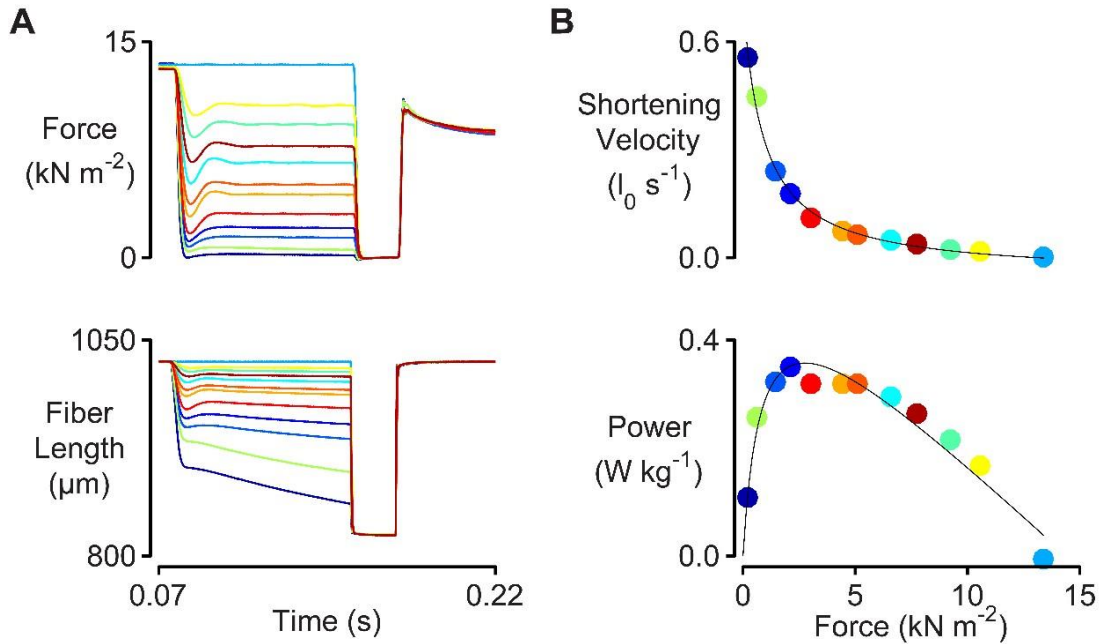


Figure 2.1.4. Representative force-velocity and force-power curves

A) Top panel, experimental records from one preparation demonstrating different forces as a result of being held at different loads. Bottom panel, fiber length records from each individual load the preparation was held at. B) Top panel, shortening velocity curve. Bottom panel, power curve.

2.3. Results

2.3.1. Heart failure has a greater effect on the Ca²⁺ sensitivity of right ventricular myocardium

Fig 2.2A shows that heart failure increased the sensitivity of the myofilaments to Ca²⁺ (main effect for disease status, $p=0.006$). pCa_{50} increased from 5.60 ± 0.03 in the non-failing samples (mean \pm sem of all non-failing samples from the LV and RV) to 5.71 ± 0.02 in the samples isolated from patients who had heart failure. The increase was greater for samples from the right ventricle ($\Delta pCa_{50} \sim 0.18$, $p < 0.001$) than for samples from the left ventricle ($\Delta pCa_{50} \sim 0.05$, not significant). These effects meant that there was a significant interaction ($p < 0.002$) between heart failure status and cardiac region for pCa_{50} . A similar statistical interaction ($p = 0.040$) was measured for the Hill coefficient n (Fig 2.2B). The effects of heart failure on the calcium sensitivity and cooperativity of the myofilament thus depend on the ventricle that is being studied.

2.3.2. Heart failure reduces maximum force and maximum power output in both ventricles

Fig 2.3 shows that preparations from the right and left ventricles generated similar contractile force in maximally activating Ca²⁺ solution. On average, the non-failing samples produced 25.3 ± 7.9 kN m⁻², whereas samples from the LV and RV of failing hearts produced 14.8 ± 5.2 kN m⁻². Heart failure, therefore, suppressed force by $\sim 40\%$ in both ventricles. Maximum power (where power was measured as the product of force and velocity during loaded shortening) was

also significantly reduced by heart failure ($p < 0.001$ for both ventricles) (Fig 2.3B). V_{\max} was not affected by ventricular region or heart failure status. On average, V_{\max} was 1.09 ± 0.36 muscle lengths per second ($l_0 s^{-1}$) (Fig 2.4A). The rate of tension development is also similar between the ventricles in non-failing and failing preparations, as the average k_{tr} was $0.88 \pm 0.15 s^{-1}$ (Fig 2.4B).

2.3.3 Heart failure alters phosphorylation of myofilament proteins

The phosphorylation status of cTnI, cardiac myosin binding protein-C (cMyBP-C), and myosin regulatory light chain (RLC) were assessed using Pro-Q Diamond and Sypro Ruby (Fig. 2.5A). Figures 2.5B and 2.5C shows that the relative phosphorylation of cTnI and RLC exhibited statistical interactions between disease status and cardiac region. In non-failing hearts, the relative phosphorylation of cTnI in the RV is ~24% higher than the LV. Conversely, in failing preparations, the relative phosphorylation of cTnI in the LV is ~22% more than the RV. The largest change in cTnI relative phosphorylation occurred in the RV where heart failure produced ~49% reduction ($p=0.029$, Fig. 2.5B).

RLC relative phosphorylation exhibited a similar statistical interaction (Fig 2.5C). Mean values were ~40% greater ($p=0.041$) in samples from non-failing RV than in preparations from non-failing LV ($p=0.041$) whereas RV samples from failing hearts had lower relative phosphorylation levels than in preparations from failing LV.

cMyBP-C relative phosphorylation levels did not demonstrate a significant statistical interaction. However, heart failure significantly ($p=0.017$) reduced the relative phosphorylation levels in both chambers (Fig. 2.5D).

Ser-23/24 is an important site for cardiac regulation on TnI. Measurements with site-specific antibodies (Figs. 2.6A & B) shows a similar statistical interaction ($p=0.039$) to the ProQ diamond cTnI (0.037) relative phosphorylation data (Figs. 2.5B).

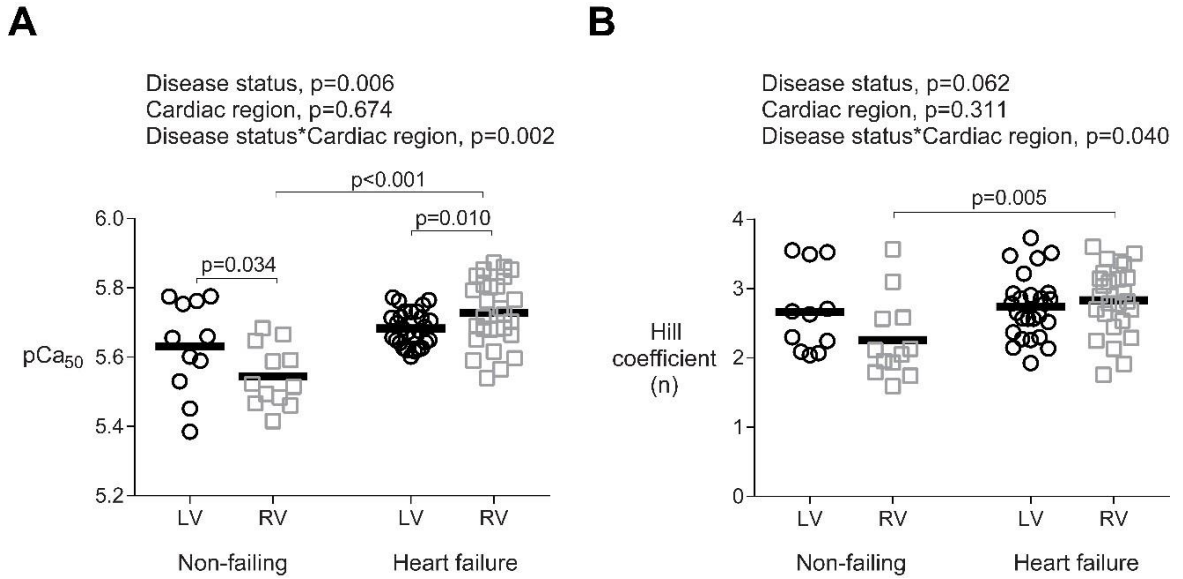


Figure 2.2. Heart failure impacts the calcium sensitivity and cooperativity of myocardium from the left and right ventricles in different ways.

A) pCa_{50} and B) Hill coefficient. Symbols show data for 79 individual preparations from $n=5$ non-failing and $n=12$ failing hearts.

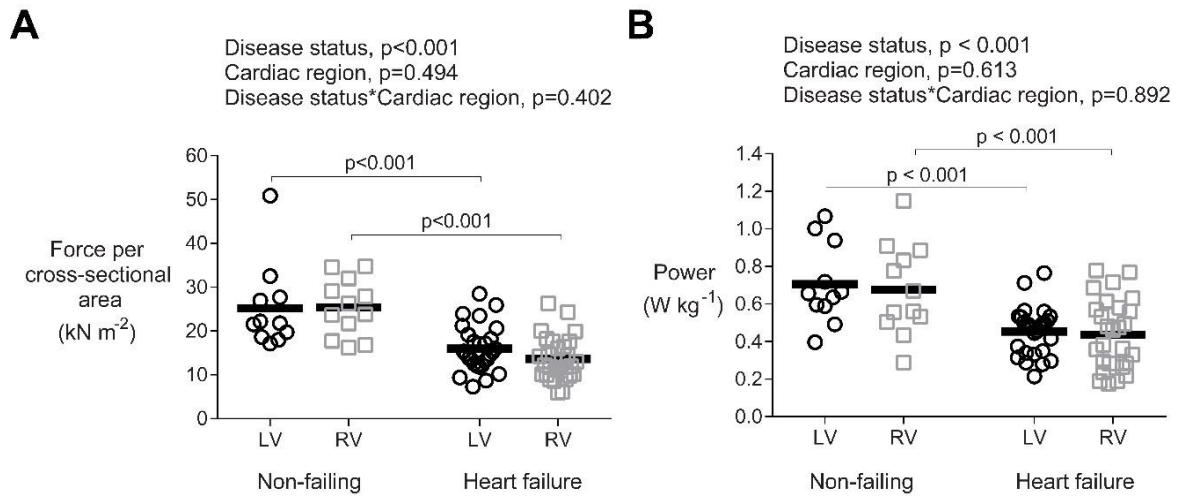


Figure 2.3. Heart failure reduces maximum force and maximum power in both ventricles.

A) Force per cross-sectional area. B) Maximum power output. All data obtained in maximally-activating solutions with $p\text{Ca}$ values of 4.5. Symbols show data for 79 individual preparations from $n=5$ non-failing and $n=12$ failing hearts.

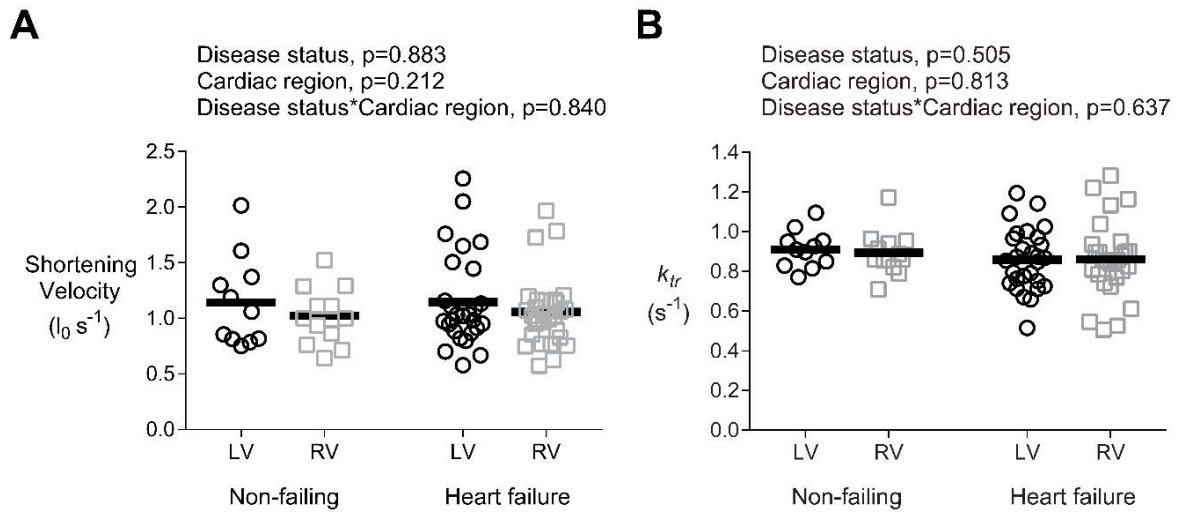


Figure 2.4. Shortening velocity and rate of force development are similar between the ventricles.

A) Shortening velocity. B) The rate of tension development, k_{tr} . All data obtained in maximally-activating solutions with pCa values of 4.5. Symbols show data for 79 individual preparations from $n=5$ non-failing and $n=12$ failing hearts.

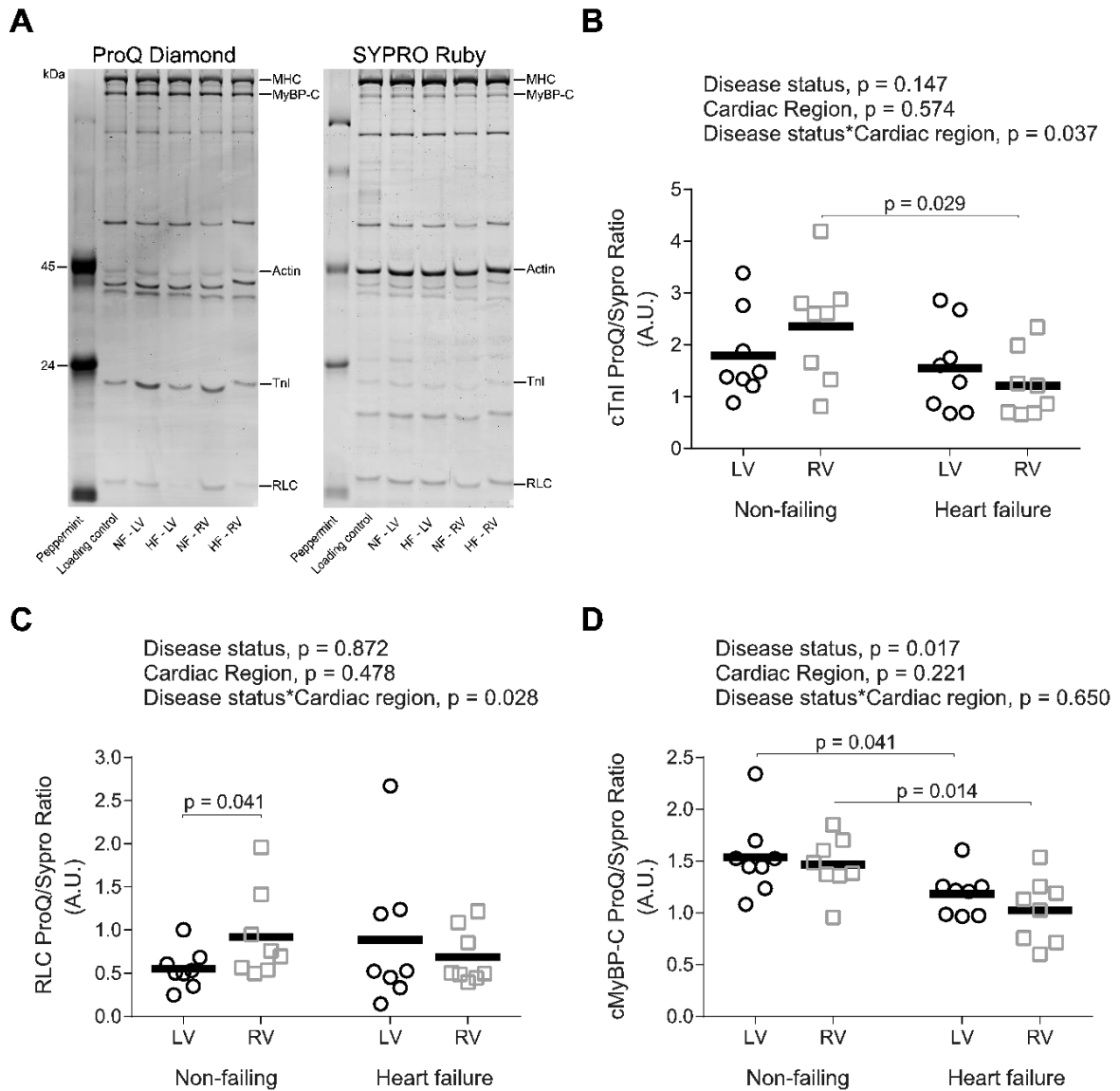


Figure 2.5. Myofilament protein phosphorylation in the RV and LV of non-failing and failing samples.

A) Images of representative gels stained with ProQ (left) and SYPRO Ruby (right). Symbols show data from 8 failing and 4 non-failing hearts (non-failing samples were run in duplicate). Individual bands were normalized to loading control. B) Relative phosphorylation of cTnl, C) cMyBP-c, and D) RLC.

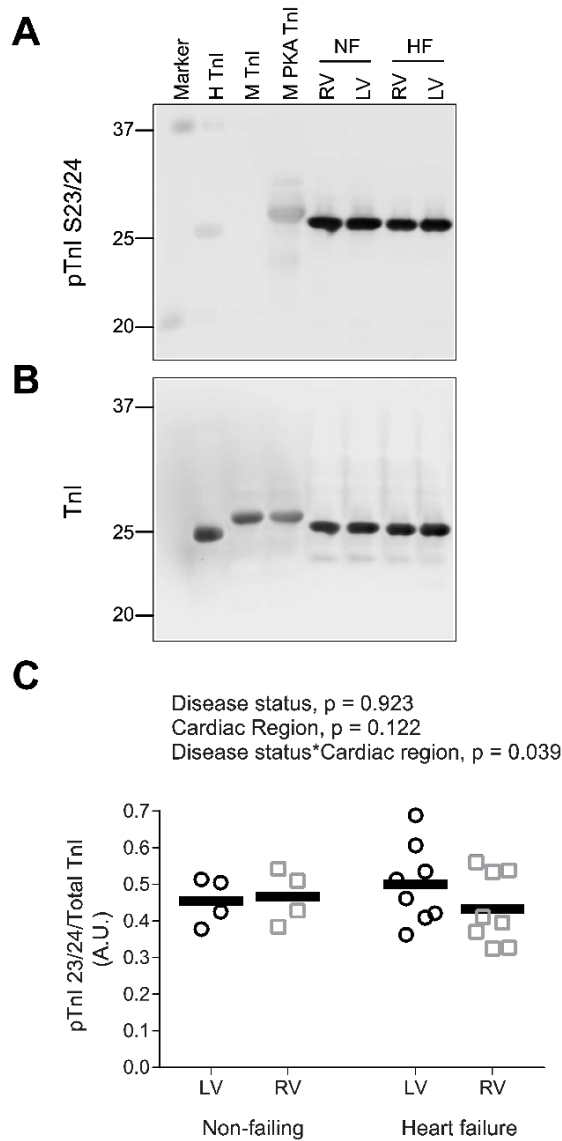


Fig 2.6. Phosphorylation of TnI Ser 23/24 exhibits a statistical interaction between heart failure status and cardiac region.

A) Representative western blot for TnI Ser-23/24 phosphorylation, and B) total TnI. C) Quantification. Gel labels for panels A and B: Marker; molecular weight marker, H TnI; purified recombinant human cardiac TnI, M TnI; purified recombinant mouse cardiac TnI, M PKA TnI; PKA-treated purified recombinant mouse cardiac TnI containing Ser-23/24 phosphorylation.

2.4. Discussion

This study shows for the first time that multicellular preparations from the RV and LV of human myocardium have similar mechanical properties when maximally activated but different sensitivities to activating Ca^{2+} . Intriguingly, in non-failing hearts, preparations from the RV are less sensitive to Ca^{2+} than preparations from the LV. In failing hearts, the opposite behavior occurs; RV samples are more sensitive to Ca^{2+} . Additional biochemical data suggest that these ventricle-specific effects are driven by changes in the relative phosphorylation of cTnl.

2.4.1. Mechanical properties of non-failing tissue

One of the long-standing questions in cardiac biology is whether interventricular differences in myocardial contraction contribute to the higher systolic pressure generated by the LV. To address this question, we measured F_{max} , maximum power output, and maximum shortening velocity, and rate of tension development in non-failing human myocardium from the RV and LV. The data show (Figs. 2.3 & 2.4) that none of these parameters exhibited significant inter-ventricular differences.

To our knowledge, our study is the first to measure these mechanical properties using permeabilized human samples. However, Belin et al. ⁷ and Perreault et al. ¹⁰ had already shown similar F_{max} results using permeabilized myocardium from rats. Other groups had compared the properties of living (electrically-excitable) human tissue. For example, Harding et al. ¹³ showed that unloaded shortening

velocity did not differ between cells from the LV and RV. Together these data suggest that myocardium from the LV and RV has similar contractile properties. Inter-ventricular differences in pressure are therefore most likely to reflect chamber-specific fiber alignments and geometries.

2.4.2. Mechanical properties in failing tissue

One of our previous studies showed that end-stage heart failure reduces F_{\max} and maximum power without altering shortening velocity in multicellular preparations from the LV of human hearts⁹⁵. The biochemical data from that study suggested that the reduction in maximal force was most likely to result from increased collagen deposition in the failing tissue. In this study, we aimed to test if heart failure has a similar impact on the mechanical properties of both ventricles. Our data (Figs. 2.3 & 2.4) show that this is indeed the case. Heart failure reduced F_{\max} by ~40% and maximum power out by ~30% in samples from both ventricles without changing shortening velocity or k_{tr} . Heart failure thus seems to produce similar contractile deficits in both chambers.

2.4.3. Calcium sensitivity

To our knowledge, this study is the first to demonstrate that human myocardium from the RV and LV has different sensitivities to activating Ca^{2+} . The pCa_{50} and Hill coefficient data both displayed an interaction between disease status and cardiac region, with the biggest change in pCa_{50} and cooperativity occurring between the RV of non-failing and failing hearts (Figs 2.2A & B). In non-failing

cardiac tissue, pCa₅₀ and Hill coefficient values were higher in the LV.

Conversely, in failing preparations, the RV had higher pCa₅₀ and Hill coefficient values.

Our data from non-failing hearts are consistent with prior measurements in rats performed by Belin and colleagues ⁷ and Perreault et al. ¹⁰. There are, however, some subtle differences between the datasets for failing tissue. Belin et al. ⁷ found that heart failure reduced pCa₅₀ in single cells isolated from the LV but had no effect on RV cells. Perreault et al. ¹⁰ used multicellular preparations and demonstrated that heart failure increased pCa₅₀ in RV myocardium but had no effect on LV tissue. It is not clear why the three studies produce slightly different results, but inter-species effects, differences between single cells and multicellular preparations, and/or statistical variation could be contributing factors.

2.4.4. Phosphorylation of myofilament proteins

It is well known that increased phosphorylation of cTnI (most notably at Ser-23/24) reduces myofilament Ca²⁺ sensitivity ^{85,86,89,92,99-103}. Our data (Figs 2.5B and 2.6C) suggest that this modification may be driving the changes we observe in pCa₅₀ (Fig 2.2A). All three datasets (pCa₅₀, global cTnI phosphorylation assessed with ProQ Diamond, and Ser-23/24 phosphorylation assessed by western blot) exhibited statistical interactions between heart failure status and cardiac ventricle, with lower pCa₅₀ values (decreased Ca²⁺ sensitivity) being associated with greater cTnI phosphorylation.

RLC relative phosphorylation also exhibited a significant statistical interaction (Fig 2.5C) with higher phosphorylation levels again being associated with reduced Ca^{2+} sensitivity. This is a more complex relationship because increased phosphorylation of RLC is known to enhance Ca^{2+} sensitivity^{104,105}. We, therefore, think that RLC phosphorylation levels may change in human heart failure to compensate for the posttranslational modifications to cTnI partially.

This interpretation is similar to that described by van der Velden and coworkers⁹³ in their study of end-stage human heart failure. Like us, these authors demonstrated parallel changes in the relative phosphorylation of RLC and cTnI. They concluded that RLC might be dephosphorylated during human heart failure to protect against enhanced Ca^{2+} sensitivity / diastolic dysfunction resulting from dephosphorylation of cTnI. Our data are consistent with this general hypothesis, but the molecular mechanisms need to be tested in additional future studies.

The data describing cMyBP-C phosphorylation are probably simpler to interpret. cMyBP-C was dephosphorylated in failing samples, but there were no inter-ventricular effects (Fig 2.5D). Reducing cMyBP-C phosphorylation increases myofilament Ca^{2+} sensitivity¹⁰⁶⁻¹⁰⁹ so this posttranslational modification may be driving the global increase in Ca^{2+} -sensitivity observed in the failing samples (Fig 2.2A).

In summary, these biochemical data suggest that changes in cTnI phosphorylation are driving inter-ventricular differences in calcium sensitivity while dephosphorylation of cMyBP-C contributes to a global increase in pCa₅₀ values during heart failure.

2.4.5. Source of intra-ventricular variation

We do not know why the LV and RV have different Ca²⁺ sensitivities. They may, for instance, be tuned to different mechanical loads. Another possibility is that they are responding differently to β-adrenergic stimulation. Molina et al.¹¹⁰ showed that the RV was more sensitive to β-adrenergic stimulation than the LV in healthy dogs and that the enhanced sympathetic response preferentially increased PKA activity in right ventricular tissue.

PKA increases phosphorylation of cTnI¹¹¹ so differential responses to β-adrenergic stimulation could contribute to the inter-ventricular effects that we measured for cTnI modifications (Fig 2.5B, 2.6C). However, region-specific changes in PKA activity would also be expected to produce enhanced cMyBP-C phosphorylation in non-failing myocardium from the right ventricle, which we did not observe. Neither is it clear whether human ventricles respond to β-adrenergic stimulation in the same way as canine tissue. More experiments in this area are required.

2.5. Conclusion

This study presents three important results. First, human heart failure increases the Ca^{2+} sensitivity of right ventricular myocardium more than the calcium sensitivity of left ventricular myocardium. Second, this contractile effect is likely to involve inter-ventricular differences in posttranslational modifications to sarcomeric proteins including TnI. Third, heart failure depresses maximum force and maximum power by similar amounts in tissue from both ventricles.

Chapter 3

Omecamtiv mecarbil increases Ca²⁺ sensitivity and decreases the rate of force development in failing human hearts

The content reported in this chapter has been published in “Swenson, A.M., Tang, W., **Blair, C.A.**, et al. Omecamtiv Mecarbil Enhances the Duty Ratio of Human beta-Cardiac Myosin Resulting in Increased Calcium Sensitivity and Slowed Force Development in Cardiac Muscle. *The Journal of biological chemistry* 292, 3768-3778, doi:10.1074/jbc.M116.748780 (2017). PMID 28082673 ¹¹².

In this dissertation, only the information about the mechanical analysis from this study will be discussed.

3.1. Introduction

Heart failure is a clinical syndrome caused by cardiac dysfunction ¹¹³. In the United States, it affects ~6 million Americans and is the main reason for hospital admissions in the elderly ¹¹⁴⁻¹¹⁶. The most common manifestation of the syndrome is systolic heart failure which is marked by decreased contractility ⁶⁴. Many factors can change the function of the heart that can lead to heart failure, including cell death, impaired calcium handling, neurohumoral disturbances, and changes in cardiac structure (hypertrophy or dilation) ¹¹⁷⁻¹¹⁹. However, in patients

suffering from systolic heart failure, sarcomeric dysfunction plays a central role
75,120.

Patients who suffer from systolic heart failure are often prescribed positive inotropes to increase contractility and cardiac output. Inotropic drugs are agents that enhance myocardial contractile force ¹²¹ (Fig. 3.1 ^{50,122}). Some of the current inotropes used in clinical practice include adrenergic receptor agonist such as Norepinephrine, and Dobutamine, as well as the phosphodiesterase inhibitor Milrinone ¹²¹. At the basic level, an increase in contractile force is accomplished by altering actin-myosin interactions. Existing Inotropic agents alter the actin-myosin interaction by indirectly activating the secondary messenger signaling pathways that increase intracellular Ca^{2+} concentrations ¹²¹. The result of these mechanisms is an increase in the number of myosin heads attached to actin filaments because of increased Ca^{2+} binding to troponin C, which increases contractile force. While these inotropes increase contractility, unfortunately, they also increase heart rate, myocardial oxygen consumption, and can produce arrhythmias, all of which can contribute to higher mortality rates ¹²²⁻¹²⁴.

To overcome the deleterious effects of current inotropes, drugs that directly target the sarcomeres of cardiac tissue are being developed. One class of such compounds that directly influence the cross-bridge cycle are called cardiac myosin activators ¹²⁵ (Fig. 3.1 ^{50,122}). The first of such drugs that have shown signs of overcoming the harmful effects of current inotropes is omecamtiv

mecarbil (OM) ¹²⁴. In studies in dogs ⁶³ and rats ⁶⁴, OM improved cardiovascular function without increasing myocardial oxygen consumption or altering Ca²⁺ transients. Thus, OM is the first cardiac myosin activator that has made it into clinical trials ¹²⁶⁻¹²⁸.

For sarcomeres to generate force, actin and myosin must interact ¹²⁹. During the actin-myosin cross-bridge cycle, actin and myosin undergo weak and strong binding (Fig.3.2 ⁶²). The cycle is initiated by the binding of ATP to myosin which allows myosin to dissociate from actin rapidly. The rapid hydrolysis of ATP to ADP and P_i by the ATPase of the cross-bridges (S1 in Fig 3.1) creates a weak or electrostatic contact between actin and myosin. Upon binding, the newly formed actin-myosin-ADP-P_i complex undergoes an activation step which concomitantly involves the release of P_i and the transition of actin and myosin from a weakly-bound to a strongly-bound state (Fig. 3.2 ⁶²) ¹³⁰. It is also during the release of P_i where the cross-bridges undergo a conformational change producing a ~10 nm power-stroke which contracts the muscle. The transition from the weak to strongly bound state between actin and myosin is a critical step, as it dictates the amount of force the muscle will be able to produce ^{131,132}. OM accelerates this transition, as the drug increases the rate of phosphate release (Fig. 3.2 ⁶²) forcing actin and myosin into the strong force producing state faster ^{64,133,134}. Thus, OM enhances the number of myosin heads in the strongly-bound force generating state at any given time during the cross-bridge cycle, which increases the amounts of cross-bridges pulling on actin.

The impact of OM on cardiac function in a dose-dependent manner in humans has not been studied. This study aims to test the hypothesis that OM alters the mechanical properties of permeabilized human cardiac tissue in a dose-dependent manner.

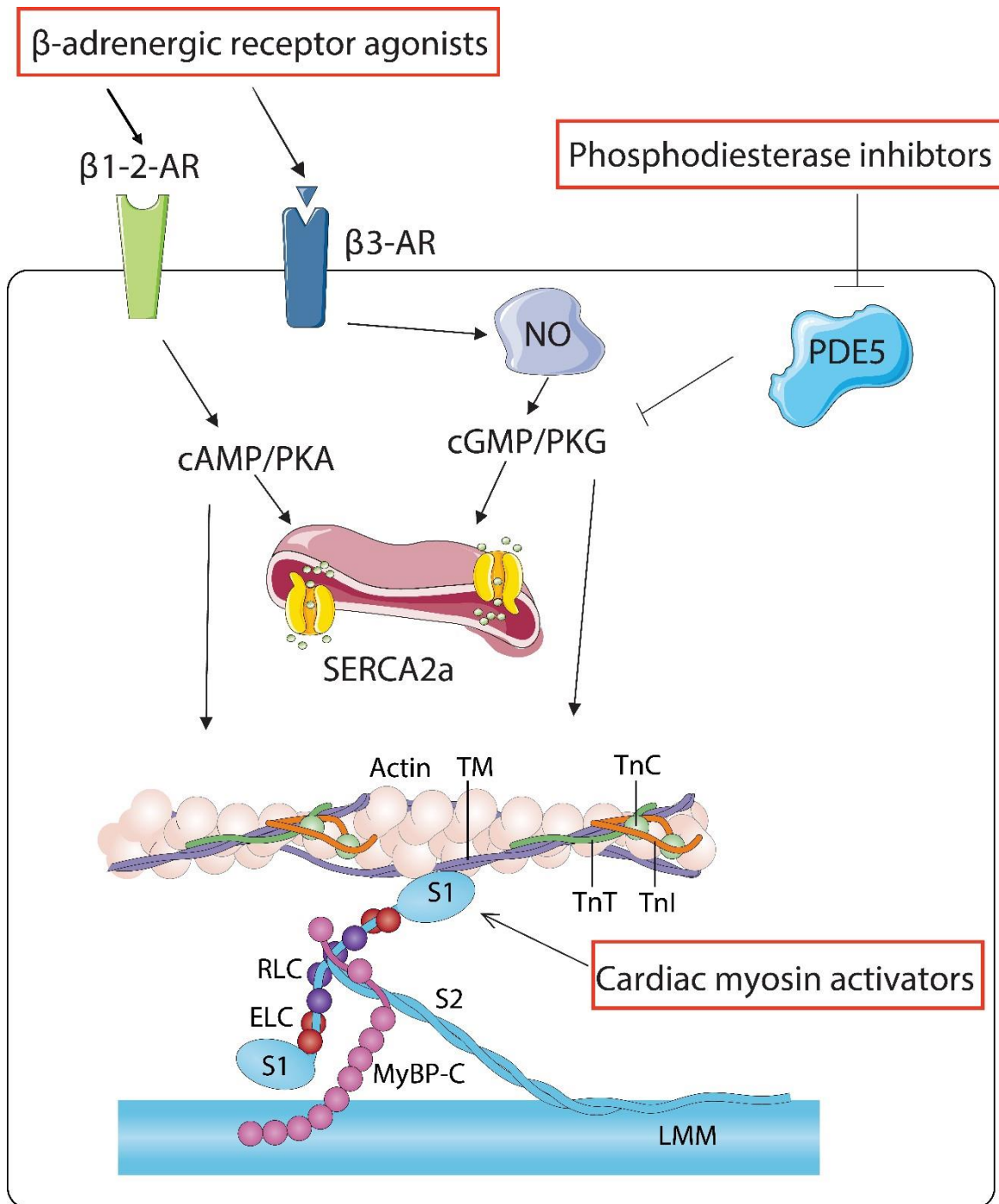


Figure 3.1. Selected inotropes mode of action

Figure was made by combining and modifying figures from Hwang & Sykes, 2015⁵⁰ and Tarone et al., 2014¹²²

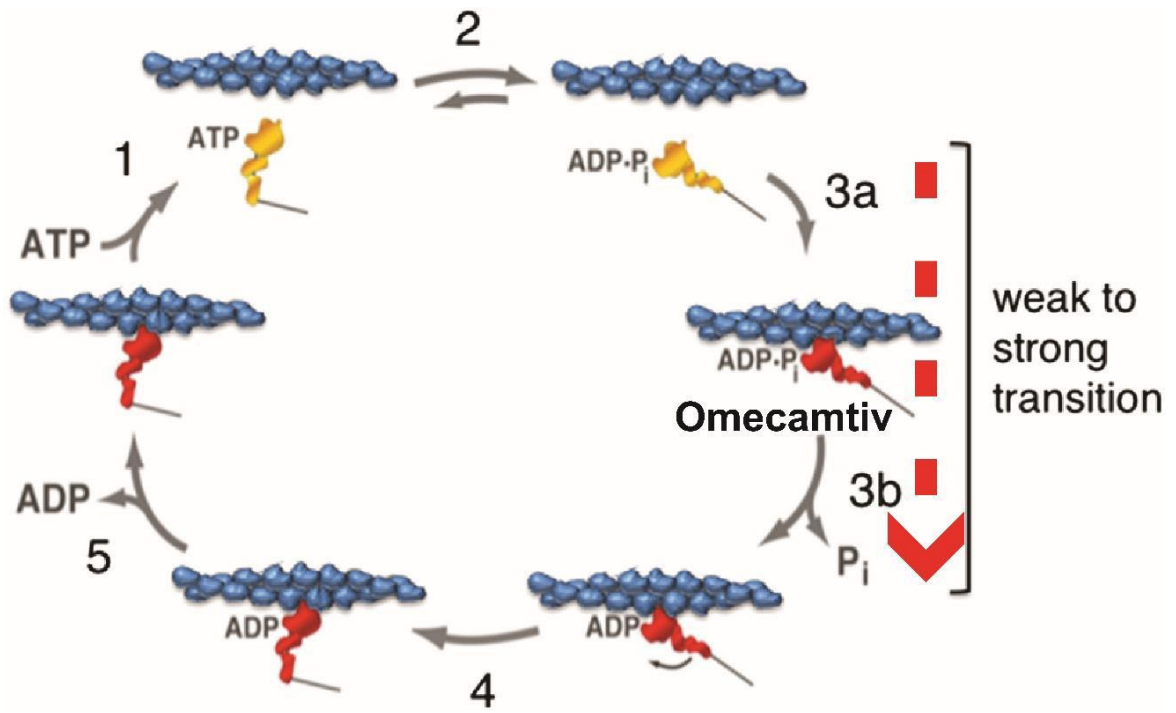


Figure 3.2. Actin-Myosin cross-bridge cycle illustrating the segment of the cycle (between 3a and 3b) omecamtiv mecarbil is proposed to influence

Figure modified from James Spudich, 2014 ⁶²

3.2. Methods

The human cardiac samples used for this study were procured as described in the methods section of chapter 2, section 2.2.1.

3.2.1. Clinical characteristics of patients with heart failure

The samples from patients undergoing heart transplants came from 3 males (classification of heart failure: 2 ischemic and 1 non-ischemic) and 5 females (classification of heart failure: 1 ischemic and 4 non-ischemic). The mean age of the patients was 50 (range 31 to 68). Patients' characteristics are summarized in table 3.1.

3.2.2. Multicellular preparations

Permeabilization of human tissue was performed as described in section 2.2.3. A total of 46 multicellular preparations from 8 patients with heart failure were analyzed. The average length of the preparations used in this study was $1055 \pm 229 \mu\text{m}$, and the average cross-sectional area was $5.87 \pm 2.42 \times 10^{-8} \text{ m}^2$. Cross-sectional area was estimated assuming a circular profile.

3.2.3. Incubation of multicellular preparations

Solutions with pCa values ranging from 9.0 to 4.5 (made as described in the methods section of chapter 2, section 2.2.5) and OM concentrations of 0.1, 1.0, or 10 μM were made by adding suitable amounts of OM dissolved in DMSO. The final percentage of DMSO in every experimental solution was 0.67%.

Preparations were immersed in a pCa 9.0 solution containing 0 (control), 0.1, 1.0, or 10.0 μM for at least 3 min between trials. The ionic strength was held constant at 180 mM in all solutions.

3.2.4. Mechanical measurements

Pilot test showed that OM is hard to wash out of the multicellular preparations. Each preparation was therefore exposed to only one concentration of OM (0, 0.1, 1.0, or 10 μM). All experimental measurements were conducted at 15°C using the experimental set-up describes in section 2.2.4 and SLControl software ⁹⁶. For this study only maximum force, the rate of tension development, and Ca^{2+} sensitivity as described in section 2.2.6 were measured. Shortening velocity and maximum power measurements were not performed in this study. The higher concentrations of OM (1.0 and 10 μM) altered the natural properties of multicellular preparations so these measurements could not be done with accuracy.

3.2.5. Statistical analysis

A linear mixed model approach that took into account drug concentration and multiple preparations from the same patient was used to analyze 46 multicellular preparations from 8 patients.

Table 3.1. Patients' characteristics

Age	Gender	Diagnosis	Cardiomyopathy
56	Female	Failing	Non-ischemic
36	Female	Failing	Non-ischemic
59	Female	Failing	Ischemic
31	Female	Failing	Non-ischemic
66	Female	Failing	Non-ischemic
37	Male	Failing	Ischemic
68	Male	Failing	Ischemic
47	Male	Failing	Non-ischemic

3.3. Results

3.3.1. OM increases Ca^{2+} sensitivity and decreases the rate of force development

The effects of OM on the mechanical properties of human myocardium were measured using chemically permeabilized multicellular preparations. The maximum isometric force was not significantly altered by OM (Fig. 3.3). In contrast, 1 and 10 μM OM significantly ($p < 0.001$) increased pCa_{50} (Fig. 3.4). In comparison to vehicle, 1 μM OM increased pCa_{50} from 5.55 ± 0.06 to 5.83 ± 0.08 ($p < 0.001$), while 10 μM OM increased pCa_{50} from 5.55 ± 0.06 to 6.05 ± 0.18 ($p < 0.001$). These concentrations of OM also slowed k_{tr} , the rate of force development (k_{tr} in OM-free medium: $1.04 \pm 0.26 \text{ s}^{-1}$; in 1 μM OM: $0.33 \pm 0.11 \text{ s}^{-1}$ ($p < 0.001$ vs OM free medium); in 10 μM OM $0.17 \pm 0.16 \text{ s}^{-1}$ ($p < 0.001$ vs OM free medium)) (Fig. 3.5).

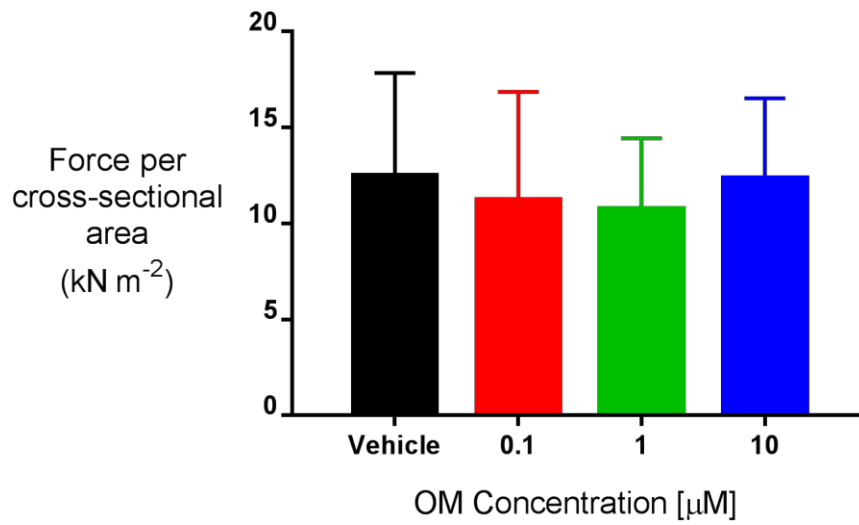


Figure 3.3. OM does not alter maximum force

All data obtained in maximally-activating solutions with pCa values of 4.5.

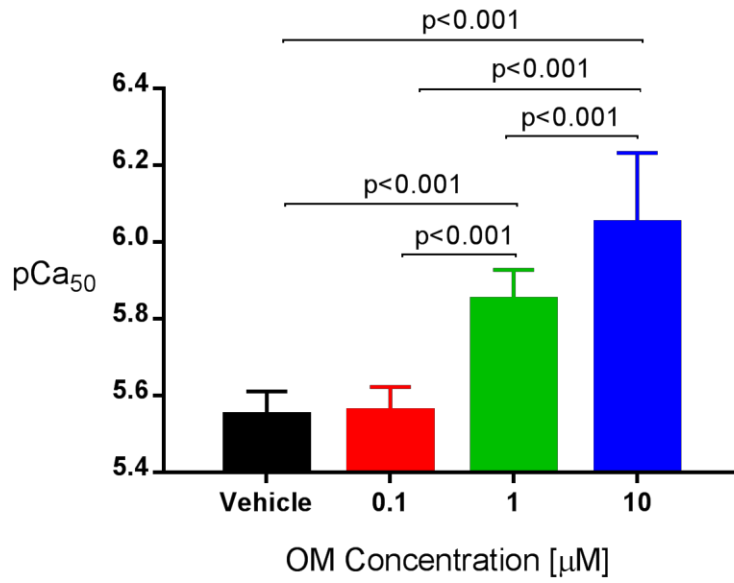


Figure 3.4. OM increases Ca²⁺ sensitivity in a dose-dependent manner

All data obtained in maximally-activating solutions with pCa values of 4.5.

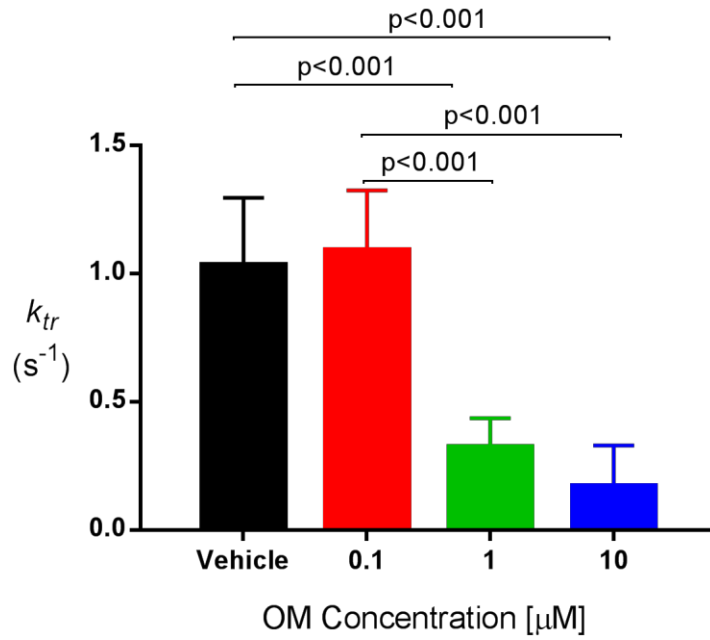


Figure 3.5. OM decreases the rate of force development

All data obtained in maximally-activating solutions with pCa values of 4.5.

3.4. Discussion

The impact of omecamtiv mecarbil on the mechanical properties of permeabilized cardiac tissue has been studied in rats ¹³⁵ and humans ¹³⁶. In rats, OM increased calcium sensitivity, slowed the rate of force development, and enhanced force in sub-maximal calcium concentrations ¹³⁵. Mamidi et al. ¹³⁶ performed similar measurements in humans, using 1 μM OM in sub-maximal calcium concentrations. They reported that OM increased isometric force and calcium sensitivity. Our study adds to the findings of Mamidi and coworkers ¹³⁶ by further examining the impact of OM on human myocardium in a dose-dependent manner. We find an increase in calcium sensitivity in a dose-dependent manner with little change in steady-state force at maximum calcium concentrations. Also, we observe a decrease in the rate of force development.

The fraction of cross-bridges bound to actin in the strongly bound state at any moment during the actin-myosin ATPase cycle is referred to as the “duty ratio” ¹³⁷. By increasing the duty ratio, you increase cooperative activation of the thin filament making it more sensitive to calcium ^{138,139}. Additionally, an increase in the duty ratio could reduce the rate of force development by slowing the detachment of strongly-bound myosin heads from actin. To determine if OM increased the duty ratio, *in vitro* motility assays were performed (not included in the dissertation, but was done by collaborator for manuscript). The results of the *in vitro* motility assays showed that OM increased the duty ratio, and trapped a population of myosin heads in a weakly-bound state with actin, slowing phosphate release ¹¹². Thus, we propose that OM increases calcium sensitivity

by increasing the duty ratio. Additionally, the data suggest that the reduction in the rate of force development could be the result of the increase in weakly bound non-force generating cross-bridges, or because of the increase in the duty ratio, which would slow the detachment of cross-bridges.

Shortening velocity measurements were attempted in the mechanical measurements. However, at OM concentrations of 1 and 10 μM , shortening velocity measurements could not be accurately obtained with the multicellular preparations. The drug drastically reduced force development; thus, when performing the load clamps (changing the length of the preparation), an accurate measurement of the force generated by multicellular preparation could not be obtained. Therefore, shortening velocity measurements were not recorded. However, sliding velocity measurements were performed by our collaborators. Using *in vitro* motility assays, they were able to measure the sliding velocity of actin with the same human cardiac samples used for the multicellular preparation experiments (all of these data are published in the manuscript by Swenson et al.¹¹²). Similar to the rate of force development, the results of the motility assay showed that OM significantly reduced sliding velocity¹¹². These findings are consistent with earlier studies in pigs^{140,141} and humans¹⁴². From the results of the motility assays, it was proposed that in the presence of OM a population of myosin heads is consistently cycling from a detached to a weakly-bound state with actin, which slows phosphate release creating a viscous drag that reduces the actin sliding velocity.

3.5. Summary

The discovery of omecamtiv mecarbil as an inotrope that can alter contractile properties without changing calcium homeostasis and myocardial oxygen consumption is promising for patients suffering from systolic heart failure. The drug has made it into clinical trials. A phase I trial demonstrated an improvement in systolic ejection time and stroke volume ¹²⁶. Similarly, a phase II trial also showed increased systolic ejection time ¹⁴³. Both studies reported that the effects are dose-dependent. Overall, it appears that dosing could be critical to receive the maximum benefits of the drug. The findings of this and previous studies also would suggest that an appropriate dose of OM is essential, as too much of the drug can slow force development and increase calcium sensitivity, which may impact the rate of relaxation.

Chapter 4

Para-Nitroblebbistatin reduces maximum force and calcium sensitivity in human myocardium

The effects of para-Nitroblebbistatin on the mechanical function of human myocardium are published in, Tang, W., **Blair, C.A.**, et al. Modulating Beta-Cardiac Myosin Function at the Molecular and Tissue Levels. *Frontiers in Physiology* **7**, doi:10.3389/fphys.2016.00659 (2017). PMID: 28119616 ¹⁴⁴.

In this dissertation, only the information about the mechanical analysis from this manuscript will be discussed.

4.1. Introduction

Hypertrophic cardiomyopathy (HCM) is the most common occurring inherited cardiac disease ¹⁴⁵. Clinically, HCM is characterized by hypertrophy of the ventricles and interventricular septum in the absence of predisposing conditions such as hypertension or aortic stenosis ¹⁴⁶. Consequently, the interior diameters of the chambers decrease, ventricular compliance is reduced, and diastolic function is impaired ¹⁴⁷. Pathologically, HCM results in disarrayed cardiomyocytes and increased fibrosis ¹⁴⁸. The disease is prevalent, as 1 in 500 individuals are affected by HCM which may result in heart failure or sudden death ¹⁴⁹.

HCM is a disease of the sarcomere, as up to 60 to 70% of HCM cases are attributed to mutations in sarcomeric proteins ¹⁵⁰⁻¹⁵². Defects in genes encoding

myosin heavy chain ¹⁵³, myosin essential ¹⁵⁴ and regulatory light chains ¹⁵⁵, myosin-binding protein C ¹⁵⁶, actin ¹⁵⁷, tropomyosin ¹⁵⁸, troponins I ¹⁵⁹, C ¹⁶⁰ and T ¹⁶¹ have all been associated with HCM. The most common being mutations in the human β -cardiac myosin heavy chain, which accounts for ~40% of HCM cases ^{162,163}. Within the entire human β -cardiac myosin (heavy and light chains), there have been over 300 pathogenic mutations described, most of which are in the motor (subfragment 1 (S1)/cross-bridges) domain ¹⁶⁴⁻¹⁶⁶.

Human β -cardiac myosin is the motor that drives contraction. Studies have shown that mutations in this protein can make the heart hypercontractile ^{167,168}. At the mechanical level, a hypercontractile heart generates more power (product of force and velocity). Thus, patients suffering from HCM as a result of myosin mutations often exhibit hyperdynamic (substantially high ejection fraction) ventricular function ¹⁶⁹. Currently, there is no pharmacological therapy for HCM. However, small molecules that can reduce the amount of force (i.e., reduce power) by lowering the duty ratio (fraction of cross-bridges bound to actin in the strongly bound state at any moment during the ATPase cycle) has the potential to help patients suffering from HCM with hyperdynamic ventricular function.

Blebbistatin is a small molecule used as an experimental tool to inhibit myosin function ^{170,171}. The molecule was first identified as a non-muscle myosin II specific inhibitor but has been shown to inhibit myosin in striated muscle as well ^{65,172}. Blebbistatin inhibits the force generating capacity of myosin by

specifically binding to the myosin-ADP-P_i complex which slows the release of inorganic phosphate, trapping cross-bridges in the weakly bound state with actin (Fig. 4.1⁶²)¹⁷⁰⁻¹⁷². Additionally, studies have shown that Blebbistatin can reduce the amount of force cardiac tissue produce by stabilizing the binding of myosin heads to the thick filament¹⁷³⁻¹⁷⁵, thus removing the ability of cross-bridges to interact with actin. The folding of myosin heads where they cannot interact with the thin filament is referred to as the super-relaxed state¹⁷⁶. The mechanism by which Blebbistatin anchors myosin heads to thick filaments is unknown¹⁷⁵. In rodents, the effects of Blebbistatin were shown to be dose-dependent, as Dou et al.¹⁷⁷ demonstrated that mice given different doses of Blebbistatin decreased twitch force and shortening velocity in a dose-dependent manner.

In its general form, the use of Blebbistatin as an experimental tool is hindered, as blue-light exposure reverses its inhibitory effects¹⁷⁸. Additionally, the molecule is cytotoxic, phototoxic, and has poor solubility¹⁷⁹. To address these issues, more soluble, non-cytotoxic, non-phototoxic, non-fluorescent derivatives called para-NitroBlebbistatin (pN-Bleb) and para-amino-blebbistatin has been synthesized^{180,181}. These derivatives exhibit the same myosin inhibitory effects.

This study aimed to test the hypothesis that pN-Bleb will alter the mechanical properties of human cardiac tissue from patients with heart failure. The results of our study show that pN-Bleb reduces maximum force, power, and calcium sensitivity without altering shortening velocity or the rate of tension development.

These data provide evidence that drugs designed to reduce myosin duty ratio by inhibiting strong attachment to actin can potentially treat patients with HCM.

4.2. Methods

The human cardiac samples used for this study were procured as described in the methods section of chapter 2, section 2.2.1.

4.2.1. Clinical characteristics of patients with heart failure

The samples from patients undergoing heart transplantation came from 2 males (classification of heart failure: 1 ischemic and 1 non-ischemic) and 2 females (classification of heart failure: 2 non-ischemic). The mean age of the patients was 45 (range 31 to 68). Patients' characteristics are summarized in Table 4.1.

4.2.2. Multicellular preparations

Permeabilization of human tissue was performed as described in section 2.2.3. A total of 46 multicellular preparations from 8 patients with heart failure were analyzed. The average length of the preparations used in this study was $1047 \pm 232 \mu\text{m}$, and the average cross-sectional area was $5.07 \pm 2.47 \times 10^{-8} \text{ m}^2$. Cross-sectional area was estimated assuming a circular profile.

4.2.3. Solutions

The ionic composition of the pCa solutions used in this study was adjusted for use at 22° C. The ionic strength was held constant at 180 mM in all solutions.

The relaxing solution used to isolate and permeabilize multicellular preparations did not change from what was described in chapter 2, section 2.2.5. To make the pCa solutions, we initially made pCa 4.5 & 9.0. Those two pCa solutions were then mixed to make intermediate concentrations ranging from pCa 5.0 to pCa 6.4. The composition of pCa 4.5 (mmol L⁻¹): 7 EGTA, 20 Imidazole, 51 KCL, 14.5 Creatine, 4.8 ATP, 7 CaCl₂, 5.22 mgCl₂, sufficient amounts of KOH to get pH to 7.0. The composition of pCa 9.0 (in mmol L⁻¹): 7 EGTA, 20 Imidazole, 68 KCL, 14.5 Creatine phosphate, 4.74 ATP, 5.48 MgCl₂, and 15.3 μM CaCl₂ plus ample amount of KOH to reach pH 7.0.

4.2.4. Incubation of samples with pN-Bleb

pN-Bleb combined with Dimethyl sulfoxide (DMSO) was added to pCa values ranging from 9.0 to 4.5 to create solutions with a final concentration of 0 (DMSO only), 1, 10, or 50 μM pN-Bleb. The final percentage of DMSO in every experimental solution was 1.33%. Each preparation was initially tested in control solutions (0 pN-Bleb) with pCa values ranging from 9.0 to 4.5. The preparation was then immersed for 5 min in a pCa 9.0 solution containing 1, 10, or 50 μM pN-Bleb. Mechanical experiments described in sections 2.2.6 and 2.2.7 were then performed to assess maximum force, calcium sensitivity, the rate of force development, shortening velocity and maximum power. These samples were only tested in pCa 4.5 solutions with 0 pN-Bleb(control) and then a chosen experimental pN-Bleb concentration. These experimental designs ensured that each preparation could act as its own control and minimized the progressive

decline in contractile force (experimental run-down) that occurs when permeabilized preparations are subjected to repeated activations.

4.2.5. Statistical analysis

The same linear mixed model used in section 3.2.5 was used to analyze the 24 multicellular preparations from the 4 patients used in this study.

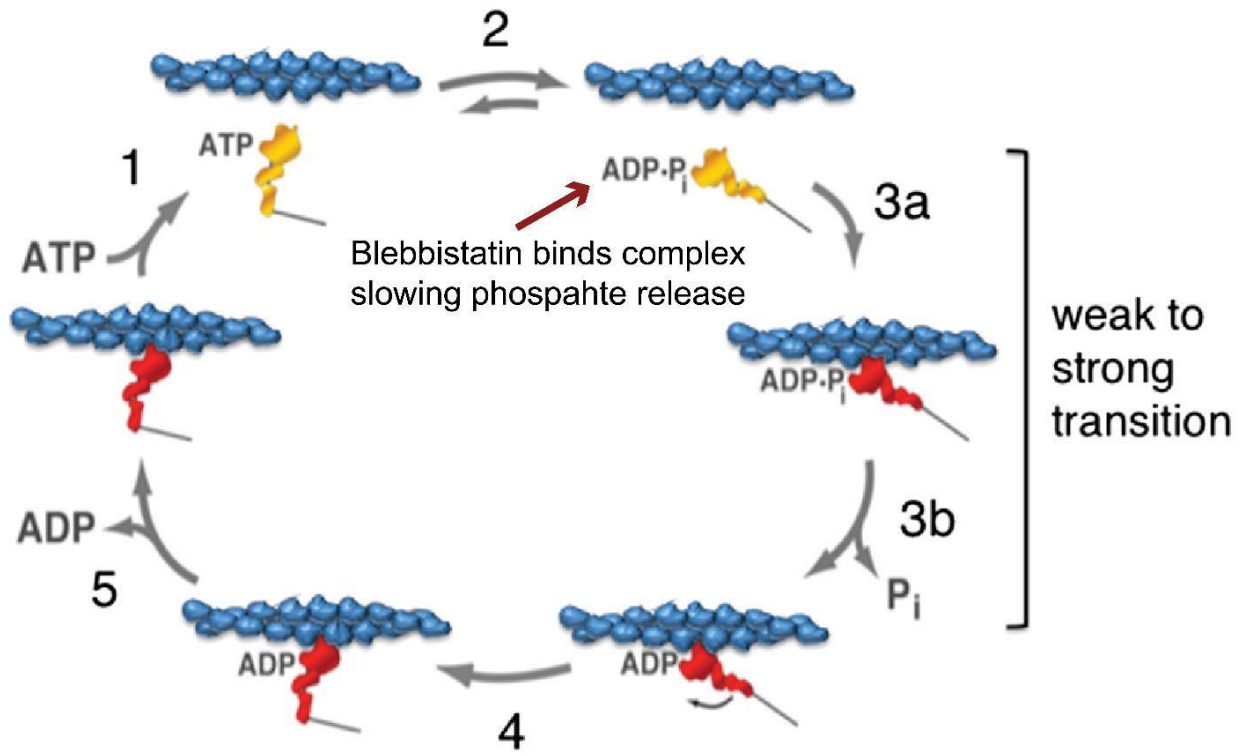


Figure 4.1. pN-Blebb mode of action

Table 4.1. Patients' characteristics

Age	Gender	Diagnosis	Cardiomyopathy
36	Female	Failing	Non-ischemic
31	Female	Failing	Non-ischemic
68	Male	Failing	Ischemic
47	Male	Failing	Non-ischemic

4.3. Results

4.3.1. pN-Bleb reduces maximum force and power

To assess the impact of pN-Bleb on the mechanical function of permeabilized human hearts, I measured force, tension recovery, Ca^{2+} sensitivity, shortening velocity and power in maximum Ca^{2+} solution containing either control (DMSO only), 1, 10, or 50 μM pN-Bleb. Figure 4.2 shows the maximum force and maximum power of 1, 10 and 50 μM pN-Bleb normalized to control. Maximum force (Fig. 4.2A) was significantly different ($p=0.020$) between the 1 μM and 50 μM pN-Bleb. The maximum power generated by the preparations were significantly reduced at 50 μM ($p<0.001$) and 10 μM ($p=0.024$) pN-Bleb (Fig. 4.2B). Neither maximum shortening velocity (Fig. 4.3A) or rate of tension recovery (k_{tr}) (Fig. 4.3B) changed between the varying concentrations of pN-Bleb.

Figure 4.4A shows tension-pCa curves of 1, 10 and 50 μM pN-Bleb normalized to control. The curves indicate that in addition to reducing relative force, pN-Bleb also reduced Ca^{2+} sensitivity ($p\text{Ca}_{50}$ values; Fig. 4.4B) and the Hill coefficient (Fig. 4.4C). The effects of pN-Bleb on $p\text{Ca}_{50}$ and the Hill coefficient were only significant at the 50 μM concentration.

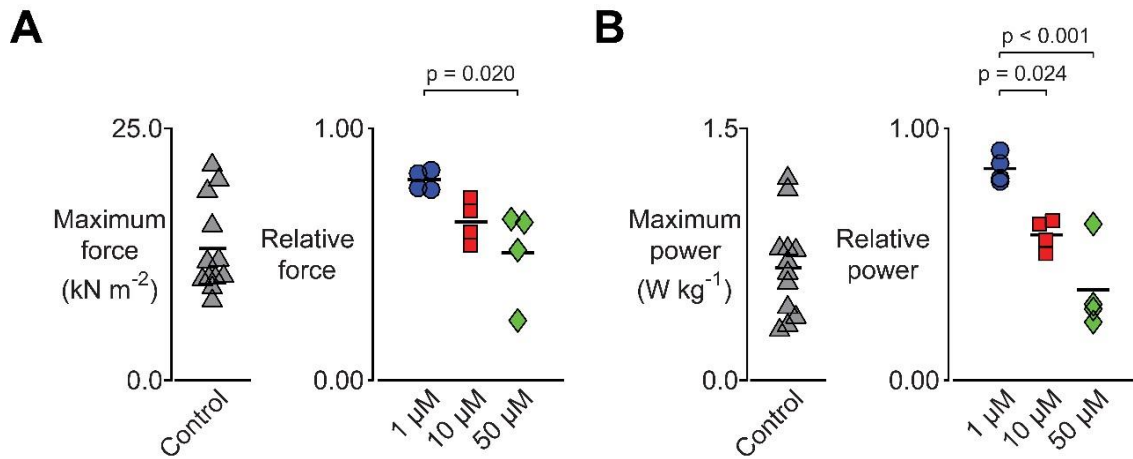


Figure 4.2. pN-Bleb reduced maximum force and maximum power.

The left- hand panels show data measured in pCa 4.5 solution plus 1.33% DMSO. Right- hand panels show data measured in pCa 4.5 solution plus 1.33% DMSO and either 1, 10, or 50 μM pN-Bleb. The different concentrations of pN-Bleb were normalized to the control (DMSO only). A) Maximum force, and B) maximum power.

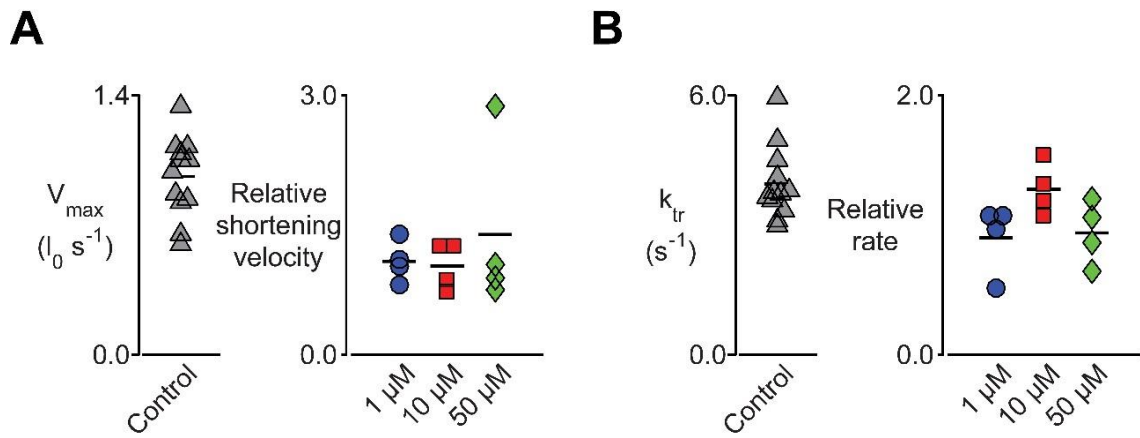


Figure 4.3. pN-Bleb does not alter shortening velocity or the rate of tension recovery.

The left-hand panels show data measured in pCa 4.5 solution plus 1.33% DMSO. Right-hand panels show data measured in pCa 4.5 solution plus 1.33% DMSO and either 1, 10, or 50 μM pN-Bleb. The different concentrations of pN-Bleb were normalized to the control (DMSO only). A) Maximum shortening velocity, and B) rate of tension redevelopment.

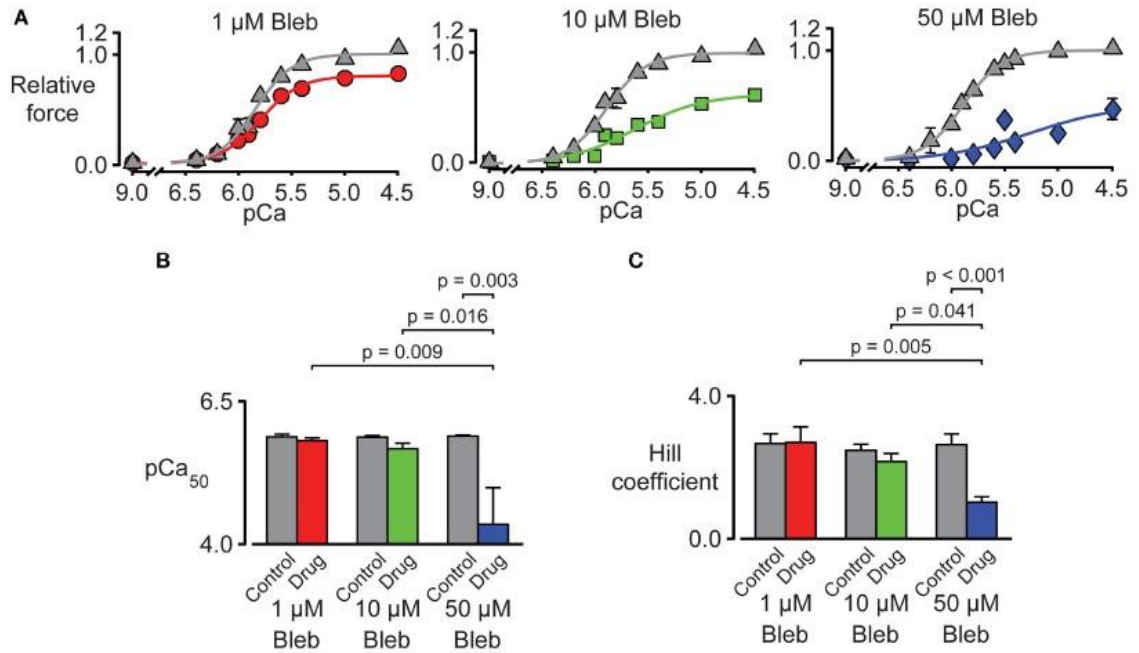


Figure 4.4. Impact of pN-Bleb on Ca^{2+} sensitivity and isometric force.

A) Representative tension-pCa curves for multicellular preparations activated in solutions with pCa values ranging from 9.0 to 4.5. The control (gray) data points show force values measured in the presence of 1.33% DMSO. The red, green, and blue data points show force measured in 1.33% DMSO plus 1, 10, or 50 μ M pN-Bleb. All force values were normalized to the control. B) pCa₅₀ and C) Hill coefficient results.

4.4. Discussion

HCM is an inherited disease that can lead to a hypercontractile heart. Currently, there is no effective treatment for the disease. However, small molecules may serve as therapy to treat the disorder, as these compounds can regulate human beta myosin, the primary protein driving the hypercontractile phenotype seen in HCM patients. Utilizing a modified version of the myosin inhibitor blebbistatin, the findings of this study suggest that small molecules designed to target human hearts with myosin inhibitory capacity similar to that of blebbistatin may serve as therapy for patients suffering from HCM. The results of the current research show that pN-Bleb reduces maximum force, maximum power, and alter Ca^{2+} sensitivity without changing the rate of cell shortening or tension recovery in human myocardium.

While the interpretation of the force and power data is unambiguous, caution should be taken when interpreting the pCa_{50} and Hill coefficient data. As shown in figure 4.4, the pCa_{50} (Fig. 4.4B) and Hill coefficient (Fig. 4.4C) values significantly decreased in the 50 μM pN-Bleb concentrations but did not demonstrate any marked effects in the 1 or 10 μM concentrations. A possible explanation for the reduction in pCa_{50} and Hill coefficients at 50 μM pN-Bleb concentrations could be the absence of the flat plateau in the tension-pCa curve at 50 μM . The absence of the flat plateau would suggest that isometric force did not completely saturate in a pCa 4.5 solution in the presence of 50 μM pN-Bleb. These data could indicate that a high concentration of pN-Bleb desensitizes the thin filaments by reducing the myosin duty ratio which would lower pCa_{50} and Hill

coefficient values. Additionally, the low Hill coefficients and pCa_{50} values measured in the presence of 50 μM pN-Bleb may also be explained by a progressive reduction in force development during the experiments. In rodent hearts, Dou et al. ¹⁷⁷ showed that the effects of blebbistatin on force development are time-sensitive, as it took ~30 min for the force to stabilize in the presence of the drug. It is possible that similar time-dependent effects in human myocardium might produce tension- pCa data similar to those shown in Figure 4.4A.

In the multicellular preparations, shortening velocity did not significantly vary between the pN-Bleb concentrations. However, it is worth noting that sliding velocity of S1 sub-fragment of myosin heads decreased in the in-vitro motility assays (data not shown here, published in Tang, Blair...et al ¹⁴⁴) exposed to the same concentrations of pN-Bleb as in the human mechanics study. In the motility assay, pN-Bleb reduced sliding velocity in a concentration-dependent manner. These data suggest that differences observed in sliding velocity at the molecular level do not translate to the tissue level. However, these differences between the molecular and tissue level may reflect the structural organization of the myosin heads at the tissue level. Within the tissue, there are many myosin heads near actin filaments, whereas in the motility assay not as many heads are interacting with actin filaments. Thus, the lowered sliding velocity observed at the molecular level could reflect the sensitivity of smaller amounts of heads to pN-Bleb inhibition that is not present in the larger organized tissue. To support this hypothesis, Fusi et al. ¹⁸² showed that only 1-4 myosin heads per thick filament

are required to sustain maximum shortening velocity in muscle fibers. Thus, even though some heads may be experiencing pN-Bleb effects in the tissue, there are enough heads that are not undergoing inhibition to maintain shortening velocity.

The use of small molecules that target the contractile apparatus to treat cardiac dysfunction has garnered a lot of attention, as some of these molecules have shown promise and have made it into clinical trials. For example, the aforementioned omecamtiv mecarbil (studies in chapter 3), and more recently, MYK-461. Similar to pN-Bleb, MYK-461 reduces the rate of phosphate release in the actin-myosin ATPase cycle. A recent study suggests that MYK-461 can diminish the hypercontractile nature of HCM hearts by reducing the myosin duty rate, thus decreasing the force and power ¹⁸³. These findings were demonstrated in rodent hearts. The results reported in this dissertation and the manuscript published by Tang, Blair, et al., ¹⁴⁴ would suggest that MYK-461 should have similar effects in humans, as the mode of action of both molecules is similar. The findings of this study and the use of MYK-461 in rodents to treat HCM are exciting, as they provide evidence that small molecules can potentially be used to treat patients with who suffer from HCM. However, more research is needed to see how effective compounds like MYK-461 are in patients with a variety of HCM mutations.

Chapter 5

Engineered troponins modulate the Ca²⁺ sensitivity of the failing human myocardium

5.1. Introduction

The troponin complex is a primary regulator of cardiac muscle contraction and relaxation. It consists of troponin C (TnC) the Ca²⁺ binding subunit, troponin I (TnI) the inhibitory subunit, and troponin T (TnT) the tropomyosin binding subunit. For a muscle to contract, free Ca²⁺ ions must bind TnC, which releases the inhibitory effects of TnI leading to a conformational shift in TnT, a cascade that ultimately enables the interaction of actin and myosin. Contrarily, the removal of Ca²⁺ ions from TnC must occur for actin and myosin to dissociate and relax the muscle. Being the Ca²⁺ sensor, TnC plays an integral role in tuning the response of the myofilament to Ca²⁺. However, the response of TnC to Ca²⁺ can be potentiated or reduced by other myofilament proteins (TnI, TnT, tropomyosin, and myosin) and by posttranslational modifications to many of these proteins ¹⁸⁴⁻¹⁸⁶.

Myofilament calcium sensitivity is a concept researcher use to describe the relationship between the concentration of free Ca²⁺ ions available to bind TnC and the amount of force the muscle generates ¹⁸⁷. While this reductionist approach reduces a complex system to two variables: free Ca²⁺ ions and force of contraction, it has provided significant insights into the mechanisms that govern cardiac contractility. Typically, hearts with increased Ca²⁺ sensitivity are

associated with increased contractility, whereas myofilaments that are less sensitive to Ca^{2+} have reduced contractility ¹⁸⁷.

A common observation in samples from patients with hypertrophic and dilated cardiomyopathy, as well as systolic and diastolic heart failure, is aberrant myofilament Ca^{2+} sensitivity ¹⁸⁸⁻¹⁹². Although it is unclear the exact mechanism by which Ca^{2+} sensitivity contributes to these etiologies, it is clear from studies in rodents that correcting cardiac Ca^{2+} sensitivity through genetic manipulation of myofilament proteins can restore normal myofilament Ca^{2+} sensitivities ¹⁹³. Specifically, Liu et al. ¹⁹³ using permeabilized tissue showed that TnC engineered to either increase or decrease Ca^{2+} sensitivity in rodent hearts with restrictive (increased Ca^{2+} sensitivity) or dilated (decreased Ca^{2+} sensitivity) cardiomyopathic mutations can restore normal Ca^{2+} sensitivity. Additionally, Shettigar and coworkers ¹⁹⁴ recently demonstrated that rationally engineered troponins could be delivered in-vivo to correct and enhance cardiac function in a rodent model.

Rodent models have shown that there are many ways to modulate the Ca^{2+} sensitivities of cardiac muscle. However, it is unclear how engineered proteins can alter Ca^{2+} sensitivities in human myocardium. In this study, engineered TnCs and TnIs (Fig 5.1) were exchanged into the permeabilized human myocardium to either increase or decrease myofilament Ca^{2+} sensitivity. The results show that compared to the wild-type troponins, our Ca^{2+} sensitizing L48Q TnC and S150D

TnI troponins exchanges both increased pCa_{50} . Conversely, the Ca^{2+} desensitizing D73N TnC and Y26E TnI troponins exchanges both decreased Ca^{2+} sensitivity. In the presence of saturating Ca^{2+} , the maximal force per cross-sectional area, shortening velocity, the rate of tension redevelopment (k_{tr}) and power were all similar for the exchanged troponins suggesting the modified troponins have a negligible direct impact on myosin function. This study demonstrates that the myofilament responsiveness of the failing human heart to Ca^{2+} can be modulated by engineering either TnC or TnI. These findings open the door to potential new troponin targeted therapies for heart disease.

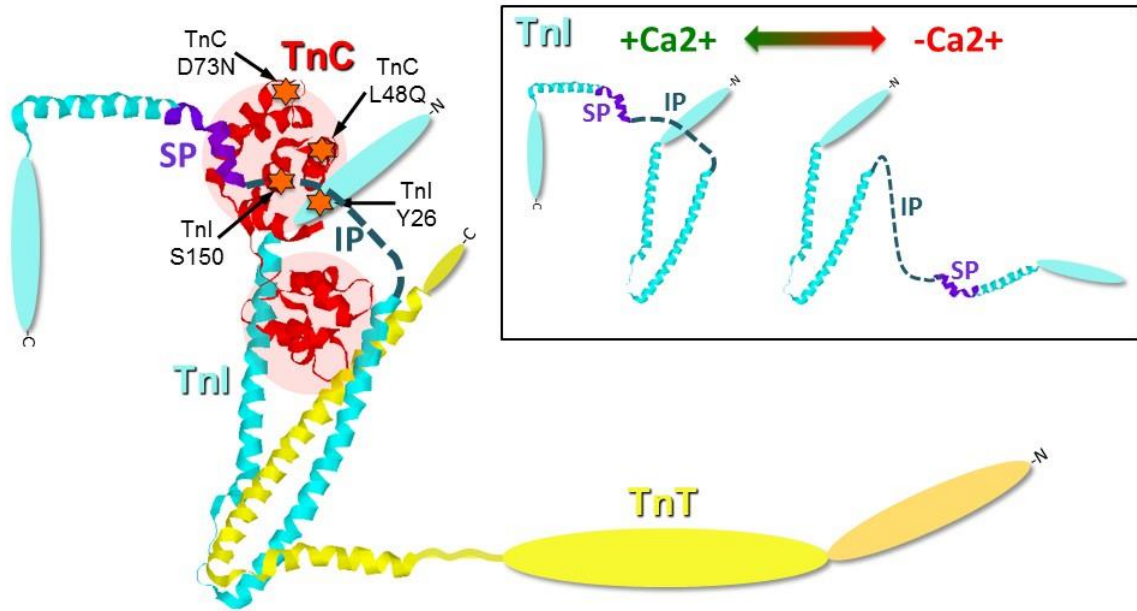


Figure 5.1. Ribbon representation of troponin complex with TnC and TnI mutations used in this study

5.2. Methods

The human cardiac samples used for this study were procured as described in the methods section of chapter 2, section 2.2.1.

5.2.1. Clinical characteristics of patients with heart failure

The samples from patients undergoing heart transplantation came from 3 females (classification of heart failure: 3 non-ischemic). The mean age of the patients was 48 (range 31 to 65). Patients' characteristics are summarized in Table 5.1.

5.2.2. Multicellular preparations

Permeabilization of human tissue was performed as described in section 2.2.3. A total of 18 multicellular preparations from 3 patients with heart failure were analyzed. The average length of the preparations used in this study was $973 \pm 138 \mu\text{m}$, and the average cross-sectional area was $4.97 \pm 2.35 \times 10^{-8} \text{ m}^2$. Cross-sectional area was estimated assuming a circular profile.

5.2.3. Solutions

The ionic composition of the pCa solutions used in this study was adjusted for use at 22° C. The ionic strength was held constant at 180 mM in all solutions. The relaxing solution used to isolate and permeabilize multicellular preparations did not change from what was described in chapter 2, section 2.2.5. To make the pCa solutions, we initially made pCa 4.5 & 9.0. Those two pCa solutions were then mixed to make intermediate concentrations ranging from pCa 5.0 to pCa

6.4. The composition of pCa 4.5 (mmol L⁻¹): 7 EGTA, 20 Imidazole, 51 KCL, 14.5 Creatine, 4.8 ATP, 7 CaCl₂, 5.22 mgCl₂, sufficient amounts of KOH to get pH to 7.0. The composition of pCa 9.0 (in mmol L⁻¹): 7 EGTA, 20 Imidazole, 68 KCL, 14.5 Creatine phosphate, 4.74 ATP, 5.48 MgCl₂, and 15.3 μM CaCl₂ plus ample amount of KOH to reach pH 7.0.

5.2.4. Incubation of samples with engineered troponins

The engineered troponins used in this study were sent to us from our collaborators at the Ohio State University. Including wild-type, there were five troponin complexes used in this study. The constructs of each troponin complex have in the abbreviation “Hc” for human cardiac, “Tn” for troponin, and either “WT” for wild-type,” or a letter with a number and another letter representing the amino acid substitution for either TnC or TnI. The constructs are as followed:

- HcTn WT (Human cardiac troponin containing TnT WT with a Myc Tag / TnC WT / TnI WT) at 10uM
- HcTn L48Q (Human cardiac troponin containing TnT WT with a Myc Tag / TnC with L48Q mutation +IAANS / TnI WT) at 11uM
- HcTn D73N (Human cardiac troponin containing TnT WT with a Myc Tag / TnC with L48Q mutation / TnI WT) at 11uM
- HcTn S150D (Human cardiac troponin containing TnT WT with a Myc Tag / TnC WT / TnI Ser-150 with Asp pseudophos) at 11uM
- HcTn Y26E (Human cardiac troponin containing TnT WT with a Myc Tag / TnC WT / TnI Tyr-26 with Glu pseudophos) at 11uM

All troponin complexes contain TnC with the fluorescent labeled T53C mutation that does not alter function and was in Exchange buffer (0.2M KCl, 5mM MgCl₂, 5mM EGTA, 1mM DTT, 20mM MOPS, pH 6.5).

To exchange the engineered troponin complexes with the native troponin complexes, two to three permeabilized preparations were placed in one of the wells in a 96 well microtiter plate. The preps were then incubated for 24-hours with 100 μ L of one of the five troponin constructs at 4°C. The next day; the preparations were then removed from the plate to carry out the mechanical experiments described in sections 2.2.6 and 2.2.7.

5.2.5. Statistical analysis

The same linear mixed model used in section 3.2.5 was used to analyze the 18 multicellular preparations from the 3 patients used in this study.

Table 5.1. Patients' characteristics

Age	Gender	Diagnosis	Cardiomyopathy
65	Female	Failing	Non-ischemic
31	Female	Failing	Non-ischemic
50	Female	Failing	Non-ischemic

5.3. Results

The results of the troponin exchange (Fig 5.2) shows that on average the engineered troponins replaced ~80% of the native troponins in the multicellular preparations, none of which significantly differed from the other (% exchange: hTnC WT = 73.2 ± 15.8 , hTnC L48Q = 84.7 ± 8.9 , hTnC D73N = 80.0 ± 15.4 , cTnI S150D = 79.3 ± 11.9 , cTnI Y26E = 84.8 ± 9.3 ; $p > 0.05$). Additionally, the Ca^{2+} sensitivity data (Fig 5.3) demonstrates that in comparison to wild-type, the Ca^{2+} sensitizing L48Q TnC and S150D TnI both increased ($p < 0.001$) pCa_{50} from ~5.48 to 5.84 and ~5.68, respectively. Conversely, the Ca^{2+} desensitizing D73N TnC and Y26E TnI both decreased ($p < 0.001$) pCa_{50} to ~5.05 and ~5.29, respectively. The Hill coefficient data also demonstrated a significant ($p = 0.015$) difference, which further post-hoc analysis shows is driven by the reduction in the Hill coefficient from ~2 to ~1 in preparations containing the TnC L48Q troponin complex (Fig 5.4). Either of the troponins had any significant impact on maximum force (Fig 5.5), maximum power (Fig 5.6), maximum shortening velocity (Fig 5.7), or the rate of tension recovery (Fig 5.8).

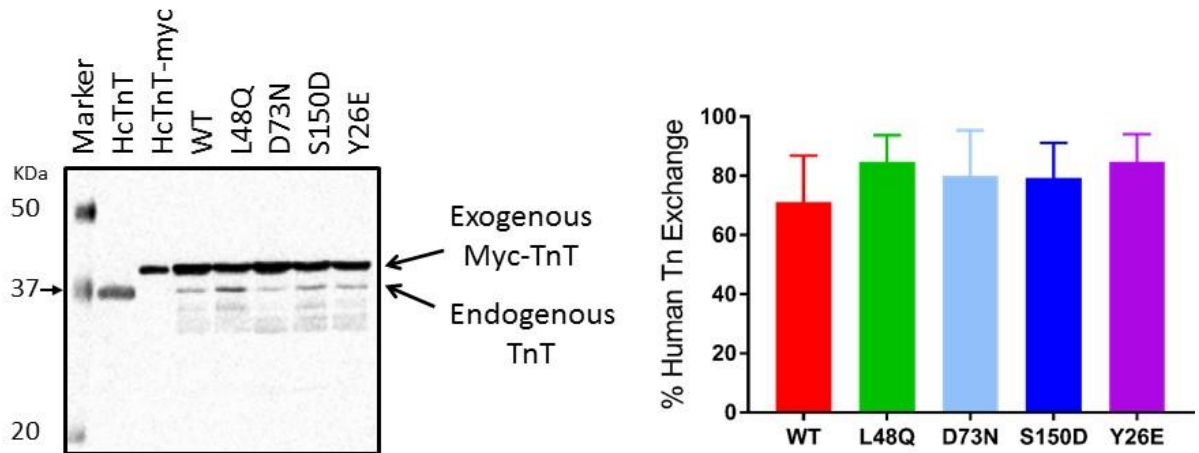


Figure 5.2. Percentage of engineered troponins exchanged into multicellular preparations from human myocardium.

Left) Western blot of TnT with Myc-tag and endogenous TnT to distinguish exchanged troponin complexes from endogenous complexes. Right)

Quantification of troponin exchange. WT = Wild-type; L48Q = TnC Ca²⁺ sensitizing mutation; D73N = TnC Ca²⁺ desensitizing mutation; S150D = TnI Ca²⁺ sensitizing mutation; Y26E = TnI Ca²⁺ desensitizing mutation. HcTnT = human cardiac TnT; HcTnT-myc = human cardiac TnT with Myc-tag

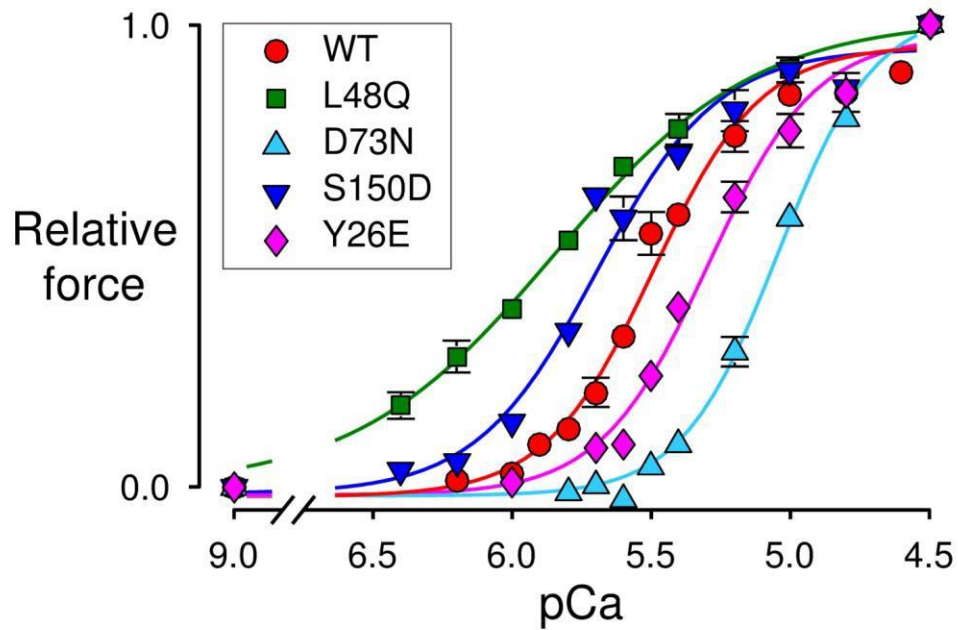


Figure 5.3. Tension-pCa curve of engineered troponins. Error bars are displayed on shapes that had multiple data points for that given pCa value.

WT = Wild-type; L48Q = TnC Ca^{2+} sensitizing mutation; D73N = TnC Ca^{2+} desensitizing mutation; S150D = TnI Ca^{2+} sensitizing mutation; Y26E = TnI Ca^{2+} desensitizing mutation

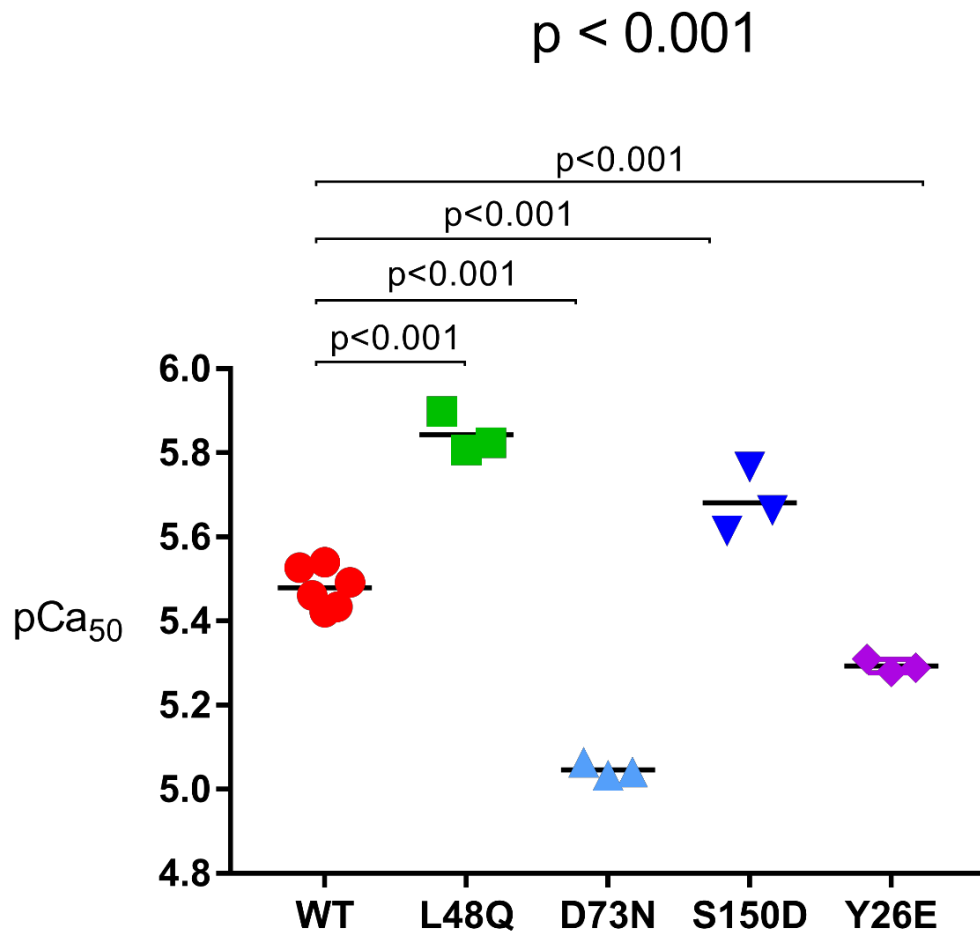


Figure 5.4. Engineered troponins can alter Ca²⁺ sensitivity in human myocardium.

Data points represent individual multicellular preparation from n=3 patients, measurements were repeated for WT. WT = Wild-type; L48Q = TnC Ca²⁺ sensitizing mutation; D73N = TnC Ca²⁺ desensitizing mutation; S150D = TnI Ca²⁺ sensitizing mutation; Y26E = TnI Ca²⁺ desensitizing mutation

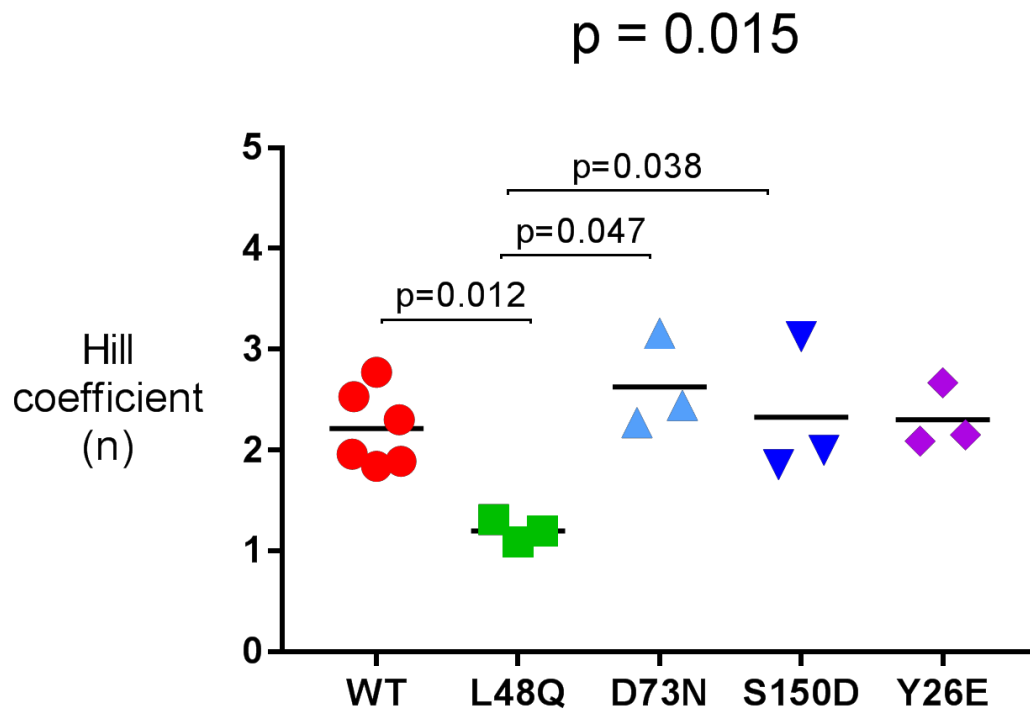


Figure 5.5. Ca²⁺ sensitizing TnC L48Q lowers Hill coefficient.

Data points represent individual multicellular preparation from n=3 patients, measurements were repeated for WT. WT = Wild-type; L48Q = TnC Ca²⁺ sensitizing mutation; D73N = TnC Ca²⁺ desensitizing mutation; S150D = TnI Ca²⁺ sensitizing mutation; Y26E = TnI Ca²⁺ desensitizing mutation

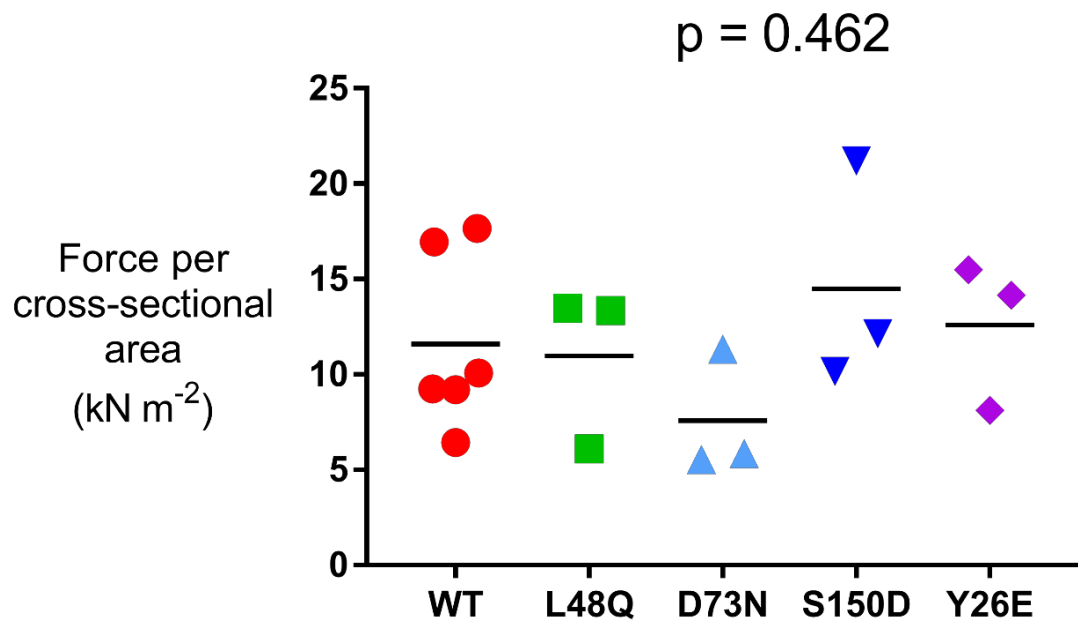


Figure 5.6. Engineered troponins does not alter maximum isometric force.

Data points represent individual multicellular preparation from n=3 patients,

measurements were repeated for WT. WT = Wild-type; L48Q = TnC Ca²⁺

sensitizing mutation; D73N = TnC Ca²⁺ desensitizing mutation; S150D = TnI Ca²⁺

sensitizing mutation; Y26E = TnI Ca²⁺ desensitizing mutation

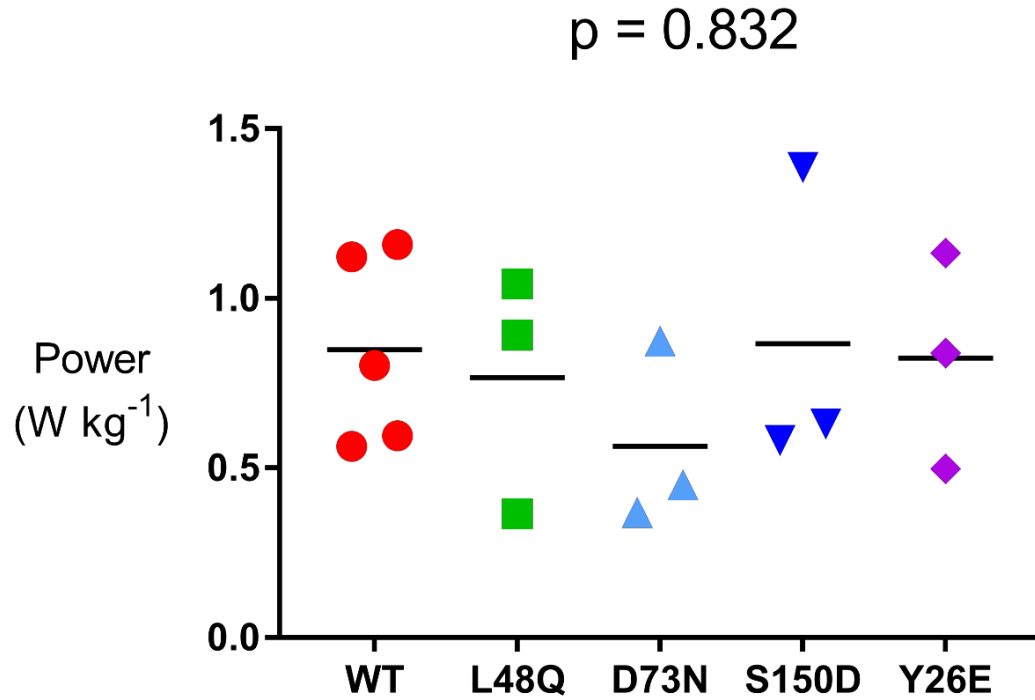


Figure 5.7. Engineered troponins do not alter maximum power.

Data points represent individual multicellular preparation from n=3 patients, measurements were repeated for WT. WT = Wild-type; L48Q = TnC Ca²⁺ sensitizing mutation; D73N = TnC Ca²⁺ desensitizing mutation; S150D = TnI Ca²⁺ sensitizing mutation; Y26E = TnI Ca²⁺ desensitizing mutation

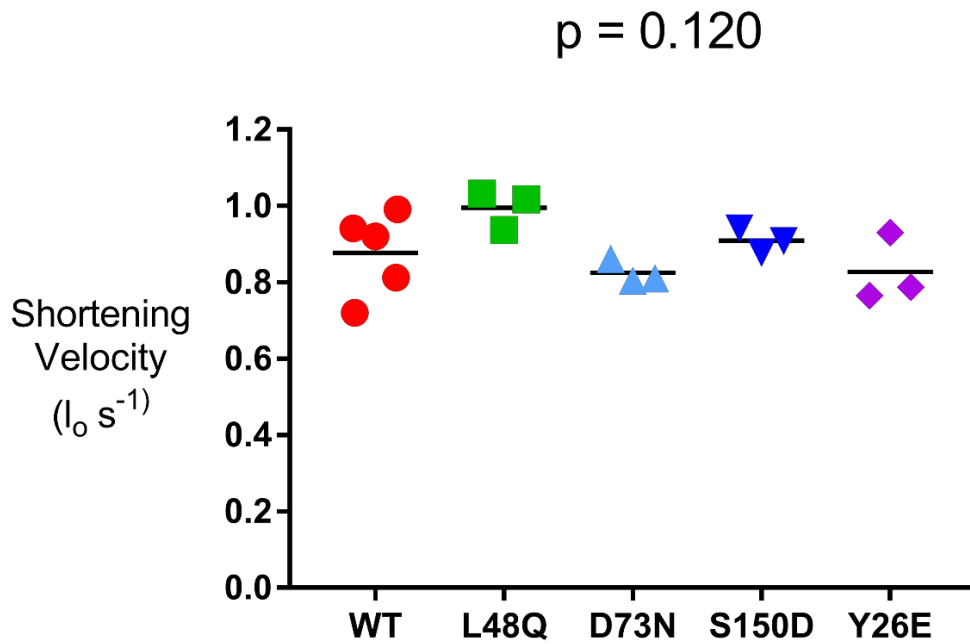


Figure 5.8. Engineered troponins do not alter maximum shortening velocity.

Data points represent individual multicellular preparation from $n=3$ patients, measurements were repeated for WT. WT = Wild-type; L48Q = TnC Ca^{2+} sensitizing mutation; D73N = TnC Ca^{2+} desensitizing mutation; S150D = TnI Ca^{2+} sensitizing mutation; Y26E = TnI Ca^{2+} desensitizing mutation

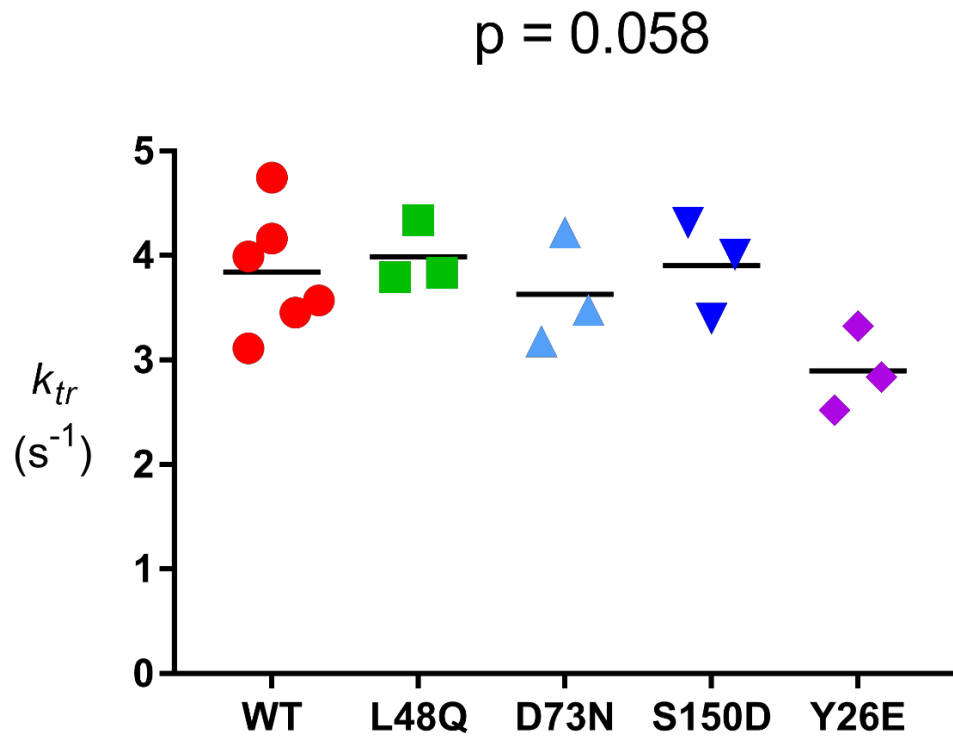


Figure 5.9. Engineered troponins do not alter the rate of force development, k_{tr} .

Data points represent individual multicellular preparation from $n=3$ patients, measurements were repeated for WT. WT = Wild-type; L48Q = TnC Ca^{2+} sensitizing mutation; D73N = TnC Ca^{2+} desensitizing mutation; S150D = TnI Ca^{2+} sensitizing mutation; Y26E = TnI Ca^{2+} desensitizing mutation

5.4 Discussion

Unusual myofilament Ca^{2+} sensitivity is a common observation with several cardiac diseases, including inherited (Hypertrophic, dilated, and restrictive) cardiomyopathies, as well as systolic and diastolic heart failure ^{192,195-197}. Rodent studies have shown that correcting the sensitivity of the myofilament to Ca^{2+} with engineered troponins can correct disease-related changes in myofilament Ca^{2+} sensitivity and improve cardiac function ^{193,194}. Thus, engineered troponins may serve as a new therapeutic strategy for treating cardiac diseases. This study aimed to test the hypothesis that engineered troponins can be used to either increase or decrease myofilament Ca^{2+} sensitivity in human myocardium.

Consistent with rodent studies ^{100,194}, engineered troponin complexes shifted the force-pCa relationship as predicted in human cardiac samples without altering maximal force production (Fig 5.3). The molecular basis for the increase or decrease in Ca^{2+} sensitivity as a result of the engineered TnCs and TnIs are not fully elucidated. However, several studies have shown that the TnC L48Q mutation increases the sensitivity of the myofilament to Ca^{2+} by causing an increase in the binding of both Ca^{2+} and TnI to TnC ¹⁹⁸⁻²⁰⁰. Interestingly, the TnC L48Q mutation reduced the Hill coefficient in comparison to wild-type in the multicellular preparations. It is unclear why the TnC L48Q mutation decreased cooperativity in our human cardiac samples. However, consistent with our findings, Kreuziger et al. ²⁰¹ reported that myocardium from rats with the TnC L48Q mutation demonstrated a similar reduction in the Hill coefficient when

compared to wild-type. In their manuscript, Kreuziger and coworkers²⁰¹ suggested that the reduction in the Hill coefficient in cardiac samples with the L48Q mutation is likely the result of a decrease in the cooperative coupling between increased Ca^{2+} binding to TnC and strong cross bridge binding to actin. It is possible that this general hypothesis can also explain the reduction in cooperativity we see in our human cardiac samples with the TnC L48Q mutation. Further studies are needed to test the molecular basis for the decrease in cooperativity in rodent and human hearts with the TnC L48Q mutation.

Recently, Shettigar et al. 2017¹⁹⁴ showed that engineered TnC could modulate cardiac function in healthy and diseased rodent hearts *in-vivo*. The results of their study demonstrated that mice transfected with TnC L48Q before and after a myocardial infarction had increased cardiac function (contractility, ejection fraction, fractional shortening). More importantly, they did not see any changes in calcium transients or saw any signs of arrhythmias, all of which have been associated with inotropic agents. Thus, the findings of their study provided evidence that precisely formulated troponins can tune the response of the myofilament to Ca^{2+} , and may serve as a novel therapy to combat heart disease. The findings of this study would suggest that similar results may be possible in human hearts, as engineered TnCs and TnIs were able to modulate myofilament responsiveness to Ca^{2+} in human cardiac samples.

Chapter 6

Summary

This chapter will provide an overview of the main findings reported in chapters 2-5. Each chapter will be divided into sections that first report the main results of each study, followed by the clinical implications. The chapter ends with a discussion tying together the main results of each chapter.

6.1. The Ca²⁺ sensitivity of right ventricular myocardium increases more than the Ca²⁺ sensitivity of left ventricular myocardium in human heart failure (chapter 2)

Summary

The results of this study presented three important results. First, human heart failure increases the Ca²⁺ sensitivity of right ventricular myocardium more than the calcium sensitivity of left ventricular myocardium. Second, this contractile effect is likely to involve inter-ventricular differences in posttranslational modifications to sarcomeric proteins including Tnl. Third, heart failure depresses maximum force and maximum power by similar amounts in tissue from both ventricles.

Clinical implication

Clinically, RV dysfunction has been under-appreciated, as it was believed that RV failure was primarily the result of impaired LV function³⁷. However, several studies have shown that left-sided and right-sided heart failure can develop

independently of each other ^{39,40,42}. The results of this study would suggest that therapeutics designed to alter myosin kinetics to enhance cardiac function does not have to be chamber specific, as mechanical parameters implicit of myosin kinetics (maximum force, maximum power, the rate of tension recovery) did not differ between the ventricles. However, these data imply that caution should be taken when considering treating patients with agents that enhance myofilament Ca^{2+} sensitivity, as the impact on the RV and LV may differ.

6.2. Omecamtiv mecarbil increases Ca^{2+} sensitivity and decreases the rate of force development in failing human hearts (chapter 3)

Summary

The results of the omecamtiv mecarbil study demonstrated that the drug increased calcium sensitivity and decreased the rate of tension development in a dose-dependent manner in human cardiac samples. Additionally, the drug did not have any significant impact on the maximally activated force. From motility assays, (not performed as a part of this dissertation, but was a part of the paper findings were reported in) it was proposed that OM increased calcium sensitivity and slowed force development by increasing the amounts of myosin heads in the strong force generating state (increased duty ratio). Additionally, the results of the motility assay suggest that the reduction in the force development could be the result of OM trapping a population of myosin heads in a detached or weakly bound state with actin.

Clinical implication

Omecamtiv mecarbil is currently in phase II clinical trial. Phase I reports showed that the drug increased left ventricular ejection time, ejection fraction, fractional shortening, and stroke volume in a dose-dependent manner in healthy subjects^{126,202}. In patients with stable heart failure (phase II) omecamtiv mecarbil increased ejection time and stroke volume¹⁴³ but did not demonstrate any of these effects in patients with acute heart failure (phase IIb)²⁰³. A clinical parameter not reported in clinical trials papers that our study suggest OM may adversely impact is relaxation. The implications from this study are that the higher the OM concentration, the longer the muscle will take to relax.

6.3. Para-Nitroblebbistatin reduces maximum force and calcium sensitivity in human myocardium (chapter 4)

Summary

In permeabilized multicellular preparations from human myocardium, para-Nitroblebbistatin reduced maximum force, maximum power, and calcium sensitivity without altering the rate of force development or shortening velocity. Results from *in vitro* motility assays (performed by collaborators) suggest that the molecule can reduce force, power and calcium sensitivity by reducing the myosin duty ratio.

Clinical implication

Para-Nitroblebbistatin is a molecule used for research purposes, but the mechanism by which the compound acts is of a large clinical significance, as a drug with similar properties may be able to treat patients suffering from hypertrophic cardiomyopathy (HCM). The molecule binds to myosin, slowing the rate of phosphate release during the actin-myosin cycle, thus reducing the myosin duty ratio. The findings of this study suggest that patients who suffer from HCM may benefit from a drug with a similar mechanism of action, as these patients often suffer from a hypercontractile heart. Recently, a small molecule named MYK-461 was identified as a novel cardiac myosin inhibitor¹⁸³. Like blebbistatin, the drug was shown to reduce myosin duty ratio. Thus it may serve as a treatment for patients suffering from HCM. The drug is currently in clinical trial²⁰⁴.

6.4. Engineered troponins modulate the Ca²⁺ sensitivity of the failing human myocardium (chapter 5)

Summary

This study aimed to determine if engineered TnCs and TnIs could alter calcium sensitivity in human myocardium. The results of the study showed that engineered TnCs and TnIs could be used to either increase or decrease myofilament calcium sensitivity in cardiac samples from humans without altering myosin kinetics.

Clinical implication

The recent advances in gene vector technology, design, and delivery modalities have made cardiovascular gene therapy a promising option for patients suffering from cardiac disease. Recently, a clinical trial was conducted using gene-based therapy to correct dysfunctional sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pump activity in patients with heart failure ²⁰⁵. The findings demonstrate that gene therapy can be used to safely deliver genetic material to the heart ²⁰⁶, as well as provide benefits to the patients ²⁰⁷. In the present study, engineered TnCs and TnIs were able to modulate calcium sensitivity in human cardiac samples. The clinical implication of these data is that engineered proteins along with gene therapy have the potential to treat patients suffering from various forms of heart disease.

6.5. Discussion

A consistent finding in each of the studies presented in this dissertation is an alternation in myofilament calcium sensitivity. In chapter 2, we saw that calcium sensitivity was higher in the LV of non-failing hearts, and that heart failure made the RV was more sensitive to calcium. In chapters 3 and 4, the data showed that the use of omecamtiv mecarbil increased myofilament calcium sensitivity whereas para-Nitroblebbistatin decreased the sensitivity of the myofilaments to calcium. Finally, in chapter 5, the findings demonstrated that engineered troponins could be used to either increase or decrease myofilament calcium sensitivity.

As a reminder, myofilament calcium sensitivity is a concept researchers use to simplify the complex dynamic contraction/relaxation processes of cardiac muscle into a two variable system that describes the relationship between the concentration of free Ca^{2+} ions available to bind TnC and the amount of force the muscle generates¹⁸⁷. In a beating heart, changes to myofilament calcium sensitivity are essential to its function, as an increase may lead to increased contractility, as well as impaired relaxation. Conversely, a decrease may result in faster relaxation rates and reduced contractility. Thus, the ability of the heart to maintain and/or alter the sensitivity of the myofilaments to Ca^{2+} is essential for normal function. The discussion sections of chapters 2-5 have outlined the observed changes in myofilament calcium sensitivity and described how each could potentially alter cardiac function. Therefore, the global cardiac implications of those findings will not be discussed any further.

A concept that has not been discussed in this dissertation is how changes in calcium sensitivity can modulate cardiac structure. This concept has grown over the years as studies have shown a correlation between changes in myofilament calcium sensitivity and cardiac diseases that alter cardiac structure^{62,208}. Hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) are cardiac diseases commonly associated with changes in myofilament calcium sensitivity²⁰⁸. Phenotypically, HCM results in a hypertrophic heart that is hypercontractile and has impaired relaxation; contrarily, DCM first causes an enlargement of the left ventricular wall followed by dilation (weakening), resulting

in systolic dysfunction (i.e., reduced ejection fraction) ^{209,210}. The cause of HCM and DCM are commonly mutations within sarcomeric proteins that either increase (HCM) or decrease (DCM) myofilament calcium sensitivity ²¹¹⁻²¹⁴. As a result, a paradigm has emerged that associates an increase in myofilament calcium sensitivity with HCM and a decrease to DCM. The results of the troponin study (chapter 5) demonstrate how mutations in sarcomeric proteins can either increase or decrease myofilament Ca²⁺ sensitivity. However, the mechanism by which changes in myofilament calcium sensitivity lead to HCM or DCM remains unclear. The question has to how do changes in myofilament Ca²⁺ sensitivity translate into cardiac disease have been asked by the field for decades, and to date, there are no definitive answers.

Recently, a study published from the laboratory of Jeffery Molkenin asked this very question. In their study, Davis et al. ²¹⁵ mutated TnC *in vivo* in mice to either increase (L48Q) or decrease (I61Q) myofilament calcium sensitivity. The results showed that L48Q enhanced myofilament calcium sensitivity, increased fractional shortening, slowed relaxation, and lead to a hypercontractile heart. However, the hearts of the mice with the L48Q mutations showed no change in cardiac growth or chamber dimensions up to 1 year of age ²¹⁵. Thus, these animals did not display the hallmark HCM phenotype. Similar results were shown by Shettigar et al. ¹⁹⁴. Unlike previous studies, Davis and coworkers ²¹⁵ believed that the effects of the L48Q mutation on cardiac growth were being masked by the high contractile state and heart rate of mouse hearts, as well as enhanced diastolic

functions. Thus, they used the β -blocker metoprolol to try and unmask these effects. When metoprolol was administered to the animals, the data showed that the L48Q mutation causes an HCM like phenotype, as left ventricular wall and septal wall thickness increased ²¹⁵. The use of isoproterenol reversed these effects the effects of metoprolol. Thus, the group concluded that the L48Q mutation could lead to HCM like phenotype; however, in a mouse, these effects are masked by the β -adrenergic drive which enhances relaxation and Ca^{2+} decay times. They also showed that the I61Q (reduced myofilament Ca^{2+} sensitivity) mutation causes DCM in mice, which can be corrected by adding a known HCM causing myosin mutation to increase Ca^{2+} sensitivity ²¹⁵.

One of the key findings in the study by Davis et al. ²¹⁵ was the correction of the I61Q-dependent dilated heart growth with the R403Q myosin mutant allele. The results of that experiment suggested that myocytes can change their growth profile by sensing aberrations in the magnitude and time of tension generated at a given cytosolic Ca^{2+} concentration ²¹⁵. The group goes on to show through a series of experiments that the decrease in myofilament tension as a result of altered myofilament Ca^{2+} binding in I61Q mice is linked to fluxes in the sarcoplasmic Ca^{2+} , which alters cytoplasmic Ca^{2+} concentration. They then proposed that the alteration in cytoplasmic Ca^{2+} initiates a Ca^{2+} -dependent signaling pathway that leads to the remodeling of the heart.

The proposed signaling pathway that leads to cardiac growth as a result of changes in myofilament Ca^{2+} sensitivities by Davis et al. ²¹⁵ is not quite clear. Additionally, it is not universal for all mutations that alter Ca^{2+} sensitivity, as they did not see morphological changes in the L48Q mice that were not given a β -blocker. Furthermore, there are mechanotransduction pathways in cardiac cells that may also lead to cardiac remodeling ²¹⁶. Further studies are needed to fully understand how changes in myofilament Ca^{2+} sensitivity leads to cardiac disease. However, the manuscript by Davis et al. ²¹⁵ has provided substantial evidence that altered myofilament Ca^{2+} sensitivity can induce cardiac growth by altering the tension generated by the myofilaments.

With the understanding that an increase or decrease in myofilament Ca^{2+} sensitivity can lead to cardiac remodeling, there are implications not discussed in the previous chapters about the Ca^{2+} sensitivity data that can be inferred. For example, in the study comparing the mechanical properties of the LV and RV (chapter 2), it may be possible that the LV is thicker than the RV and can generate more pressure as a result of the myofilaments of the LV being more sensitive to Ca^{2+} . A more concerning implication of the Ca^{2+} sensitivity data comes from the drugs (chapters 3 & 4) and engineered troponin (chapter 5) studies. In the drugs and troponin studies, it was shown that small molecules and engineered proteins could be used to either increase or decrease myofilament Ca^{2+} sensitivity. Considering the tension generation idea described by Davis et al. ²¹⁵ it may be possible that continuous administration of omecamtiv mecarbil or

MYK-461 could lead to cardiomyopathy, as these drugs can increase or decrease myofilament tension generation respectively. Considering this theory, it would be interesting to perform a study to see how the continuous administration of these drugs over a long period modulate the morphology of the heart. The use of engineered proteins to either increase or decrease myofilament Ca^{2+} sensitivity also pose the same possibility of causing cardiomyopathy, which was demonstrated in the study by Davis et al. ²¹⁵. Thus caution should be taken when considering this form of therapy.

The findings in this dissertation have provided information addressing a general cardiac biology question (chapter 2), as well as information on how potential therapies (chapters 3, 4, and 5) may alter the mechanical properties of human hearts. These data will provide a significant contribution to the field, as the results are either being revised for publication (chapter 2), being prepped for submission (chapter 5) or have already been published (chapters 3 & 4). As it pertains to future studies, an interesting study would be to repeat the potential therapeutics studies (chapters 3, 4, and 5) with different myofilament lengths. This would be a unique study, as it would address two question, 1) how does length-dependent activation (activation of the myofilament at different lengths) influence the mode of action for each of these therapies, and 2) how does each treatment impact length-dependent activation.

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Vita
Cheavar A. Blair

EDUCATION:

- 2012-2017 **University of Kentucky**
Graduate Program: Physiology
Ph.D. Thesis: "The mechanical properties of non-failing and failing human hearts."
Advisor: Dr. Kenneth Campbell
- 2010-2016 **Southern Illinois University Carbondale**
Master of Science: Plant Biology
Master's Thesis: "*Biochemical response of Brachypodium distachyon to ultraviolet B-radiation.*"
Advisor: Dr. Andrew Wood
- 2004-2010 **Southern Illinois University Carbondale**
Bachelor of Science; Major: Biological Sciences, Minor: Chemistry

RESEARCH EXPERIENCE:

- 3/2013-Present Department of Physiology, **University of Kentucky**
PhD research
- ❖ Assessed fibrosis in samples from patients with heart failure using histology (sectioned, stained, and analyze)
 - ❖ Isolated RNA from cardiac samples obtained from non-failing and failing human hearts
 - ❖ Analyzed mRNA and miRNA data from human cardiac samples
 - ❖ Member of a human tissue procurement team; experience in handling, transportation, and inventory of human tissue
- 10/2012-12/2012 Department of Physiology, **University of Kentucky**
8-week lab rotation in the lab of Dr. Karyn Esser.
- ❖ Performed western blot analysis on skeletal troponin I in mice who have had *Bmal1*, a key circadian rhythm gene knocked-out.
- 1/2013-3/2013 Department of Physiology, **University of Kentucky**
8-week lab rotation in the lab of Dr. Michael Reid.
- ❖ Isolated, prepped and analyzed force output from the extensor digitorum longus muscle from wild-type mice.

- 8/2010-8/2012 Department of Plant Biology, **Southern Illinois University Carbondale**
Masters Research
- ❖ Examining the molecular, biochemical, and physiological responses of *Brachypodium distachyon* (Purple false brome) to ultraviolet radiation
 - ❖ Performed DNA and RNA extractions
 - ❖ Executed several molecular and biochemical assays
 - ❖ Completed numerous transformations and ligations
 - ❖ Researched *Panicum Virgatum* (Switch grass) biofuel capacity

TEACHING AND WORK EXPERIENCE:

1/2016 – Present Adjunct Faculty, Bluegrass Community & Technical College, Lexington, KY. Teach Anatomy and Physiology & General Biology

8/2012 – Present Research Assistant, University of Kentucky, Lexington, KY. Studies heart failure

8/2011-5/2012 - Teaching Assistant, Southern Illinois University, Carbondale, IL. Taught plant biology

8/2010-8/2011 - Teaching Assistant, Southern Illinois University, Carbondale, IL. Taught general biology

MENTORING:

10/2013-present **University of Kentucky**
Mentored 15 undergraduates, 4 high school students, and 4 master's students

5/2013-Present **Big Brother, Big Sister of the Bluegrass**
Mentor to a young man name De'Montavious Smith, a high school student.

5/2014 – Present **National Institute of Health (NIH) Bridge to the Doctorate Mentor**
Mentor 3 master's student from Kentucky State University

COMMUNITY OUTREACH:

Volunteer: (2014) YMCA Black Achievers Program in Lexington, KY

- ❖ Help in the design of the experiments students performs during our workshops on Saturdays. Accompany students on tours, tutor, and offer college advice to junior and senior high school students.

Master of Ceremony: (2012) International Festival at Southern Illinois University Carbondale

- ❖ Invited by the SIUC International Student Council to preside over a community-wide showcase to highlight the cultures and traditions of the diverse SIUC student body.

PROFESSIONAL DEVELOPMENT:

2014-Present **Graduate Student Advisory Committee for LSAMP**
University of Kentucky

Serve as the graduate student advisor for the Kentucky-West Virginia Louis Stokes Alliance for Minority Participation (LSAMP) program. Attend meetings and conferences to enhance undergraduate minority participation in Science, Technology, Engineering, and Mathematics (STEM) programs. As a minority in a STEM program and the only African American male student in the department of physiology, I offer a unique perspective on the development and implementation of the program. I work side by side with Dr. Judy Jackson, Vice President for Institutional Diversity to develop programs to enroll, recruit, and retain minority students in STEM programs.

January 2016 **American Physiology Society (APS) Writing and Reviewing for Scientific Journals Course**

Selected to attend the writing and reviewing for scientific journals course held in Orlando, FL presented by APS. A four-day course where students were required to bring a manuscript that was critiqued by former presidents of APS as well as current editors of scientific journals. Students were taught how to properly put together a manuscript, as well as the process for submitting, revising, and resubmitting papers to journals. Participants were also given the opportunity to review other members documents to understand the review process.

October 2015 **Guest Speaker at Centre College**

Invited to Centre College in Danville, KY to speak to a small group of minority student in STEM majors. Shared my story on the route I took towards getting into graduate school and answered questions.

September 2015 **Student Panel at GEM GRAD Lab Symposium**

Serve on a graduate student panel to answer questions about graduate school to underrepresented undergraduate students in STEM fields.

July 2015 **Selection Committee Member for LSAMP Program Director**

Selected to serve on a six persons committee to make suggestions on who should be the new Louis Stokes Alliance for Minority Participation (LSAMP) program director at the University of Kentucky.

April 2015 **Graduate Student Discussion Panel Member for LSAMP Conference**

One of three graduate student selected to serve on the Kentucky-West Virginia Louis Stokes Alliance for Minority Participation (KY-WV LSAMP) mini-conference. Spoke to ~100 student and attendees about my experience in graduate school and my journey to get into graduate school. Answered questions related to my experience and offered constructive feedback.

AWARDS & FELLOWSHIPS:

Stanford ChEM-H Post-Doc Fellowship **Awarded May 2017**

Awarded a two-year \$100,000 (\$50,000 a year) post-doctoral fellowship to perform research at Stanford University as a member of the Chemistry, Engineering & Medicine for Human Health (ChEM-H) program.

Lyman T. Johnson Diversity Fellowship **Awarded April 2015**

Two-year fellowship awarded to graduate student who maintains a 3.3 GPA and contributes to the University of Kentucky's compelling interest in diversity while demonstrating leadership and community service. Award provides tuition as well as a \$7,500 per year stipend (15,000 total).

FASEB/MARC Travel Award **Awarded February 2016**

Awarded \$1850 to attend the 2016 Experimental Biology conference in San Diego, CA.

FASEB/MARC Minority Travel Award **Awarded December 2015**

Awarded \$1300 to attend the American Physiological Society Professional Skills Training Course in Orlando, FL.

Brian Harding Award **Awarded November 2015**

The Brian Harding award is awarded to a member of the physiology department that makes coming to work fun, is hard working, and pushes everyone else to do better science. I was awarded this award because my peers felt that I displayed these characteristics.

Biophysical Society Diversity Travel Award **Awarded November 2015**

Awarded a \$1000 travel award from the Biophysical Society to attend the 2016 Biophysical conference in Los Angeles, Ca.

PUBLICATIONS:

Swenson, A. M., Tang, W., **Blair, C. A.**, Fetrow, C. M., Unrath, W. C., Previs, M. J., Campbell, K. S. & Yengo, C. M. (2017). Omecamtiv Mecarbil Enhances the Duty Ratio of Human Beta Cardiac Myosin Resulting in Increased Calcium Sensitivity and Slowed Force Development in Cardiac Muscle. *J Biol Chem*, In press. PMID not available. [PMID 28082673](#).

Tang W, **Blair C. A.**, Walton S, Málnási-Csizmadia A, Campbell K, and Yengo C. Modulating beta-cardiac myosin function at the molecular and tissue levels. *Frontiers in Physiology* 7: 2016. doi: 10.3389/fphys.2016.00659

Blair, C. A., Haynes, P., Campbell, S. G., Chung, C., Mitov, M. I., Dennis, D., Bonnell, M. R., Hoopes, C. W., Guglin, M. & Campbell, K. S. (2016). A Protocol for Collecting Human Cardiac Tissue for Research. *VAD J* 2, Article 12. <http://uknowledge.uky.edu/vad/vol12/iss11/12>. [PMCID PMC5199025](#).

Campbell K. S and **Blair C. A.**: F1000Prime Recommendation of [Zhu X et al., *J Cell Mol Med* 2013, 17(9):1173-87]. In F1000Prime, 10 Mar 2014; DOI: 10.3410/f.718098345.793489967. F1000Prime.com/718098345#eval793489967

Campbell K. S and **Blair C. A.**: F1000Prime Recommendation of [Koshman YE et al., *J Card Fail* 2013, 19(4):283-94]. In F1000Prime, 27 Aug 2013; DOI: 10.3410/f.718018238.793478149. F1000Prime.com/718018238#eval793478149

ORAL ABSTRACT PRESENTATIONS:

Biophysical Society 61ST Annual Meeting, New Orleans, LA

Blair, C. A., Davis, J. P., Biesiadecki, B., Campbell, K, S. (2017) Engineered troponins modulate the Ca²⁺ sensitivity of the failing human myocardium

4th Annual Kentucky American Physiological Society (APS) Meeting, Lexington, KY

Blair, C. A., Guglin, M., Stromberg, A., Campbell, K. S. (2016) Myocardium from the left and right ventricles of human hearts has similar mechanical properties.

Saha Cardiovascular Research Day, Lexington, KY

Blair, C. A., Guglin, M., Stromberg, A., Campbell, K. S. (2016) Myocardium from the left and right ventricles of human hearts has similar mechanical properties.

POSTER ABSTRACT PRESENTATIONS:

Biophysical Society 61ST Annual Meeting, New Orleans, LA

Wanjian T., **Blair, C. A.**, Campbell, K. S., Yengo, C. M. (2017) Impact of Para-Nitroblebbistatin on human beta-cardiac myosin at the molecular and tissue levels

Biophysical Society 61ST Annual Meeting, New Orleans, LA

Awinda, P., **Blair, C. A.**, Guglin, M., Campbell, K. S., Tanner, B, CW. (2017) Maximal force increases at physiological temperature in myocardial strips from non-failing and failing human hearts

Experimental Biology, San Diego, CA

Blair, C. A., Guglin, M., Stromberg, A., Campbell, K. S. (2016) Myocardium from the left and right ventricles of human hearts has similar mechanical properties.

Biophysical Society 60th Annual Meeting, Los Angeles, CA

Blair, C. A., Guglin, M., Stromberg, A., Campbell, K. S. (2016) Myocardium from the left and right ventricles of human hearts has similar mechanical properties.

Biophysical Society 60th Annual Meeting, Los Angeles, CA

Swenson, A. M., **Blair, C. A.**, Fetrow, C., Tang, W., Kenneth, M. G., Campbell, S., et al. (2016). Omecamtiv mecarbil enhances actin affinity and slows force production in human beta-cardiac myosin.

Center for Muscle Biology Annual Fall Retreat, Lexington, KY

Blair, C. A., Haynes, P., Campbell, K. S., Hoopes, C. W., Sadayappan, S., Stromberg, A. J., Nava, K. E., Lawson, B. A., Mitov, M. I., Campbell, S. G., Bonnell, M. R. (2014) Transmural heterogeneity of cellular level power output is reduced in human heart failure.

Kentucky APS Conference, Louisville, KY

Blair, C. A., Haynes, P., Chung, C. S., Nava, K. E., Lawson, B. A., Mitov, M.I., Campbell, S. G., Bonnell, M. R., Hoopes, C. W., Campbell, K. S. (2014) Myocyte power output in humans with end-stage heart failure: Effects of LVAD treatment.

Center for Clinical and Translational Science Conference, University of Kentucky

Blair, C. A., Haynes, P., Campbell, K. S., Hoopes, C. W., Sadayappan, S., Stromberg, A. J., Nava, K. E., Lawson, B. A., Mitov, M. I., Campbell, S. G., Bonnell, M. R. (2014) Transmural heterogeneity of cellular level power output is reduced in human heart failure.

Annual plant biology research day, Southern Illinois University Carbondale

Blair, C. A. and Wood, A. (2011) Characterization of Chalcone Synthase (CHS) and CHS-like genes in *Brachypodium distachyon*.