Changes in Skeletal Muscle Sarcoplasmic Reticulum Calcium Handling and Regulatory Protein Content in Congestive Heart Failure

By
Emily E. Allen

Submitted to the Faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of
Master of Science
in the
Department of Human Nutrition, Foods, and Exercise

Dr. Jay Williams – Committee Chair

Dr. Michael E. Houston – Committee Member

Dr. William E. Newton – Committee Member

April 17th, 2002
Blacksburg, Virginia

Abstract

Fatigue and skeletal muscle weakness are problems associated with congestive heart failure. Research does not support the theory that the affected cardiac function is responsible for the fatigue. During skeletal muscle fatigue, calcium handling is altered. Thus, the fatigue associated with congestive heart failure could be attributed to altered calcium handling. The main proteins involved in calcium release are the ryanodine receptor (RyR) and the dihydropyridine receptor (DHPR). The main proteins involved in calcium uptake are the fast and slow isoforms of sarco(endo)plasmic reticulum calcium ATPase (SERCA 1 and SERCA 2 respectively). Calsequestrin (Csq) and calmodulin (CaM) play regulatory roles in calcium handling. Changes in the levels of these proteins could explain alterations in calcium handling and subsequent muscle function. The purpose of this study was to use a genetic model of heart failure, the SHHF rat, to examine the levels of regulatory calcium handling proteins to determine if changes in the amounts of RyR, DHPR, SERCA1, SERCA2, Csq and CaM are altered in congestive heart failure.

A significant decrease was found in the amounts of RyR, DHPR, and SERCA 1 of the SHHF gastrocnemius and diaphragm samples in comparison to the control. There was no significant difference found in the amounts of CaM or SERCA 2 between the two groups. Csq was not found to be statistically different between the two groups of the gastrocnemius samples. However, there was an increase in Csq in the SHHF diaphragm samples in comparison to the control. In conclusion, the calcium handling proteins are affected in the genetic model of

heart failure. These changes could explain previous reports of altered calcium handling within the skeletal muscles of congestive heart failure animals.

Acknowledgements

There are many people who have helped get me to where I am today and that I would like to thank, first and foremost, my parents Jeff and Susan Allen. They started me off with good genes and then followed that up with encouragement and enthusiasm. They also always had an ear open to listen when I need it. For all that they've done, I am forever indebted to them.

The second group I would like to thank for helping me in my academic career is my committee, Dr. Williams, Dr. Houston and Dr. Newton. I would like to thank them for making me push beyond face value of things and get the most out of my thesis. To Dr. Williams, my advisor, I'd like to extend an additional thank you not just for all of your patience with the revisions, my questions and lab problems but also for being so approachable and friendly to his students. I would also like to thank Dr. Susan Kandarian who generously supplied the skeletal muscle vesicles used in this study.

Another important group to thank would be all of the lab guys. I don't think I would have enjoyed working in the lab nearly as much if I didn't have a great group of people to work with. Thanks especially to Simon Lees and Jeff Otis who taught me the procedures I needed to know and answered the many questions I threw at them.

I'd also like to thank all of my family and friends who have endured my conversations about my research. I'd especially like to thank Nicole for our countless walks (aka, thesis venting sessions) and to Amanda for giving me someone to visit during the many breaks in lab procedures.

Last, but certainly not least, I'd like to thank my fiancé Dave. Not only did he put up with two years of countless weekend trips that included a very boring drive, but he was also there to encourage me and to listen when I needed it. He also didn't complain too much about the countless journal article piles lying around the apartment. For all he's done I'm thankful.

Table of Contents

List of Tables

List of Figures

Chapter 1:

Introduction

Introduction

Congestive heart failure (CHF) not only puts a strain on the heart and lungs of patients, but also results in debilitating skeletal muscle weakness that makes everyday activities difficult to complete. Many explanations for this fatigue have been explored. One explanation currently being studied is the possibility of changes occurring within the skeletal muscle itself making it a more fatigable muscle.

The sarcoplasmic reticulum is the storage site of calcium within a muscle. Through regulation of release and uptake of calcium, muscle contraction and relaxation can be controlled. There are a series of proteins that work together in order to closely regulate this process.

Excitation-contraction coupling (ECC) is the process of releasing calcium from the sarcoplasmic reticulum (Groh et al., 1999). It begins with a message from the neuromuscular junction to the muscle cell membrane, which initiates an action potential (Stephenson et al., 1998). The two main proteins involved in changing this electrical signal into action are the dihydropyridine receptor (DHPR) and the ryanodine receptor (RyR). Through their interaction, calcium is released from the sarcoplasmic reticulum and is free to bind to troponin. Cross bridge cycling can then occur and force development of the muscle takes place (Hamilton et al., 2000). Relaxation is due mainly to the action of sarco(endo)plasmic reticulum calcium ATP-ase (SERCA). This works by hydrolyzing ATP in order to pump free myoplasmic calcium back into the sarcoplasmic reticulum (Schulte et al., 1993). Both the release and uptake of

calcium are affected by regulatory proteins that influence the rates of calcium transport.

Alterations in the proteins involved in the movement of calcium can affect the function of the muscle as is seen in certain disease states associated with changes to calcium handling proteins. Brody's disease occurs because of a defect in SERCA 1 expression (Danon et al., 1988). Muscle relaxation is compromised because of the inability to quickly clear out myoplasmic calcium without the function of SERCA 1. Malignant hyperthermia is a condition affecting the RyR (Fill et al., 1990). The RyR is mutated so that it becomes sensitive to many substances causing calcium release. When an affected person is put under general anesthesia, the anesthesia causes the constant release of calcium, which leads to rigidity of muscles along with a large increase in body temperature (Gronert, 1994). Thus the proteins involved in calcium handling are critical for the proper functioning of the muscle.

Statement of Problem

With CHF, one debilitating symptom is skeletal muscle fatigue (Lunde et al., 2001b). This fatigue has been attributed to diminished cardiovascular function. However, when the severity of symptoms was tested against left ventricular function, little correlation was found (Harrington and Coats, 1997). Even with pharmacological interventions that increased oxygen delivery to the muscle, the symptoms were not alleviated (Drexler et al, 1989).

Studies of skeletal muscles during CHF have found that a decrease in Type I, slow twitch fibers, and an increase in Type II, fast twitch fibers, occurs (Perreault et al., 1993). This change in fiber type composition is indicative of progression towards a more fatigable muscle. The change in fiber type was speculated to be a result of inactivity of CHF patients. Yet research has not supported this theory (Simonini et al, 1996; Vescovo et al., 1996).

It has been found that a key component to fatigue is altered calcium handling. When an animal is subjected to fatiguing contractions, sarcoplasmic reticulum uptake and release rates are altered. The resultant decrease in myoplasmic calcium leads to a decrease in force production (Williams and Klug, 1995). This observation raises the possibility that the fatigue seen in skeletal muscles after CHF could be due to altered calcium handling.

Within the muscle cell, there are many proteins that affect calcium handling. The RyR and DHPR have been shown to be the main proteins involved in ECC and calcium release from the sarcoplasmic reticulum (Hamilton et al., 2000; Pereon et al.,1997c). The skeletal isoforms are the RyR1 and the $\alpha 1_s$ subunit of the DHPR. Cardiac isoforms of these proteins also exist (RyR2 and the $\alpha 1_c$ subunit of the DHPR) (Froemming et al., 2000). SERCA 1 and SERCA 2, the fast and slow isoforms, are the main proteins shown to be involved in calcium uptake into the sarcoplasmic reticulum (Spangenburg et al., 2002). Other proteins play regulatory roles in the movement of calcium into and out of the sarcoplasmic reticulum. Specifically, calsequestrin (Csq) helps bind free calcium once it is pumped into the sarcoplasmic reticulum (Kobayashi et al.,

2000). Calmodulin (CaM) appears to interact with the RyR to affect calcium release (Hamilton et al., 2000).

In the present study, SHHF/Mcc-cp (SHHF) rats will be used as the model of CHF. This strain has been shown to exhibit both hypertension and CHF. The rats exhibit many clinical signs of CHF including enlarged hearts, subcutaneous edemas and hyperemia of the lungs (McCune et al., 1990). Previous measurements of calcium uptake and release of isolated sarcoplasmic reticulum vesicles from these animals found release rates for the SHHF animals decreased in both the diaphragm and gastrocnemius. However, uptake rates increased in the SHHF gastrocnemius but decreased in the SHHF diaphragm (Appendix B). These results raise the question as to what specific mechanisms mediate these changes. There are two possibilities. First, changes in the RyR and SERCA expression could directly alter calcium uptake and release. Second, changes in the levels of proteins that regulate the RyR and SERCA function could indirectly alter calcium handling.

The purpose of this study is to examine the levels of regulatory calcium handling proteins to determine if changes in the protein content of the RyR1, DHPR, SERCA 1, SERCA 2, Csq and CaM are consistent with the changes observed in calcium uptake and release. If so, these results could provide more validation for the ECC explanation of muscle weakness and fatigue experienced by individuals with CHF.

Significance of Study

Altered calcium handling is known to be associated with fatigue. Under conditions such as denervation, hindlimb suspension and chronic low frequency stimulation, the proteins that affect calcium handling have been altered at the protein level and the gene level (Hicks et al., 1997; Loirat et al., 1988; Pereon et al., 1997c; Schulte et al., 1993; Simonini et al., 1999). The research on changes in protein levels and the subsequent effect on calcium handling is conflicting. Under pathological conditions other than CHF, a correlation has been found between changes in protein expression and subsequent changes in calcium uptake and release rates (Kandarian et al., 1994; Schulte et al., 1993). However, under a different set of conditions, though a change in protein expression and content occurred, it did not correlate with calcium uptake and release rates (Kandarian et al., 1996; Spangenburg et al., 2002). Using SHHF animals as a model of heart failure, it has already been found that an alteration in the calcium handling occurs in the gastrocnemius and diaphragm. Investigating possible changes in the specific proteins responsible for calcium handling may put some of the puzzle pieces into place leading to a better understanding of what is occurring within these muscles during CHF. In turn, this understanding could lead to either preventative measures or pharmacological treatments that could greatly improve the quality of life for those CHF patients who currently have their everyday activities hindered due to skeletal muscle fatigue.

Specific Aims

1) To determine if the content of the RyR1 is different in the diaphragm and gastrocnemius of an SHHF rat in comparison to a control rat.
2) To determine if the content of the $\alpha 1_s$ subunit of DHPR is different in the diaphragm and gastrocnemius of an SHHF rat in comparison to a control rat.
3) To determine if the content of SERCA 1 is different in the diaphragm and gastrocnemius of an SHHF rat in comparison to a control rat.
4) To determine if the content of SERCA 2 is different in the diaphragm and gastrocnemius of an SHHF rat in comparison to a control rat.
5) To determine if the content of Csq is different in the diaphragm and gastrocnemius of an SHHF rat in comparison to a control rat.
6) To determine if the content of CaM is different in the diaphragm and gastrocnemius of an SHHF rat in comparison to a control rat.

Research Hypotheses

The following are the null hypotheses tested in this study:

H_{O1}: There will be no difference in the content of the RyR1 between SHHF rats and control rats.

H_{O2}: There will be no difference in the content of the $\alpha 1_s$ subunit of DHPR between SHHF rats and control rats.

H_{O3}: There will be no difference in the content of SERCA 1 between SHHF rats and control rats.

H_{O4}: There will be no difference in the content of SERCA 2 between SHHF rats and control rats.

H_{O5}: There will be no difference in the content of Csq between SHHF rats and control rats.

H_{O6}: There will be no difference in the content of CaM between SHHF rats and control rats.

Limitations and Basic Assumptions

The following are limitations and basic assumptions made by the investigator:

1. SHHF rats do not have an ideal control group so Wistar-Furth rats were used as the control animal.
2. Muscles were removed from SHHF rats when it was assumed they were near death due to CHF.
3. The optical density of bands achieved with Western blot procedures, stains-all procedures and dot blots are thought to reflect protein content of the particular protein tested.
4. The measurement of the amount of protein is proportional to the optical density and the binding of specific antibodies.
5. The study is limited to the gastrocnemius and diaphragm of male rats
6. Rats were well fed and hydrated.
7. The SR vesicles were prepared properly.
8. Control rats were disease free.

Chapter 2:

Review of the Literature

Introduction

Skeletal muscle weakness and fatigue along with a decrease in exercise tolerance are among the debilitating symptoms associated with CHF (Lunde et al., 2001b; Simonini et al., 1999; Simonini et al., 1996; Williams and Ward, 1998). CHF is characterized by an inability of the heart to pump blood at a sufficient volume to meet the needs of the body tissues (Braunwald, 1997). It would seem that fatigue seen in CHF patients could be attributed to the affected cardiovascular function. A reduction in oxygen delivery to the muscles was thought to be one result of affected cardiovascular function that could be a factor in fatigue. Yet when CHF patients were tested, it was found that by increasing the number of exercising muscles, peak oxygen consumption was increased (Jondeau et al., 1992). Further studies of exercise in CHF patients have found no correlation between the left ventricular ejection fraction and the peak oxygen consumption (Minotti et al., 1991; Volterrani et al., 1994). This result indicates the delivery of oxygen to the exercising muscles is not a limiting factor. Other studies have explored the connection between CHF and exercise and have found there is no correlation between a persons exercise capacity and the left ventricular performance tested while at rest (Franciosa et al., 1981; Benge et al., 1980). Pharmacological intervention was tested in the form of administering angiotensin converting enzyme inhibitors, which increase blood flow by decreasing peripheral resistance. These substances did not have an immediate effect on exercise capacity (Drexler et al., 1989). When vasodilators were administered to increase cardiac output in CHF patients, an increase in exercise

capacity and peak oxygen consumption was not immediately seen (Wilson et al., 1983). Another study used dobutamine to increase cardiac output and leg blood flow in CHF patients. However, this increased output did not increase the duration of exercise for the patients (Wilson et al., 1984). This results raises the question as to the extent which myocardial function affects exercise capacity.

Due to the inability of the altered cardiac function to sufficiently explain the symptoms of CHF, skeletal muscle is being examined for correlations between changes in function and the fatigue seen in CHF. Fatigue is a reduction in the force production and shortening of the muscle that develops gradually from repeated contractions (Stephenson et al., 1998). Research done on skeletal muscles in animals that have CHF has shown a decrease in Type I, slow-twitch fibers and an increase in Type II, fast-twitch fibers (Lipkin et al., 1988; Perreault et al., 1993). This change in fiber type composition is indicative of a progression to a more fatigable muscle. Inactivity was presented as an explanation for the change in fiber type. However, Simonini et al. (1993) found that the activity level of surgically induced CHF rats and sham control rats was not significantly different. Vescovo et al. (1996) examined myosin heavy chain content in CHF patients, stroke patients and an inactive control group. The changes in the myosin heavy chain (MHC) fibers from MHC I, the slow isoform found in slow twitch muscles, to MHC IIx, the fast glycolytic isoform found in fast twitch muscles, were seen only in the CHF patients despite the inactivity of the other groups. These studies suggest inactivity alone cannot explain the skeletal muscle changes observed.

Skeletal muscle atrophy was then approached as the reason for the changes in muscle fiber content of the muscle. Yet a study that compared the soleus and extensor digitorum longus in a CHF model and a sham control showed after 17 weeks there was no difference found in the muscle weights or water content between the two groups (Lunde et al., 1997). Another study found no change in muscle weight in the soleus, extensor digitorum longus or tibialis anterior between a CHF model and control (Bernocchi et al., 1996). Thus atrophy alone does not appear to be a factor in the CHF models tested.

Fatigue

The findings described above suggest that changes occur within the skeletal muscle itself during CHF causing the fatigue. According to Williams and Klug (1995), altered calcium handling is a key component to fatigue. When skeletal muscle is subjected to fatiguing contractions, the myoplasmic calcium levels decrease as well as the rates of sarcoplasmic reticulum calcium uptake and release. This change results in a decrease in contractile activation and force. In a study in which rats were subjected to fatiguing exercise, a modification in the release mechanism resulted in a reduction in calcium release from the sarcoplamic reticulum (Favero et al., 1993). Fatigue conditions have also been studied in CHF models. In a coronary ligation model, the rats were subjected to fatiguing stimulation. This resulted in a reduction in the relaxation rate of the soleus when compared to the control. This difference is not due to inactivity as that parameter was not significantly different between the model and

control (Lunde et al., 2001a). In another study, intact CHF skeletal muscles were examined and were found to have decreased calcium release and uptake kinetics (Perreault et al., 1993). These results are quantitatively similar to those discussed by the Williams and Klug (1995) study of calcium handling and fatigue. Due to the many studies on altered calcium handling and fatigue and due to the fact that muscle fatigue occurs rapidly and severely in CHF patients, it seems possible that alterations in calcium handling may play a part in the decreased exercise tolerance and fatigue in CHF patients.

Excitation Contraction Coupling – DHPR and RyR

The process of calcium release from the sarcoplasmic reticulum into the myoplasm for use in contraction occurs through a process known as ECC (Groh et al., 1999). At the neuromuscular junction, acetylcholine binds to receptors on the sarcolemma (Stephenson et al., 1998) and results in the generation of an action potential that is then propagated along the muscle fiber membrane (Hamilton et al., 2000). When it reaches the t-tubule, the depolarization alters the conformation of the DHPR (Pereon et al., 1997a) (Figure 1). DHPR, named because of its high binding affinity to dihydropyridine, is a protein consisting of five subunits (Pereon et al., 1997b; Stephenson et al., 1998). The $\alpha 2$, β, δ and γ subunits all help modulate the activity of the $\alpha 1$ subunit which acts as the voltage sensor and L-type calcium channel (Pereon et al., 1997b). In skeletal muscle, the $\alpha 1_S$ subunit is present whereas in cardiac muscle the $\alpha 1_C$ subunit is present (Froemming et al., 2000). The $\alpha 1$ subunit has four transmembrane segments

that are positively charged and help detect the depolarization of the t-tubule (Stephenson et al., 1998). Once the change in membrane potential has been detected, a conformational change occurs in DHPR. This conformational change then acts to trigger the opening of the RyR or calcium release channel, located in the sarcoplasmic reticulum cisternae (Hamilton et al., 2000; Pereon et al., 1997c) (Figure 1).

The RyR, which is named for its ability to bind the plant alkaloid ryanodine with high affinity, is thought to be physically coupled with the DHPR at the cytosolic II-III loop of the DHPR (Hamilton et al., 2000; Stephenson et al., 1998; Strange et al., 2001). The RyR is a homotetramer consisting of four identical subunits. The large cytoplasmic portion of the protein is known as the foot structure and is thought to connect exterior membranes to the sarcoplasmic reticulum membrane (Stephenson et al., 1998; Franzini-Armstrong, 1999). The RyR1 is the isoform found predominantly in skeletal muscle, RyR2 is the cardiac isoform and RyR3 is the isoform found in the brain (Pereon et al., 1997b; Froemming et al., 2000). It has been shown that the DHPRs are arranged in groups of four called a tetrad. Every tetrad is arranged across from a RyR to form a triadic junction (Hamilton et al., 2000; Margreth et al., 1999; Stephenson et al., 1998). Each DHPR in the triadic junction appears to be linked to one subunit of the RyR (Franzini-Armstrong, 1999). However, only half of the RyRs face a tetrad (Stephenson et al., 1998). During t-tubule depolarization, the RyRs linked to the DHPRs are opened causing calcium release. This release in turn is thought to activate the adjacent RyRs that are not linked to a tetrad to also

release calcium in a process known as “calcium-induced calcium release” (Hamilton et al., 2000; Margreth et al., 1999). The release of calcium from the sarcoplasmic reticulum through the RyR causes an increase in myoplasmic calcium which activates troponin to cause cross bridge cycling resulting in muscle contraction (Hamilton et al., 2000; Kandarian et al., 1996).

Many studies have been done to determine the role played by these two main proteins of ECC when skeletal muscles are subjected to different conditions. Denervation and hindlimb unloading are two procedures used to induce a shift in skeletal muscles from predominantly slow twitch to fast twitch fibers whereas chronic low frequency stimulation (CLFS) causes a change from fast twitch to slow twitch fibers (Bastide et al., 2000; Kandarian et al., 1992; Pereon et al., 1997a; Pereon et al., 1997c). Although there are not different isoforms of the proteins for fast versus slow muscles, it has been shown that there are more DHPRs and RyRs in fast twitch muscles than in a slow twitch muscles (Klug and Tibbits, 1988).

In a study on denervated rats, the mRNA levels for the RyR1 isoform and for the $\alpha 1_S$ DHPR subunit of the denervated rats were found to increase in comparison to the control in the soleus. Interestingly, in the extensor digitorum longus, mRNA for the $\alpha 1_C$ DHPR subunit was found to increase in comparison to the control (Pereon et al., 1997c). In a hindlimb unloading experiment with rats, an increase in the mRNA of the DHPR was found in the unloaded limbs, yet with CLFS, a decrease in the DHPR mRNA of the treated limb was seen (Kandarian et al., 1992; Pereon et al., 1997a). These studies show a change in gene

expression and this change appears to be reflected in the protein content of skeletal muscles under the differing conditions. Another hindlimb unloading study with rats showed that after four weeks there was an increase in the amount of the RyR1 in the soleus (Bastide et al., 2000).

Interestingly, CLFS on rabbits resulted in a reduction of the RyR1 and the $\alpha 1_S$ subunit of the DHPR but caused an increase in RyR2 and the $\alpha 1_C$ subunit of the DHPR (Froemming et al., 2000). Two other studies, one with dogs and one with rabbits, both found a significant reduction in the amount of both the RYR1 and the $\alpha 1_S$ DHPR subunit after the muscles were subjected to CLFS (Hicks et al., 1997; Ohlendieck et al., 1991). An additional CLFS study found the same results, a reduction in the amount of the $\alpha 1_S$ DHPR and RyR1 after fourteen days. However, when a thirty-day recovery period was added, the down regulation of the RyR1 and the $\alpha 1_S$ DHPR was reversed (Froemming et al., 2000). All of these studies show evidence of the plasticity of not only the genes regulating the DHPR and RyR expression but also of their content in skeletal muscles.

Calcium Uptake – SERCA

While the DHPR and RyR play a part in the release of calcium from the sarcoplasmic reticulum into the myoplasm, one protein plays a major role in pumping the free calcium back into the sarcoplasmic reticulum. The protein is known as SERCA (Figure 2). This pump moves two calcium ions into the sarcoplasmic reticulum for every molecule of ATP it uses (Schulte et al., 1993).

SERCA has a high affinity for binding calcium on its myoplasmic side; however, on the lumenal side it has a low affinity for binding calcium (Stephenson et al., 1998). This asymmetry helps move the calcium from the myoplasm to the lumen of the sarcoplasmic reticulum, regulating free calcium levels in the myoplasm resulting in control of muscle relaxation (Schulte et al., 1993; Stephenson et al., 1998). In skeletal muscle this protein is present in two isoforms. SERCA 1 is the fast isoform and is found mainly in fast-twitch muscle (Wu and Lytton, 1994). SERCA 2 has a lower specific activity and calcium uptake rate. This property, along with it being present in the slow-twitch muscles has caused it to be known as the slow isoform (Lytton et al., 1992). There is also a difference in the amount of the protein in the sarcoplasmic reticulum that helps account for the difference in uptake rates. The fast isoform is present at an amount that is five fold greater than the amount of the slow isoform (Wu and Lytton, 1993; Zubrzycka-Gaarn et al., 1982). The two isoforms are similar in protein structure, with the SERCA 2 protein being 84% identical to SERCA 1. The two isoforms are also similar in their affinities for binding calcium (Lytton et al., 1992).

In a hindlimb unloading study on rats, the SERCA 1 mRNA was increased over the duration of unloading (4,7,14,28 days) and the amount of SERCA 1 protein was also increased. With SERCA 2 however, the mRNA decreased slightly, yet the protein levels did not show a decrease until day 28 (Schulte et al., 1993). In a study using CLFS as the treatment on rabbits, the amount of SERCA 1 was significantly reduced in the treated limbs. The decrease in SERCA 1 levels began after only three days of stimulation. The amount of SERCA 2 did

increase but the increase was less than the amount of SERCA 1 lost (Ohlendieck et al., 1999).

SERCA and CHF

Several studies have examined SERCA levels in skeletal muscles of animals with CHF. Spangenburg et al. (2002) used the coronary ligation model to induce moderate CHF and also added an additional treatment of functional overload. The control consisted of sham-operated animals that underwent the same surgery as the treated animals, yet did not have the coronary ligation inflicted. No difference was found in the amount of SERCA 2 or total SERCA between CHF and sham rats. In a study by Simonini et al. (1999), a coronary ligation model was used to induce severe CHF. A reduction in both the amount of SERCA 2 protein and mRNA was observed in the CHF animals compared to the sham animals. The difference in the results achieved by these two groups is attributed to a difference in severity of CHF (Spangenburg et al., 2002). Peters et al. (1997) used a genetic model of CHF with rats bred for spontaneous hypertension and heart failure. When compared to the Wistar-Furth rats used as a control, the amount of SERCA 1 was decreased in the tibialis anterior, diaphragm, and gastrocnemius of the SHHF group. The amount of SERCA 2 was found to be unchanged in the diaphragm and was below detection in the tibialis anterior and the gastrocnemius. All of these studies show that plasticity of the mRNA and the amount of the SERCA isoforms does exist.

Calsequestrin

Within the sarcoplasmic reticulum, Csq acts to bind the free calcium pumped in by the SERCA (Kobayashi et al., 2000; Szegedi et al., 1999) (Figure 1 and Figure 2). Csq is a highly acidic glycoprotein. It has a high capacity for calcium and, therefore, acts to lower the amount of free calcium within the sarcoplasmic reticulum. However, it only binds calcium with an intermediate affinity. This property allows the calcium to be released when needed (Szegedi et al., 1999). Csq is located in the vicinity of the RyR. The proteins junctin and triadin are believed to act as anchoring strands to bind the RyR and Csq together (Kobayashi et al., 2000). With these two proteins coupled, a conformational change in one can affect the other protein. Thus, the opening of the RyR might cause a conformational change in Csq causing it to release bound calcium (Szegedi et al., 1999). The plasticity of this protein was shown in a denervation study on rats. After cutting the sciatic nerve, the extensor digitorum longus and soleus were examined for Csq levels at thirty-one days post surgery. In both the extensor digitorum longus and soleus the amount of Csq increased in the denervated muscle when compared to the control (Loirat et al., 1988).

Calmodulin

Though the DHPR and RyR are the main proteins involved in ECC, there are other proteins that can play a regulatory role in the release of calcium. One of interest is CaM (Figure 1). This protein exists in a calcium free form (ApoCaM) and a calcium bound form (Ca^{++}CaM). CaM has the ability to bind four calcium

ions. Two binding sites are located at the N-terminal and the remaining two binding sites are located at the C-terminal. As the calcium binds to CaM, it causes a conformational change that results in exposure of a hydrophobic binding pocket, which in turn can bind to target proteins (Hamilton et al., 2000; Rodney et al., 2000). Interestingly, ApoCaM is also able to bind to some targets. The RyR is one target that both ApoCaM and Ca^{++}CaM can bind. It appears both forms can bind to four possible sites on each RyR, one per subunit (Hamilton et al., 2000; Rodney et al., 2000).

The exact way CaM helps regulate calcium release is not known. With calcium-induced calcium release, micromolar calcium concentrations activate the RyR, yet at millimolar calcium concentrations, the action of the RyR is inhibited calcium is not released (Rodney et al., 2000). However, in vitro studies have shown that with CaM bound to the RyR, activation occurs at nanomolar calcium concentrations whereas inhibition occurs at micromolar calcium concentrations. This result indicates that with CaM bound, activation and inhibition of the RyR occurs at lower calcium concentrations than would occur without CaM (Hamilton et al., 2000; Rodney et al., 2000).

The dual regulation (regulation of activation and inhibition) was further tested in another in vitro study, which showed ApoCaM acted to cause the RyR1 activation by increasing the receptor's sensitivity to calcium. However, Ca^{++}CaM acted as an inhibitor of the RyR1. It is speculated that at very low calcium concentrations, ApoCaM is bound to the RyR1. As the release channel opens and the concentration of calcium rises in the myoplasm, calcium begins to bind

CaM causing a conformational change that then acts to inhibit the RyR1 and terminate calcium release (Rodney et al., 2000). This hypothesis provides a possible explanation as to why CaM acts as both an activator and inhibitor of the RyR.

Calcium Handling

The relationship between changes in the amount of calcium handling proteins and calcium uptake and release rates is unclear. In a study of functional overload on the plantaris, no change was seen in the amount of either the RyR1 or the $\alpha 1_S$ subunit of the DHPR, yet calcium release rates decreased (Kandarian et al.,1996). A coronary ligation study showed calcium uptake rates increased in the CHF model, yet the amount of SERCA isoforms did not change (Spangenburg et al., 2002). These two studies seem to indicate changes in the calcium uptake or release rates are not necessarily due to changes in the amount of protein. However, in a hindlimb unloading experiment, both SERCA 1 mRNA and protein content showed a large increase and only a small decrease in both SERCA 2 mRNA and protein content. The ATPase activity rates increased reflecting the increase in the overall amount of SERCA (Schulte et al., 1993). A study of a functionally overloaded plantaris showed a decrease in the overall amount of the SERCA pump that was caused by a decrease in SERCA 1, which was not counteracted by the increase in SERCA 2. This loss in SERCA was reflected by a 15% decrease in the calcium uptake rate (Kandarian et al., 1994).

The difference between the calcium uptake and release studies presented above may be summarized as follows. When a change in protein content was observed, a change in uptake activity occurred. However, a change in uptake or release activity did not necessarily translate into a change in protein content.

Conclusion

The current research indicates that the fatigue that occurs with CHF is not related to altered cardiovascular function. The skeletal muscles themselves are altered. Altered calcium handling is known to be a factor in fatigue of skeletal muscles and may be involved in the fatigue of CHF patients. The main proteins and regulatory proteins involved in calcium handling have been shown to be plastic; the amounts present can change depending on the condition to which the muscle is subjected. Changes in the amounts of these proteins could provide a key component to understanding calcium handling in the skeletal muscle of CHF patients and perhaps provide more information to elucidate the reasons for fatigue associated with CHF.

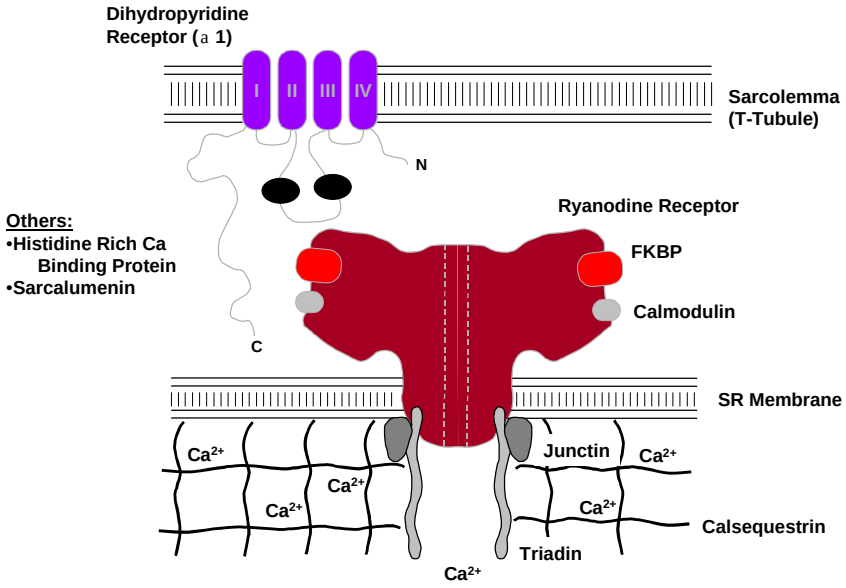

Figure 1: Diagram of the t-tubule and sarcoplasmic reticulum structure and the proteins involved in calcium release.

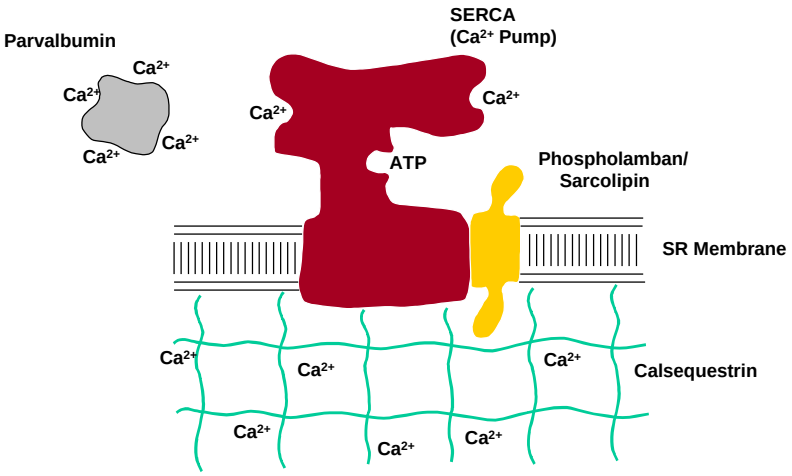

Figure 2: Diagram of the sarcoplasmic reticulum structure and the proteins involved in calcium uptake.

Chapter 3:

Methods

Animals

SHHF/Mcc-cp rats were used as the model of CHF (SHHF, n=4). Wistar-Furth rats were used as the control (n=4). The samples used were obtained from Dr. Susan Kandarian. When the rats were determined to be in end-stage heart failure, they were anesthetized and the gastrocnemius and diaphragm were removed. The tissue was collected and prepared as described below and the rats were maintained in Dr. Susan Kandarian's laboratory. Sarcoplasmic reticulum vesicle samples were then shipped on dry ice to Virginia Tech.

Preparation of the Samples

The isolated heavy sarcoplasmic reticulum (HSR) vesicles and isolated light sarcoplasmic reticulum (LSR) vesicles of the gastrocnemius and diaphragm were prepared in Dr. Susan Kandarian's laboratory. The procedure for vesicle isolation consisted of homogenizing the muscle, spinning at 8000g and discarding the pellet. The supernatant was then spun at 12000g. The pellet collected contained the HSR vesicles. The remaining supernatant was then spun at 60000g. The pellet collected contained the LSR vesicles. The vesicles were frozen and sent to Virginia Tech where they were stored at -80°C.

The protein concentrations obtained in a preliminary study (E. Spangenburg and M. Stockunas, unpublished data) were used to determine the amount of sample needed to give 250 μg of protein for each LSR gastrocnemius and diaphragm sample, 200 μg of protein for each HSR gastrocnemius sample and 190 μg for each HSR diaphragm sample. The samples were vortexed and

added to test tubes. Distilled water was added to bring the total volume of the protein and water to 100 μl. 100 μl of 2x buffer (Table 1) was then added to give a final volume in the test tubes of 200 μl. The final concentrations for the LSR gastrocnemius and diaphragm samples were 1.25 μg/μl. The final concentrations for the HSR were 1.0 μg/μl for the gastrocnemius and 0.95 μg/μl for the diaphragm. The test tubes were then placed in a boiling water bath for two minutes. After removing the test tubes from the boiling water bath the tubes were covered with parafilm and stored at -80°C.

Electrophoresis - SERCA 1, SERCA 2, RyR, DHPR, Csq

7.5% acrylamide SDS-PAGE gels (Table 2) were used to examine SERCA 1, SERCA 2, and DHPR content, a 5% acrylamide SDS-PAGE gel (Table 2) was used to examine RyR content, and a 10% acrylamide SDS-PAGE gel (Table 2) was used to examine Csq content. A 4% acrylamide stacking gel (Table 2) was used for all gels. For SERCA1 and Csq the spacing of the plates was 0.75 mm. For SERCA 2, RyR and DHPR the spacing of the plates was 1.5 mm. Both the stacking and separating gel solutions were prepared and degassed by pulling a vacuum on the solution. After twenty minutes, the separating gel solution was removed from the vacuum. TEMED and a 10% ammonium persulfate solution (Table 2) were added, quickly swirled and then poured using a glass pipette to a level 1 cm below the bottom of the gel comb. The gel was then be overlaid with 20% ethanol and allowed to polymerize for thirty minutes. The alcohol was poured off and the gel was briefly overlaid with a

1% SDS solution. The 1% SDS solution was then be poured off and the plates were dried with filter paper. The stacking gel solution was removed from the vacuum and TEMED and the 10% ammonium persulfate solution (Table 2) were added. The gel solution was swirled and poured with a glass pipette until the solution was coming over the top of the short plate. The comb was then gently inserted. The gel was allowed to polymerize for thirty minutes at which time the previously prepared samples were removed to allow time to thaw. The combs were removed and the electrophoretic apparatus was set up. 5x electrode buffer (Table 2) was mixed with distilled water to form 1x running buffer (Table 2). The running buffer was added to the inner chamber to cover the shorter plates. Each lane was washed with the buffer using a syringe. The prepared samples were vortexed and loaded into the lanes. 10 μg of LSR sample were used to examine SERCA1 content, 50 μg of LSR sample were used to examine SERCA 2 content, 25 μg of LSR sample were used to examine Csq content, and 40 μg of HSR sample were used to examine RyR and DHPR content. Running Buffer was added to the outer chamber and the setup was packed in ice. SERCA1, SERCA2, Csq, and DHPR gels were run at a constant voltage of 200V until the blue dye contained in the 2x buffer used in sample preparation was seen coming out of the bottom of the gel. The RyR gel was run at a constant current of 45mA until the dye ran off the gel.

Western Blot - SERCA1, SERCA2, DHPR, RyR

The gels were removed from the plates and notches were made for identification purposes. They were then placed in transfer buffer (Table 3) prepared that day. The 0.75mm thick gels were soaked for fifteen minutes, and the 1.50mm thick gels for thirty minutes. A nitrocellulose sheet and three filter pads for each gel were allowed to equilibrate in transfer buffer for ten minutes. A mini-blot apparatus was used. The components were stacked in the following order: two sheets of filter paper, nitrocellulose sheet, gel, and filter paper. Transfer buffer was added to the chamber. The setup was packed in ice and ran overnight at 30V in a room kept at 4°C. The nitrocellulose sheets were removed from the transfer setup and placed in TBS (Table 3) for fifteen min. Next, the sheets were placed in blocking buffer (Table 3) for forty-five minutes. The sheets were then washed three times with TTBS (Table 3) for ten minutes each. The primary antibody (Table 6) was applied and the nitrocellulose sheets were kept overnight in the presence of the antibody in a room kept at 4°C.

After the transfer, the gels were removed from the transfer setup and placed in Coomassie Blue solution (Table 3). After remaining in the Coomassie Blue solution overnight, the Coomassie Blue solution was removed from the gels, which were then washed with a destaining solution (Table 3). The absence of protein bands in the gel after destaining indicated successful transfer of the proteins from the gel to the nitrocellulose sheet. The presence of protein bands

was evidence of incomplete transfer of the protein from the gel to the nitrocellulose at which point the experiment was repeated.

After incubating the nitrocellulose sheets with the primary antibody overnight, they were then washed with TTBS twice for five minutes each. The secondary antibody (Table 6) was applied at a concentration of 1:30000 for sixty minutes. The nitrocellulose sheets were then washed two times with TTBS for five minutes each. Finally, the sheets were washed for five minutes in TBS. 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) was placed onto the nitrocellulose sheets to colorize the protein bands. The nitrocellulose sheets were covered during the light sensitive colorizing. After bands appeared, the sheets were washed with distilled water. The nitrocellulose sheets were then scanned using the MultiImage™ Light Cabinet from Alpha Innotech Corporation. The optical densities of the bands were analyzed using AlphaImager™ 2000 Documentation and Analysis Systems.

Stains-all - Csq

The 10% acrylamide SDS-PAGE gels that were run to measure Csq were removed from the glass plates and placed in a 25% isopropyl alcohol solution overnight. After six washes in 25% isopropyl alcohol solution at thirty minutes each time, the gels were placed in freshly prepared stains-all solution (Table 4) for forty-eight hours during which time the gels remained covered due to the light sensitive nature of stains-all. The gels were briefly destained in 25% isopropyl alcohol and scanned. The scanned images were analyzed using the Scion

program obtained from the National Institutes of Health. Stains-all stained all glycoproteins (such as Csq) blue whereas the other proteins stained pink.

Dot Blot – CaM

Two nitrocellulose sheets were divided into eight sections. 5µg of HSR sample from each test animal for the gastrocnemius and diaphragm were added, one sample per section, and were allowed to air dry. The sheets then underwent the same procedure listed in the section on western blotting with the procedures beginning after the transfer of the proteins from the gel to the nitrocellulose sheets and including only the procedures pertaining to the nitrocellulose sheets.

Gels Stained with Coomassie Blue

A 7.5% acrylamide SDS-PAGE gel (See Table 5) was run according to the same procedures listed in the section on electrophoresis with the following specifications: 0.75mm plates were used, 20µg of each sample were added and the gel was run at 200V until the dye ran off. The gels were then removed from the glass plates. The stacking gel was removed and the gel was placed in Coomassie Blue solution (Table 5). The gel was allowed to agitate in the solution overnight. The Coomassie Blue solution was poured off of the gels and a series of washes with destaining solution (Table 5) took place until a clear picture of the protein pattern could be seen.

Statistics

Separate Student's t-tests were used to compare the effects of the treatment (SHHF) and control on the optical density of the RyR1, DHPR, SERCA1, SERCA2, Csq and CaM in the gastrocnemius and diaphragm muscles. Differences will be considered statistically significant at the $p < 0.05$ level.

Table 1: Composition of the 2x buffer.

1 g SDS
5 ml Glycerol
0.47 ml Beta-Mercaptoethanol
0.003 g Bromophenol blue
5 ml of 0.5 M Tris-HCl (pH 6.8)
Distilled H_2O to a final volume – 25 ml

Table 2: Composition of solutions used for SDS-PAGE.

5% Acrylamide SDS-PAGE Separating Gel

12.30 ml Distilled H_2O
5.00 ml 1.5 M Tris (pH 8.8)
0.200 µl 10% SDS
2.500 ml 40% Acrylamide
Final Volume – 20 ml
After vacuum add:
100 µl 10% Ammonium Persulfate Solution
20 µl TEMED

4% Acrylamide SDS-PAGE Stacking Gel

6.34 ml Distilled H_2O
2.50 ml 0.5 M Tris (pH 6.8)
0.100 µl 10% SDS
1.000 ml 40% Acrylamide
Final Volume – 10 ml
After vacuum add:
50 µl 10% Ammonium Persulfate Solution
10 µl TEMED

7.5% Acrylamide SDS-PAGE Separating Gel

10.93 ml Distilled H_2O
5.00 ml 1.5 M Tris (pH 8.8)
0.200 µl 10% SDS
3.750 ml 40% Acrylamide
Final Volume – 20 ml
After vacuum add:
100 µl 10% Ammonium Persulfate Solution
20 µl TEMED

5x Running Buffer

15g Tris
72g Glycine
5g SDS
Distilled H_2O to a final volume – 1 L

1x Running Buffer

100 ml 5x Running Buffer
400 ml Distilled H_2O
Final Volume – 500 ml

10% Acrylamide SDS-PAGE Separating Gel

9.68 ml Distilled H_2O
5.00 ml 1.5 M Tris (pH 8.8)
0.200 µl 10% SDS
5.000 ml 40% Acrylamide
Final Volume – 20 ml
After vacuum add:
100 µl 10% Ammonium Persulfate Solution
20 µl TEMED

Table 3: Composition of solutions used for Western blotting.

Transfer Buffer – SERCA1 and SERCA2

5.82 g Tris
2.93 g Glycine
Distilled H_2O to a final volume – 1 L

Transfer Buffer – DHPR and RyR

3.03 g Tris
14.4 g Glycine
Distilled H_2O to a final volume – 1L

TBS

4.84 g Tris
58.4 g NaCl
Distilled H_2O to a final volume – 2 L
pH – 7.5

TTBS

100 ml TBS
50 µl Tween

Blocking Buffer

100 ml TBS
50 µl Tween
3-5 g Non-Fat Dry Milk

Coomassie Blue Solution

0.1% Coomassie Blue R-250
40% Methanol
10% Acetic Acid
49.9% Distilled H_2O

Destaining Solution
40% Methanol
10% Acetic Acid
50% Distilled H_2O

Table 4: Composition of Stains-all solution

75 ml Isopropyl Alcohol
22.5 ml Formamide
1.09 g Tris
Distilled H_2O to a final volume – 300 ml
pH – 8.8
Add 0.0075 g Stains-all

Table 5: Composition of solutions used for Coomassie Blue stained gels.

7.5% Acrylamide SDS-PAGE Separating Gel

10.93 ml Distilled H_2O
5.00 ml 1.5 M Tris (pH 8.8)
0.200 µl 10% SDS
3.750 ml 40% Acrylamide
Final Volume – 20 ml
After vacuum add:
100 µl 10% Ammonium Persulfate Solution
20 µl TEMED

4% Acrylamide SDS-PAGE Stacking Gel

6.34 ml Distilled H_2O
2.50 ml 0.5 M Tris (pH 6.8)
0.100 µl 10% SDS
1.000 ml 40% Acrylamide
Final Volume – 10 ml
After vacuum add:
50 µl 10% Ammonium Persulfate Solution
10 µl TEMED

Coomassie Blue Solution

0.1% Coomassie Blue R-250
40% Methanol
10% Acetic Acid
49.9% Distilled H_2O

Destaining Solution
40% Methanol
10% Acetic Acid
50% Distilled H_2O

Table 6: Antibodies used for Western blots and dot blots

Primary Antibodies

Ryanodine Receptor 1 (Affinity BioReagents, Inc.)

Clone – 34C
Host – Mouse IgG1
Dilution – 1:5000 (diluted in TBS)

Dihydropyridine Receptor ($\alpha 1_s$ subunit) (Sigma)

Clone – 1A
Host – Mouse IgG
Dilution – 1:500 (diluted in TBS)

SERCA 1 ATPase (Affinity BioReagents, Inc.)

Clone – IIH11
Host – Mouse IgG
Dilution – 1:2500 (diluted in TBS)

SERCA 2 ATPase (Affinity BioReagents, Inc.)

Clone – 2A7-A1
Host – Mouse IgG2a
Dilution – 1:2000 (diluted in TBS)

Calmodulin (Sigma)

Clone – 6D4
Host – Mouse IgG1
Dilution – 1:2000 (diluted in TBS)

Secondary Antibodies

Alkaline Phosphatase Conjugated Goat Anti-Mouse IgG (Sigma)

Dilution – 1:30000 (diluted in TBS)

Chapter 4:

Results

Calcium Uptake Rates, Release Rates and ATPase Activities

Calcium uptake and release rate measurements revealed a lower calcium release rate and ATPase rate of the SHHF samples of both the diaphragm and gastrocnemius when compared to the control (E. Spangenburg and M. Stockunas, unpublished data). However, whereas the SHHF diaphragm samples showed a lower calcium uptake rate than the control, the SHHF gastrocnemius samples showed a higher calcium uptake rate than the control (Appendix B). Although these data were not collected as a part of this thesis, the results are included here to provide insight into how the structural change may affect sarcoplasmic reticulum function.

Western Blotting

Western blots of RyR1 using the 34C clone antibody typically yield two distinct bands. One (molecular mass ~ 565 kDa) is the skeletal isoform (RyR1) and the second (molecular mass ~ 410 kDa) is referred to as the 410 kDa proteolytic fragment (Wu et al., 1997). Though a fragment, it is thought to be part of the functional RyR. Both bands added together constitute total RyR1 for each sample. RyR1 was significantly less in both the gastrocnemius and the diaphragm samples (71% and 83% reductions, respectively) in the SHHF samples in comparison to the control (Figure 3). The 410 kDa proteolytic fragment was significantly less in the diaphragm samples of the SHHF animals (86%). In the gastrocnemius samples, there was a trend towards less of the fragment of the SHHF animals, yet it was not significantly different from the

control animals. In both the gastrocnemius and the diaphragm samples, the total amount of RyR1 protein was significantly less in the SHHF rats in comparison to the controls. In the gastrocnemius samples, there was 64% less in the SHHF animals than in the control. In the diaphragm samples, the difference in the SHHF animals was 84% less than the control. A quarter of the SHHF samples from the gastrocnemius and from the diaphragm showed no detectable RyR1 or the 410 kDa proteolytic fragment. There were also several other samples in which a 410 kDa proteolytic fragment was not detected. This result does not necessarily mean that there was no calcium release channel present. It could be present either in quantities that could not be detected by the method used or as an isoform that does not react to the 34C clone (e.g., the cardiac isoform RyR2).

The amount of DHPR protein in the gastrocnemius samples was 82% lower in the SHHF animals than the control as detected using the 1A clone antibody (Figure 4). There was no detectable DHPR signal in one half of these SHHF samples. However, the signal in the remaining SHHF samples was well below that of the control. In the diaphragm samples, no detectable DHPR from the SHHF animals was observed resulting in significantly less DHPR in the SHHF samples than the control. Again, no detectable signal does not mean there is no DHPR present. Either it may not be detectable with the method used or it is of an isoform that cannot react to the 1A clone (e.g., the cardiac isoform with the $\alpha 1_c$ subunit).

SERCA 1 was also found to be significantly less in the SHHF animals when compared to the control in both the diaphragm and gastrocnemius samples

by using the IIh11 clone antibody. In the gastrocnemius samples, the amount of SERCA 1 was 57% less whereas, in the diaphragm samples, there was 94% less in the SHHF animals when compared to the control (Figure 5). In three quarters of the SHHF diaphragm samples, no SERCA 1 was detected.

In both the diaphragm and gastrocnemius vesicles, there was no statistical difference in the SERCA 2 levels between the SHHF and control samples using a 2A7-A1 clone antibody (Figure 5). In half of the SHHF samples and a quarter of the control, no SERCA 2 could be detected and, in the remaining samples, the signal was very faint.

Stains-all

Csq signal was detected in all of the samples. In the gastrocnemius samples of the SHHF animals, there was a tendency for the amount of Csq to be lower in comparison to the control (Figure 6). Nevertheless, this difference was not statistically significant. However, in the diaphragm samples of the SHHF animals, 146% more Csq was observed compared to the control. This difference was found to be statistically significant.

Dot Blot

In all of the samples, a CaM signal was detected using a 6D4 clone antibody. In both the gastrocnemius and the diaphragm samples of the SHHF animals, there was a slight tendency towards more CaM detected when

compared to the control. However, neither of these differences was significant (Figure 7).

Correlations

The optical densities for the Western blot protein signals, stains-all, and dot blot protein signals were all examined for any correlations that might exist between proteins of the same muscle. In the gastrocnemius samples, significant positive correlations were observed between DHPR and total RyR1, SERCA 1 and total RyR1, and SERCA 1 and DHPR (Table 7). In the diaphragm samples, significant positive correlations were observed between DHPR and total RyR1, SERCA 1 and total RyR1, and SERCA 1 and DHPR. However, in the diaphragm vesicles only, negative correlations were observed between Csq and total RyR1, Csq and DHPR, and between Csq and SERCA 1 (Table 8).

Coomassie Staining

In half of the SHHF diaphragm samples (samples 2009 and 2011), there was no detectable amount of both SERCA 1 and SERCA 2. For this reason, a 7.5% gel was run and stained with Coomassie Blue to determine the total SERCA present in the samples. A band at the molecular weight corresponding to SERCA was observed in sample 2009. In sample 2011, although a faint band was visible, it was not measurable by the AlphaImager program. When the intensity of the bands at the molecular weight marker of SERCA were measured for all of the diaphragm samples, the bands for the SHHF animals were found to

be 60% less when compared to the control (Figure 5). The same procedure was done for the gastrocnemius samples to determine total SERCA levels. Significantly less (42%) was found in the SHHF samples in comparison to the control.

Very low intensity bands corresponding to the RyR could also be detected in the Coomassie Blue stained gels (data not shown). However, they could not be quantified reliably. Nevertheless, RyR appeared to be present in all samples, but was considerably reduced in SHHF samples.

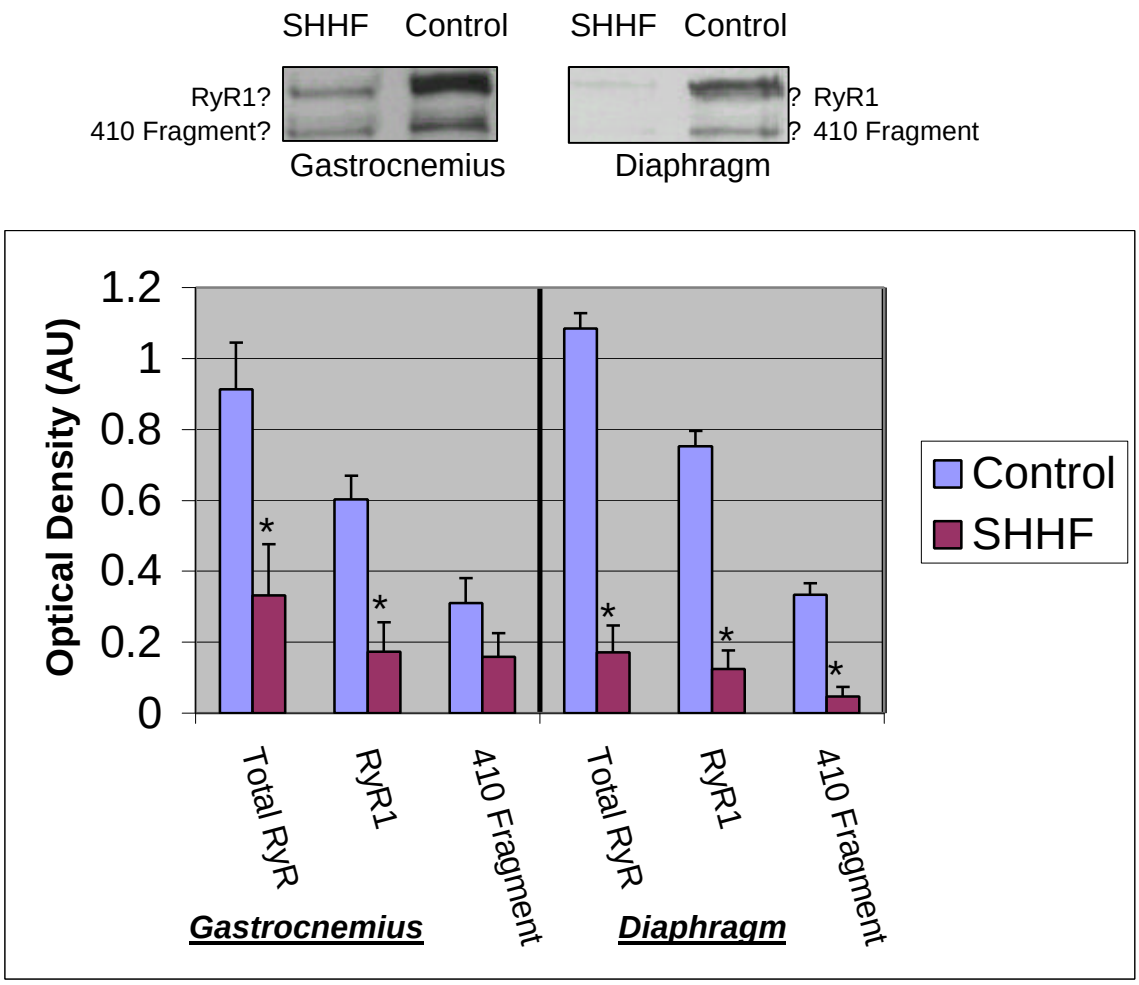

Figure 3: Western blot results for RyR1 in control and SHHF gastrocnemius and diaphragm samples. Top: Representative blots using the 34C clone antibody. Bottom: Mean optical densities ± standard error (SE) of the western blot signals (measured in arbitrary units, AU). * Significantly different from control ($p<0.05$) (See Table 10 for raw data)

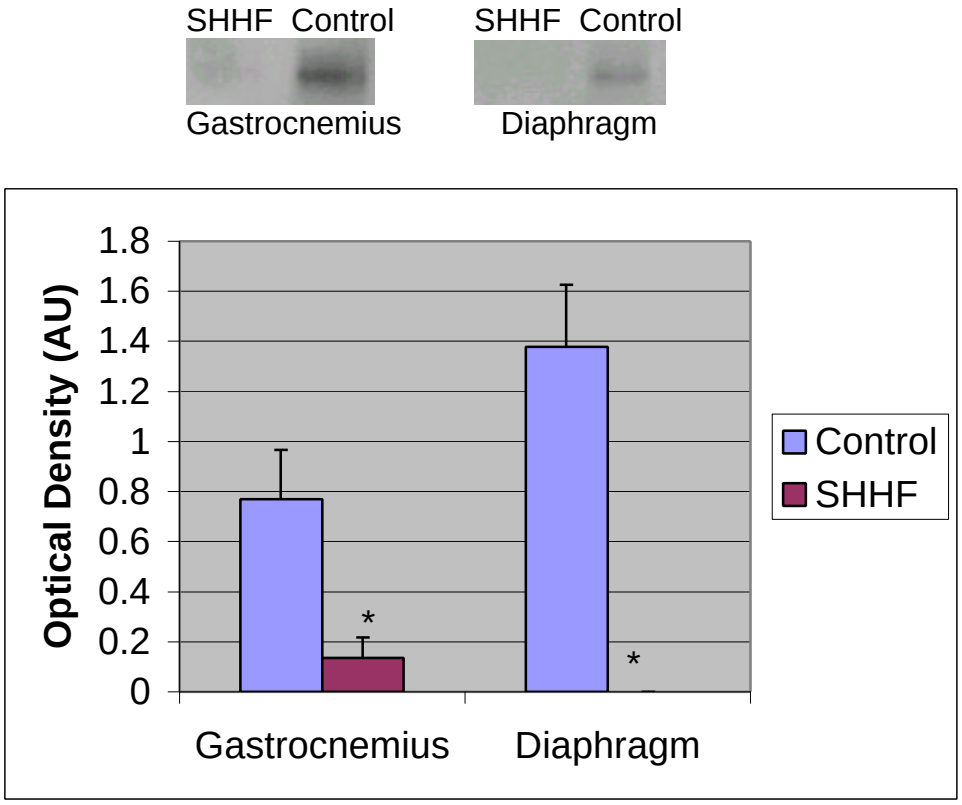

Figure 4: Western blot results for DHPR in control and SHHF gastrocnemius and diaphragm. Top: Representative blots using the 1A clone antibody. Bottom: Mean optical densities ± SE of the Western blot signal. * Significantly different from control ($p<0.05$) (See table 11 for raw data)

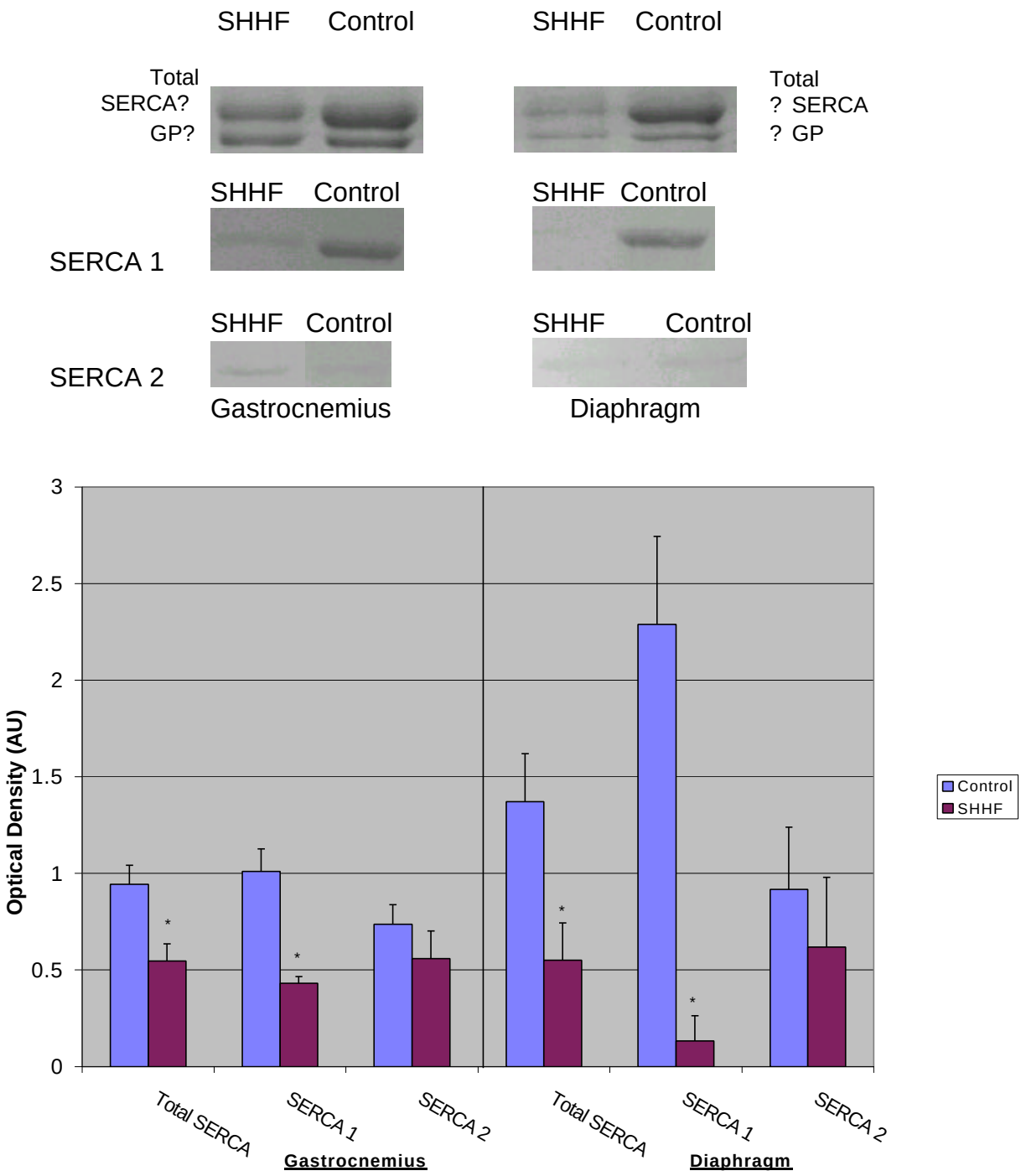

Figure 5: Coomassie Brilliant Blue and western blot results for total SERCA, SERCA 1 and SERCA 2 in control and SHHF gastrocnemius and diaphragm samples. Top: Representative lanes for total SERCA and representative blots for SERCA 1 and SERCA 2 using the IIH11 clone and 2A7-A1 clone antibodies respectively. Bottom: Mean optical densities ± SE of the stained gel and western blot signal. GP- Glycogen Phosphorylase; * Significantly different from control ($p<0.05$) (See Table 12 for raw data)

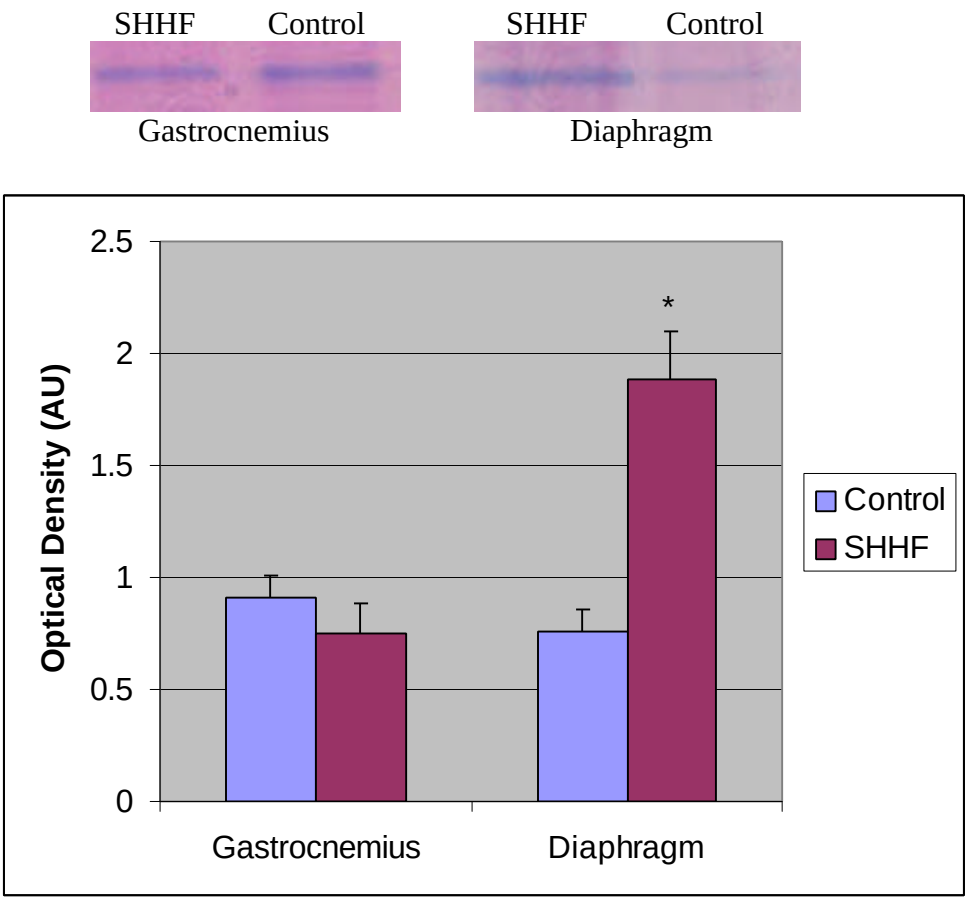

Figure 6: Stains-all results for Csq in control and SHHF gastrocnemius and diaphragm. Top: Representative lanes. Bottom: Mean optical densities ± SE of the Stains-all signal. *Significantly different from control ($p<0.05$) (See Table 13 for raw data)

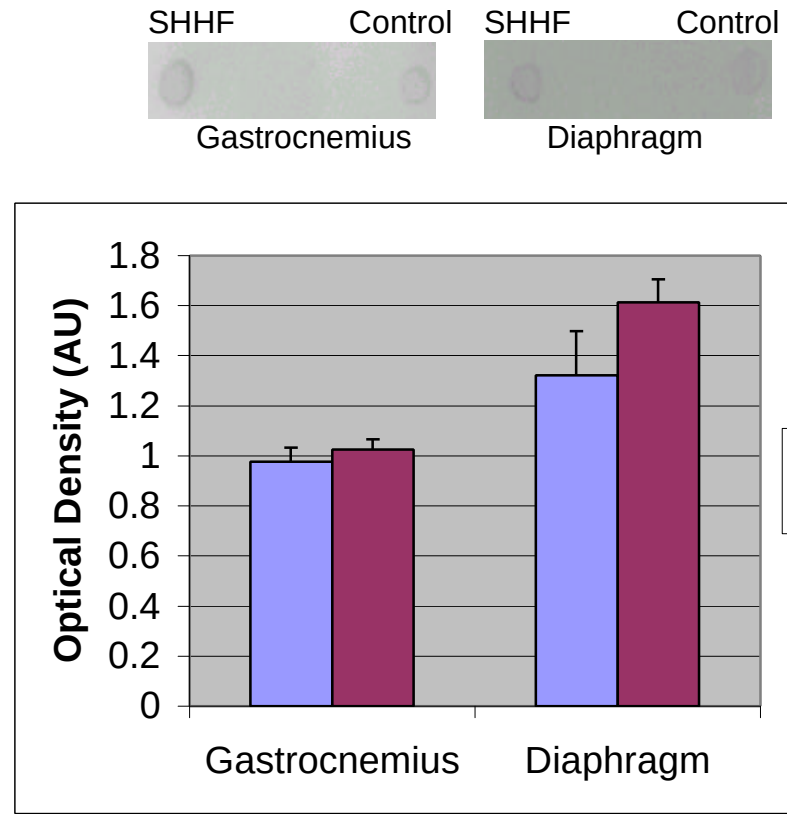

Figure 7: Dot blot results for CaM in control and SHHF gastrocnemius and diaphragm. Top: Representative blots using the clone 6D4 antibody. Bottom: Mean optical densities ± SE of the Dot Blot Signal. (See Table 14 for raw data)

	RyR	DHPR	SERCA 1	SERCA 2	Csq	CaM
RyR	-----	0.950 *	0.896 *	0.089	0.634	-0.386
DHPR		-----	0.944 *	0.238	0.706	-0.261
SERCA 1			-----	0.240	0.631	-0.264
SERCA 2				-----	0.295	0.402
Csq					-----	0.279
CaM						-----

Table 7: Correlations for the optical densities of the proteins in the gastrocnemius. R-values are given.
* Denotes significant correlation between the two proteins ($p<0.05$)

	RyR	DHPR	SERCA 1	SERCA 2	Csq	CaM
RyR	-----	0.904 *	0.899 *	0.397	-0.811 *	-0.506
DHPR		-----	0.925 *	0.012	-0.807 *	-0.356
SERCA 1			-----	0.173	-0.824 *	-0.137
SERCA 2				-----	-0.082	-0.330
Csq					-----	0.315
CaM						-----

Table 8: Correlations for the optical densities of the proteins in the diaphragm. R-values are given. * Denotes significant correlation between the two proteins ($p<0.05$)

Chapter 5:

Discussion

Summary

In this study, optical densities of western blots, coommasie and stains-all stained gels and dot blots were used to determine the relative protein isoform contents of control and SHHF sarcoplasmic reticulum muscle samples. Significantly less RyR1, DHPR, and SERCA 1 were found in the SHHF gastrocnemius and diaphragm samples in comparison to the control. There were no significant differences found in the amounts of both CaM and SERCA 2 between the two groups. Csq amounts were not found to be statistically different between the two groups of the gastrocnemius samples. However, in the samples of the diaphragm, there was more Csq in the SHHF in comparison to the control. These results are summarized in Table 9. They indicate that SHHF animals, which are in end-stage CHF, exhibit alterations in the amount and/or isoform of proteins involved in calcium handling.

Calcium Release

The changes in the amounts of the main proteins responsible for calcium release are consistent with the decrease in the calcium release rates found for the gastrocnemius and diaphragm in the SHHF rats (E. Spangenburg and M. Stockunas, unpublished data). In the gastrocnemius, significantly less RyR1 was found in the SHHF group when compared to the control. However, there was no significant difference in the amount of the 410 kDa proteolytic fragment between the groups. Yet in the diaphragm, a significant reduction in both RyR1 and the 410 kDa proteolytic fragment occurred in the SHHF group when compared to the

control. In both the gastrocnemius and diaphragm samples, there was significantly less total RyR1 in the SHHF group when compared to the control. The reduction in total RyR1 could be accompanied by a change in the RyR isoform from the skeletal muscle isoform RyR1 to the cardiac isoform RyR2. In a study by Froemming et al. (2000), it was found that during chronic low frequency stimulation, the RyR1 isoform was significantly decreased whereas the RyR2 isoform was up regulated showing a change in protein isoform was occurring. However, in the present study, the Coomassie stained gel results are consistent with the idea that there is less total RyR in the SHHF samples.

In the gastrocnemius samples, there was significantly less DHPR protein in the SHHF group when compared to the controls. In half of the SHHF samples, DHPR was not detected. In the diaphragm of the SHHF group, no DHPR was detected. It is not clear if this represents a true absence of the DHPR protein in the muscle. It is possible that either the DHPR in the samples does not react to the antibody used (e.g., it is the cardiac isoform with the $\alpha 1_c$ subunit) or less is recovered during isolation. In the study by Froemming et al. (2000), a decrease in the skeletal muscle isoform and an increase in the cardiac muscle isoform of the DHPR were found with chronic low frequency stimulation.

In both muscles examined, a positive correlation was found between the total RyR1 and DHPR content. This correlation could be due to a possible physical coupling between the two proteins that may occur during vesicle isolation. One point of interest is that the RyR is the only protein tested that could have directly affected the calcium release rates. The vesicles used to test

the calcium release rates do not require the DHPR's action as the voltage sensor to start off ECC. Instead, the RyR itself was stimulated chemically using $AgNO_3$. The reduction in the amount of total RyR in the SHHF animals could lead to decreased release of calcium into the myoplasm. Whereas either a reduction in the amount of DHPR or its isoform would not affect the calcium release rates measured in isolated vesicles, the reduction in the amount of DHPR could cause a further decrease in the calcium release rate in an *in vivo* model when ECC occurs and DHPR is acting as the voltage sensor for the release of calcium.

Calcium Uptake

In both the gastrocnemius and diaphragm samples of the SHHF group, there was significantly less SERCA 1 when compared to the control, but no significant difference in the amount of SERCA 2 between the two groups. Differences in the total amount of SERCA for the SHHF samples were also seen in both muscles. SERCA 1 is the more abundant protein of the two and a reduction in the amount of SERCA 1 would be expected to have a very evident effect on calcium uptake rates. Lower ATPase activities in samples from both muscles of the SHHF animals were observed when compared to controls (E. Spangenburg and M. Stockunas, unpublished data). This difference can be explained by the lower amounts of total SERCA and SERCA 1 present. ATP is used by SERCA to pump free calcium into the sarcoplasmic reticulum. A reduction in the amount of SERCA present would decrease total ATPase activity expressed per total protein independent of changes in the specific activity of this

enzyme. In the gastrocnemius, there is a 42% decrease in the amount of total SERCA in the SHHF muscles in comparison to the controls. The difference (37%) in ATPase activity in this same muscle between the SHHF animals and controls is relatively similar. Thus, a reduction in total SERCA seems to be reflected in the decreased ATPase activity.

The reduction in SERCA 1 without a compensatory increase in SERCA 2 also provides an explanation for the reduced calcium uptake rate measured in the diaphragm samples of the SHHF group (E. Spangenburg and M. Stockunas, unpublished data). The increase in the calcium uptake rate found in the gastrocnemius samples of SHHF rats (E. Spangenburg and M. Stockunas, unpublished data) is not consistent with significantly less total SERCA, SERCA 1 and no change in SERCA 2 found in the gastrocnemius samples. It should be emphasized that the measurement of calcium uptake was actually net calcium uptake rates (i.e. rate of uptake minus spontaneous release or leak rates). The SERCA will continue to pump calcium into the sarcoplasmic reticulum vesicle either until there is no calcium left outside of the vesicle or until its capacity to hold calcium is reached. Even without activation of the RyR, some calcium may be released during uptake. In addition, some calcium could leak across the membrane. Either case would contribute to the amount of calcium outside of the vesicle. This increase in calcium outside of the vesicle would cause the measured net uptake rate to be reduced. If for some reason, a reduction in the rate of calcium either released or leaked across the membrane occurred, the net calcium uptake rate would increase. This increase could result from either a

decrease in the permeability of the membrane to calcium or a decrease in the amount of RyR. Thus, a decrease in calcium uptake rates could be masked by an even greater relative decrease in the efflux rate. The amount of RyR1 was significantly less in the gastrocnemius of the SHHF group in comparison to the control. There was also a reduction in the ATPase activity (E. Spangenburg and M. Stockunas, unpublished data) and in the amount of SERCA 1 in the SHHF group. These results are consistent with the idea that the absolute calcium uptake rate was reduced, but the reduction in the amount of RyR1 present had a greater effect on the net calcium uptake rate.

To test this theory, measurements would need to be done on vesicles that were treated either with a strong intravesicular precipitating agent, such as oxalate, or with a compound that inhibits calcium release, such as ruthenium red. These measurements should produce a better understanding of calcium uptake within the SHHF gastrocnemius.

Regulatory Proteins

The two regulatory proteins examined, CaM and Csq, presented interesting results. The two main calcium release proteins, RyR1 and DHPR, were greatly affected in the SHHF model, but there was no significant difference in CaM between the two models in either of the muscles. CaM can bind to the RyR and is thought to influence release. It was expected that changes in CaM would reflect changes in RyR. This expectation may still be the case given the possibility that there were changes in the RyR isoform. Yet the function of CaM

is still under debate and this situation makes it difficult to presume the effect CaM is having on the SHHF model.

There was no significant difference in the amount of Csq of the gastrocnemius samples between the SHHF model and the control. However, in the diaphragm samples, significantly more Csq was found in the SHHF model as compared to the control. When examining correlations, a negative correlation was present between total RyR1 and Csq, DHPR and Csq and between SERCA 1 and Csq in the diaphragm. Thus as RyR, DHPR and SERCA 1 tend to decrease, Csq tends to increase. The increase in Csq might be to compensate for the changes in other proteins. The coupling of the RyR and Csq seems to offer a possibility that the two would affect each other. Yet Csq could be acting to protect all of the calcium handling proteins. With the reduction in both calcium release and calcium uptake proteins, the increase in Csq, a calcium binding protein within the sarcoplasmic reticulum, could allow free calcium to be quickly bound once it is in the sarcoplasmic reticulum and to leave adequate space for excess calcium. This function may help keep resting free myoplasmic calcium concentrations low whereas excess calcium could activate calcium-activated proteases. High calcium concentrations have been shown to activate proteases that could attack calcium handling proteins (Belcastro, 1993). In support of this theory, in the diaphragm, a reduction in the 410 kDa proteolytic fragment of the RyR was seen in the SHHF group when compared to the control. This result raises the possibility that there was a reduction in the amount of proteolysis occurring within the diaphragm.

Previous Work

Previous studies that examined protein levels in CHF models have found conflicting results. Spangenburg et al. (2002) found no difference in either SERCA 2 protein levels or total SERCA between the CHF ligation model and the control. Yet Simonini et al. (1999) observed a reduction in the amount of SERCA 2. The two studies used the same model, however, the former induced moderate CHF whereas the latter induced severe CHF. The difference in severity was the reason given for the difference in results (Spangenburg et al., 2002). The results of the present study agree with Spangenburg et al (2002) in that no difference in the amount of SERCA 2 protein was found between the conditions, however, a difference in total SERCA was found in the present study, which does not agree with the results of Spangenburg et al. (2002). The difference in results of the present study and the two coronary ligation model studies could be attributed to the difference in the model of CHF. Peters et al. (1997) used the genetic model (SHHF) as the model of CHF. In this study, they found a significant decrease in SERCA 1 in the SHHF model as compared to the control in the gastrocnemius and diaphragm. They also found that levels of SERCA 2 were not significantly different in the diaphragm. The levels in the gastrocnemius were below detectable limits. Peters et al. (1997) used the same model as was used in the current study. The same relative differences or lack of differences in the results involving SERCA proteins were identical between the two studies using the same model of CHF.

Mechanism of Adaptation

The reason for the changes in the amount of protein within skeletal muscles is of interest. In CHF, the muscle type changes to a more fast twitch muscle (Perreault et al, 1993; Lipkin et al., 1988), the calcium handling is altered (Williams and Ward, 1998) and it appears the calcium handling proteins are also altered. Inactivity has been presented as a possible explanation for the changes that occur in the muscle, but research has not supported this theory. A study showed no change in activity levels between CHF and control groups, yet changes in muscle fiber type occurred in the CHF group (Simonini et al., 1996). Another study showed that the changes that occur in the muscles of CHF patients are different from those occurring in other disease and inactivity models (Vescovo et al., 1996). In the present study, activity levels between the SHHF group and the control were not tested. Thus, inactivity cannot be ruled out as a possible factor contributing to the changes seen with the calcium handling, yet with the past research showing no correlation with the changes caused by CHF and activity (Simonini et al., 1996; Vescovo et al., 1996), it is unlikely inactivity would be a factor.

Another possible factor causing the changes within the skeletal muscle is hormonal signals. Tumor necrosis factor-alpha (TNFα) is especially of interest. Elevated levels of TNFα have been found in CHF patients (Levine et al., 1990; McMurray et al., 1991). This cytokine has also been shown to decrease the contractility of myocardial muscle (Finkel et al., 1992). Studies have shown TNFα decreases muscle protein synthesis while increasing muscle protein

proteolysis (Charters and Grimble, 1989; Flores et al., 1989). The increase in TNFα is not only seen in CHF in humans. A study has shown the SHHF strain of animals secrete significantly more TNFα than the control group (Bergman et al., 1999). The fact that the amount of this cytokine circulating in the blood increases in CHF patients and causes catabolic effects on muscle protein makes it a prime candidate for further testing to find out the exact effect of TNFα and to see if preventative measures can be taken in order to protect the heart and skeletal muscle.

In all of the calcium handling proteins that showed a significant reduction in amount between control and SHHF (RyR1, DHPR and SERCA1), a greater percentage reduction occurred in the diaphragm than in the gastrocnemius. The reason for this is most likely due to the difference in function of the muscle. With CHF, there is a buildup of fluid on the lungs. This additional load puts added stress on the diaphragm during respiration. Similar effects should not occur in the gastrocnemius as this muscle is used for mobility and there is no added stress on the muscle. Thus, differences in the responses of the diaphragm and gastrocnemius during CHF are likely due to the added stress placed on the former.

Conclusion

Using a genetic model of CHF (SHHF), changes in calcium handling occurred. The amount of calcium handling proteins, RyR1, DHPR and SERCA 1, in both the gastrocnemius and diaphragm, and Csq in the diaphragm was

significantly affected by CHF. However, the amounts of SERCA 2 and CaM in the gastrocnemius and diaphragm, and Csq in the gastrocnemius showed no significant difference in the SHHF group.

There is still no clear picture of the relationship between CHF and calcium handling, calcium handling proteins and sarcoplasmic reticulum function. It has been shown that calcium handling in a CHF model has been altered and the main calcium handling proteins are affected in a way that does explain those changes. The signals causing the changes are still unknown. There are other proteins that could be altered that have not been explored. Different muscles also may respond differently based on their function. The present study is a small step in the research that could lead to many more steps helping to find a way to fight the fatigue associated with CHF.

Research Hypotheses Conclusions

The null hypotheses H_{O1}, H_{O2}, and H_{O3} were rejected for both the gastrocnemius and diaphragm samples, as the amounts of RyR1, DHPR and SERCA 1 were significantly different between conditions ($p<0.05$). The null hypotheses H_{O4} and H_{O6} were not rejected for both the gastrocnemius and diaphragm samples, as the amounts of SERCA 2 and CaM were not significantly different between conditions ($p\geq0.05$). The null hypothesis H_{O5} was rejected for the diaphragm samples, as the amount of Csq was significantly different between conditions ($p<0.05$), but was not rejected for the gastrocnemius samples, as the amount of Csq was not significantly different between conditions ($p\geq0.05$)

Future Directions

The following are directions for future research in the area of calcium handling and CHF.

1. Determine if changes in the amount of cardiac isoforms of calcium handling proteins occur in CHF samples.
2. Determine the effects of CHF on other regulatory proteins, such as sarcolipin and parvalbumin, involved in calcium handling.
3. Determine the calcium uptake rates in CHF muscle after minimizing both the calcium leakage through the membrane and calcium release by way of the RyR.
4. Determine the physical activity levels of the SHHF model and control.
5. Determine if the same pattern of change in calcium handling proteins occurs in other muscles.
6. Determine the effect of TNFα on calcium handling and calcium handling proteins in the skeletal muscle.

	RyR1	DHPR($\alpha 1_S$ Subunit)	SERCA 1	SERCA 2	Csq	CaM
Gastrocnemius	? 64%	? 82%	? 57%	NS	NS	NS
Diaphragm	? 84%	? 100%	? 94%	NS	? 146%	NS

Table 9: Relative differences in protein amounts between control and SHHF samples. Values are calculated as (SHHF-Control)/Control *100. Values given represent a significance of $p<0.05$. NS represents no significance.

Chapter 6:

References

Bastide, B., A. Conti, V. Sorrentino, and Y. Mounier. Properties of ryanodine receptor in rat muscles submitted to unloaded conditions. *Biochem. Biophys. Res. Commun.* 270: 442-447, 2000.

Belcastro, A. N. Skeletal muscle calcium-activated neutral protease (calpain) with exercise. *J. Appl. Phsyiol.* 74: 1381-1386, 1993.

Benge, J. W., R. L. Litchfield, and M. L. Marcus. Exercise capacity in patients with severe left ventricular dysfunction. *Circulation*. 61: 955-959, 1980.

Bergman, M. R., R. H. Kao, S. A. McCune, and B. J. Holycross. Myocardial tumor necrosis factor-α secretion in hypertensive and heart failure-prone rats. *Am. J. Physiol.* 277: H543-H550, 1999.

Bernocchi, P., C. Ceconi, P. Pedersini, E. Pasini, S. Curello, and R. Ferrari. Skeletal muscle metabolism in experimental heart failure. *J. Mol. Cell. Cardiol.* 28: 2263-2273, 1996.

Braunwald, E. Pathophysiology of heart failure. In: *Heart disease. A textbook of cardiovascular medicine,* Edited by E. Braunwald. Philadelphia: W.B. Saunders Company, pp. 394-418, 1997.

Charters, Y., and R. F. Grimble. Effect of recombinant human tumor necrosis factor alpha on protein synthesis in liver, skeletal muscle and skin of rats. *Biochem. J.* 258: 493-497, 1989.

Danon, M. J., G. Karpati, J. Charuk, and P. Holland. Sarcoplasmic reticulum adenosine triphosphatase deficiency with probable autosomal dominant inheritance. *Neurology* 38: 812-815, 1988.

Drexler, H., U. Banhardt, T. Meinertz, H. Wollschlager, M. Lehmann, and H. Just. Contrasting peripheral short-term and long-term effects of converting enzyme inhibition in patients with congestive heart failure. A double-blind, placebo-controlled trial. *Circulation*. 79: 491-502, 1989.

Favero, T. G., I. N. Pessah, and G. A. Klug. Prolonged exercise reduces Ca^{2+} release in rat skeletal muscle sarcoplasmic reticulum. *Pflugers Arch.* 422: 472-475, 1993.

Fill, M., R. Coronado, J. R. Mickelson, J. Vilven, J. J. Ma, B. A. Jacobson, and C. F. Louis. Abnormal ryanodine receptor channels in malignant hyperthermia. *Biophys. J.* 57: 471-475, 1990.

Finkel, M. S., C. V. Oddis, T. D. Jacob, S. C. Watkins, B. G. Hattler, and R. L. Simmons. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science*. 257: 387-389, 1992.

Flores, E. A., B. R. Bistrian, J. J. Pomposelli, C. A. Dinarello, G. L. Blackburn, and N. W. Istfan. Infusion of tumor necrosis factor/cachectin promotes muscle catabolism in the rat. *J. Clin. Invest.* 83: 1614-1622, 1989.

Franciosa, J. A., M. Park, and T. B. Levine. Lack of correlation between exercise capacity and indexes of resting left ventricular performance in heart failure. *Am. J. Cardiol.* 47: 33-39, 1981.

Franzini-Armstrong, C. The sarcoplasmic reticulum and the control of muscle contraction. *FASEB J.* 13(Suppl): S266-S270, 1999.

Froemming, G. R., B. E. Murray, S. Harmon, D. Pette, and K. Ohlendieck. Comparative analysis of the isoform expression pattern of calcium regulatory membrane proteins in fast-twitch, slow-twitch, cardiac, neonatal, and chronic low-frequency stimulated muscle fibers. *Biochim. Biophys. Acta.* 1466: 151-168, 2000.

Groh, S., I. Marty, M. Ottolia, G. Prestipino, A. Chapel, M. Villaz, and M. Ronjat. Functional interaction of the cytoplasmic domain of triadin with the skeletal ryanodine receptor. *J. Biol. Chem.* 274: 12278-12283, 1999.

Gronert, G.A. Malignant hyperthermia. In: *Myology. Basic and Clinical* (2nd ed.), Edited by A. G. Engel, and C. Franzini-Armstrong. New York: McGraw-Hill, pp. 1661-1678, 1994.

Hamilton, S. L., I. Serysheva, and G. M. Strasburg. Calmodulin and excitation-contraction coupling. *News in Physiological Sciences.* 15: 281-284, 2000.

Harrington, D., and A. J. Coats. Skeletal muscle abnormalities and evidence for their role in symptom generation in chronic heart failure. *Eur. Heart J.* 18: 1865-1872, 1997.

Hicks, A., K. Ohlendieck, S. O. Gopel, and D. Pette. Early functional and biochemical adaptations to low-frequency stimulation of rabbit fast-twitch muscle. *Am. J. Physiol.* 273: C297-C305, 1997.

Jondeau, G., S. D. Katz, L. Zohman, M. Goldberger, M. McCarthy, J. P. Bourdarias, and T. H. LeJemtel. Active skeletal muscle mass and cardiopulmonary reserve. Failure to attain peak arobic capacity during maximal bicycle exercise in patients with severe congestive heart failure. *Circulation*. 86: 1351-1356, 1992.

Kandarian, S. C., S. O'Brien, K. Thomas, L. Schulte, and J. Navarro. Regulation of skeletal muscle dihydropyridine receptor gene expression by biomechanical unloading. *J. Appl. Physiol.* 72: 2510-2514, 1992.

Kandarian, S. C., D. G. Peters, T. G. Favero, C. W. Ward, and J. H. Williams. Adaptations of the skeletal muscle calcium-release mechanism to weight-bearing condition. *Am. J. Physiol.* 270: C1588-C1594, 1996.

Kandarian, S. C., D. G. Peters, J. A. Taylor, and J. H. Williams. Skeletal muscle overload upregulates the sarcoplasmic reticulum calcium pump gene. *Am. J. Physiol.* 266: C1190-C1197, 1994.

Klug, G. A., and G. F. Tibbits. The effect of activity on calcium-mediated events in striated muscle. In: *Exercise and Sport Science Reviews*, Edited by K. B. Pandolf. New York: Macmillan, pp.1-59, 1988.

Kobayashi, Y. M., B. A. Alseikhan, and L. R. Jones. Localization and characterization of the calsequestrin-binding domain of Triadin 1. *J. Biol. Chem.* 275: 17639-17646, 2000.

Levine, B., J. Kalman, L. Mayer, H. M. Fillit, and M. Packer. Elevated circulating levels of tumor necrosis factor in sever chronic heart failure. *N. Engl. J. Med.* 223: 236-241, 1990.

Lipkin, D., D. Jones, J. Round, and P. PooleWilson. Abnormalities of skeletal muscle in patients with chronic heart failure. *Int. J. Cardiol.* 18: 187-195, 1988.

Loirat, M. J., B. Lucas-Heron, B. Ollivier, and C. Leoty. Calcium binding protein changes of sarcoplasmic reticulum from rat denervated skeletal muscle. *Biosci. Rep.* 8: 369-378, 1988.

Lunde, P. K., A. J. Dahlstedt, J. D. Bruton, J. Lännergren, P. Thorén, O. M. Sejersted, and H. Westerblad. Contraction and intracellular Ca^{2+} handling in isolated skeletal muscle of rats with congestive heart failure. *Circ. Res.* 88: 1299-1305, 2001a.

Lunde, P. K., I. Sjaastad, H. M. Schiøtz Thorud, and O. M. Sejersted. Skeletal muscle disorders in heart failure. *Acta Physiol. Scand.* 171: 277-294, 2001b.

Lunde, P. K., T. Tønnesen, G. Christensen, and O. M. Sejersted. Skeletal muscle membrane proteins in ischemic heart failure in rats. *N. Y. Acad. Sci. Meeting*. PII-13 (Abstract), 1997.

Lytton, J., M. Westlin, S. E. Burk, G. E. Shull, and D. H. MacLennan. Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *J. Biol. Chem.* 267: 14483-14489, 1992.

Margreth, A., E. Damiani, and E. Bortoloso. Sarcoplasmic reticulum in aged skeletal muscle. *Acta Physiol. Scand.* 1167: 331-338, 1999.

McCune, S. A., P. B. Baker, and H. F. Stills. SHHF/Mcc-cp rat: model of obesity, non-insulin-dependent diabetes and congestive heart failure. *ILAR News.* 32: 23-27, 1990.

McMurray, J., I. Abdullah, H. J. Dargie, and D. Shapiro. Increased concentrations of tumor necrosis factor in “cachectic” patients with severe chronic heart failure. *Br. Heart J.* 66:356-358, 1991.

Minotti, J. R., I. Christoph, R. Oka, M. W. Weiner, L. Wells, and B. M. Massie. Impaired skeletal muscle function in patients with congestive heart failure. *J. Clin. Invest.* 88: 2077-2082, 1991.

Ohlendieck, K., F. N. Briggs, K. F. Lee, A. W. Wechsler, and K. P. Campbell. Analysis of excitation-contraction-coupling components in chronically stimulated canine skeletal muscle. *Eur. J. Biochem.* 202: 739-747, 1991.

Ohlendieck, K., G. R. Froemming, B. E. Murray, P. B. Maguire, E. Leisner, I. Traub, and D. Pette. Effects of chronic low-frequency stimulation on calcium regulatory membrane proteins in rabbit fast muscle. *Pflugers Arch*. 438: 700-708, 1999.

Péréon, Y., J. Navarro, M. Hamilton, F. W. Booth, and P. Palade. Chronic stimulation differentially modulates expression of mRNA for dihydropyridine receptor isoforms in rat fast twitch skeletal muscle. *Biochem. Biophys. Res. Commun.* 235: 217-222, 1997a.

Péréon, Y., J. Navarro, V. Sorrentino, J. P. Louboutin, J. Noireaud, and P. Palade. Regulation of dihydropyridine receptor and ryanodine receptor gene expression in regenerating skeletal muscle. *Pflugers Arch.* 433: 221-229, 1997b.

Péréon, Y., V. Sorrentino, C. Dettbarn, J. Noireaud, and P. Palade. Dihydropyridine receptor and ryanodine receptor gene expression in long-term denervated rat muscles. *Biochem. Biophys. Res. Commun.* 240: 612-617, 1997c.

Perreault, C. L., H. Gonzalez-Serratos, S. E. Litwin, X. Sun, C. Franzini-Armstrong, and J. P. Morgan. Alterations in contractility and intracellular Ca^{2+} transients in isolated bundles of skeletal muscle fibers from rats with chronic heart failure. *Circ. Res.* 73: 405-412, 1993.

Peters, D. G., H. L. Mitchell, S. A. McCune, S. Park, J. H. Williams, and S. C. Kandarian. Skeletal muscle sarcoplasmic reticulum calcium ATPase gene expression in congestive heart failure. *Circ. Res.* 81: 703-710, 1997.

Rodney, G. G., B. Y. Williams, G. M. Strasburg, K. Beckingham, and S. L. Hamilton. Regulation of RyR1 activity by calcium and calmodulin. *Biochemistry.* 39: 7807-7812, 2000.

Schulte, L. M., J. Navarro, and S. C. Kandarian. Regulation of sarcoplasmic reticulum calcium pump gene expression by hindlimb unweighting. *Am. J. Physiol.* 264: C1308-C1315, 1993.

Simonini, A., K Chang, P Yue, C. S. Long, and B. M. Massie. Expression of skeletal muscle sacroplasmic reticulum calcium-ATPase is reduced in rats with postinfarction heart failure. *Heart.* 81: 303-307, 1999.

Simonini, A., C. S. Long, G. A. Dudley, P. Yue, J. McElhinny, and B. M. Massie. Heart failure in rats causes changes in skeletal muscle morphology and gene expression that are not explained by reduced activity. *Circ. Res.* 79: 128-136, 1996.

Stephenson, D. G., G. D. Lamb, and G. M. M. Stephenson. Events of the excitation-contraction-relaxation (E-C-R) cycle in fast- and slow-twitch mammalian muscle fibres relevant to muscle fatigue. *Acta Phsiol. Scand.* 162: 229-245, 1998.

Strange, M., A. Tripathy, and G. Meissner. Two domains in dihydropyridine receptor activate the skeletal muscle Ca^{2+} release channel. *Biophys. J.* 81: 1419-1429, 2001.

Spangenburg, E. E., S. J. Lees, J. S. Otis, T. I. Musch, R. J. Talmadge, and J. H. Williams. Effects of moderate heart failure and functional overload on rat plantaris muscle. *J. Appl. Physiol.* 92: 1-7, 2002.

Szegedi, C., S. Sárközi, A. Herzog, I. Jóna, and M. Varsányi. Calsequestrin: more than ‘only’ a luminal calcium buffer inside the sarcoplasmic reticulum. *Biochem. J.* 337: 19-22, 1999.

Vescovo, G., F. Serafini, P. Tenderini, U. Carraro, L. Dalla Libera, C. Catani, and G. B. Ambrosio. Specific changes in skeletal muscle myosin heavy chain composition in cardiac failure: differences compared with disuse atrophy as assessed in microbiopsies by high resolution electrophoresis. *Heart.* 76: 337-343, 1996.

Volterrani, M., A. L. Clark, P. F. Ludman, J. W. Swan, S. Adamopoulos, M. Piepoli, and A. J. S. Coats. Predictors of exercise capacity in chronic heart failure. *Eur. Heart J.* 15: 801-809, 1994.

Williams, J. H and G. A. Klug. Calcium exchange hypothesis of skeletal muscle fatigue. *Muscle Nerve.* 18: 421-434, 1995.

Williams, J. H. and C. W. Ward. Changes in skeletal muscle sarcoplasmic reticulum function and force production following myocardial infarction in rats. *Exp. Physiol*. 83: 85-94, 1998.

Wilson, J. R., J. L. Martin, and N. Ferraro. Impaired skeletal muscle nutritive flow during exercise in patients with congestive heart failure: role of cardiac pump dysfunction as determined by the effect of dobutamine. *Am. J. Cardiol.* 53: 1308-1315, 1984.

Wilson, J. R., J. L. Martin, N. Ferraro, and K. T. Weber. Effect of hyralazine on perfusion and metabolism in the leg during upright bicycle exercise in patients with heart failure. *Circulation*. 68: 425-432, 1983.

Wu, K. D. and J. Lytton. Molecular cloning and quantification of sarcoplasmic reticulum Ca2+-ATPase isoforms in rat muscles. *Am. J. Physiol.* 264: C333-C341, 1993.

Wu, Y., B. Aghdasi, S. J. Dou, J. Z. Zhang, S. Q. Liu, and S. L. Hamilton. Functional interactions between the cytoplasmic domains of the skeletal muscle Ca^{2+} release channel. *J. Biol. Chem.* 272: 25051-25061, 1997.

Zubrzycka-Gaarn, E., B. Korczak, H. Osinska, and M.G. Sarsala. Studies on sarcoplasmic reticulum from slow-twitch muscle. *J. Muscle Res. Cell Motil.* 3: 191-212, 1982.

Appendix A:

Data

Total RyR1

	Gastrocnemius	
Samples	SHHF	Control
(2009;2010)	0.204	1.000
(2011;2012)	0.000	0.921
(2013;2014)	0.468	1.179
(2015;2016)	0.658	0.554
Mean	0.332	0.913
SEM	0.145	0.132
t-test		0.025

	Diaphragm	
Samples	SHHF	Control
(2009;2010)	0.105	1.000
(2011;2012)	0.000	1.048
(2013;2014)	0.227	1.203
(2015;2016)	0.353	1.088
Mean	0.171	1.085
SEM	0.076	0.043
t-test		0.000

RyR1

	Gastrocnemius	
Samples	SHHF	Control
(2009;2010)	0.110	0.636
(2011;2012)	0.000	0.569
(2013;2014)	0.195	0.761
(2015;2016)	0.392	0.445
Mean	0.174	0.603
SEM	0.083	0.066
t-test		0.007

	Diaphragm	
Samples	SHHF	Control
(2009;2010)	0.105	0.624
(2011;2012)	0.000	0.804
(2013;2014)	0.147	0.805
(2015;2016)	0.247	0.773
Mean	0.125	0.752
SEM	0.051	0.043
t-test		0.000

410 kDa Proteolytic Fragment

	Gastrocnemius	
Samples	SHHF	Control
(2009;2010)	0.094	0.364
(2011;2012)	0.000	0.353
(2013;2014)	0.273	0.418
(2015;2016)	0.266	0.108
Mean	0.158	0.311
SEM	0.067	0.069
t-test		0.164

	Diaphragm	
Samples	SHHF	Control
(2009;2010)	0.000	0.376
(2011;2012)	0.000	0.244
(2013;2014)	0.080	0.397
(2015;2016)	0.106	0.315
Mean	0.047	0.333
SEM	0.027	0.034
t-test		0.001

Table 10: Total RyR1, RyR1 and 410 kDa proteolytic fragment raw data. Optical densities for the Western blots of individual SHHF and control gastrocnemius and diaphragm samples for total RyR1, RyR1, and the 410 kDa proteolytic fragment using the 34C clone antibody. (Refer to Figure 3 for presentation of data)

Samples	*Gastrocnemius*	
	SHHF	Control
(2009;2010)	0.000	1.000
(2011;2012)	0.000	0.813
(2013;2014)	0.221	1.062
(2015;2016)	0.322	0.201
Mean	0.136	0.769
SEM	0.081	0.197
t-test		0.025

Samples	*Diaphragm*	
	SHHF	Control
(2009;2010)	0.000	1.000
(2011;2012)	0.000	1.971
(2013;2014)	0.000	1.596
(2015;2016)	0.000	0.947
Mean	0.000	1.379
SEM	0.000	0.246
t-test		0.001

Table 11: DHPR raw data. Optical densities for the Western blots of individual SHHF and control gastrocnemius and diaphragm samples for DHPR using the 1A clone antibody. (Refer to Figure 4 for presentation of data)

Total SERCA

	Gastrocnemius	
Samples	SHHF	Control
(2009;2010)	0.587	1.000
(2011;2012)	0.284	0.657
(2013;2014)	0.689	1.126
(2015;2016)	0.625	0.991
Mean	0.546	0.943
SEM	0.090	0.100
t-test		0.026

	Diaphragm	
Samples	SHHF	Control
(2009;2010)	0.579	1.000
(2011;2012)	0.000	0.887
(2013;2014)	0.816	1.876
(2015;2016)	0.807	1.718
Mean	0.551	1.370
SEM	0.192	0.250
t-test		0.040

SERCA 1

	Gastrocnemius	
Samples	SHHF	Control
(2009;2010)	0.327	1.000
(2011;2012)	0.445	1.091
(2013;2014)	0.475	1.252
(2015;2016)	0.475	0.694
Mean	0.430	1.009
SEM	0.035	0.117
t-test		0.003

	Diaphragm	
Samples	SHHF	Control
(2009;2010)	0.000	1.000
(2011;2012)	0.000	3.124
(2013;2014)	0.000	2.416
(2015;2016)	0.527	2.615
Mean	0.132	2.289
SEM	0.132	0.455
t-test		0.004

SERCA 2

	Gastrocnemius	
Samples	SHHF	Control
(2009;2010)	0.615	1.000
(2011;2012)	0.822	0.740
(2013;2014)	0.154	0.510
(2015;2016)	0.644	0.697
Mean	0.559	0.737
SEM	0.143	0.101
t-test		0.348

	Diaphragm	
Samples	SHHF	Control
(2009;2010)	0.000	1.000
(2011;2012)	0.000	0.000
(2013;2014)	1.082	1.155
(2015;2016)	1.391	1.509
Mean	0.618	0.916
SEM	0.362	0.323
t-test		0.562

Table 12: Total SERCA, SERCA 1 and SERCA 2 raw data. Optical densities for the Coomassie Blue stained gel and Western blots of individual SHHF and control gastrocnemius and diaphragm samples for total SERCA, SERCA 1 and SERCA 2. SERCA 1 and SERCA 2 blots were done using IIH11 clone and 2A7-A1 clone antibodies respectively. (Refer to Figure 5 for presentation of data)

Samples	*Gastrocnemius*	
	SHHF	Control
(2009;2010)	0.408	1.000
(2011;2012)	0.871	1.014
(2013;2014)	0.713	1.017
(2015;2016)	1.024	0.612
Mean	0.754	0.911
SEM	0.132	0.100
t-test		0.379

Samples	*Diaphragm*	
	SHHF	Control
(2009;2010)	2.058	1.000
(2011;2012)	1.231	0.793
(2013;2014)	2.179	0.725
(2015;2016)	2.053	0.535
Mean	1.880	0.763
SEM	0.218	0.096
t-test		0.003

Table 13: Csq raw data. Optical densities for the stains-all gel lanes of individual SHHF and control gastrocnemius and diaphragm samples for Csq. (Refer to Figure 6 for presentation of data)

Samples	*Gastrocnemius*	
	SHHF	Control
(2009;2010)	0.953	1.000
(2011;2012)	1.128	1.113
(2013;2014)	0.974	0.847
(2015;2016)	1.048	0.942
Mean	1.026	0.976
SEM	0.040	0.056
t-test		0.490

Samples	*Diaphragm*	
	SHHF	Control
(2009;2010)	1.560	1.000
(2011;2012)	1.697	1.703
(2013;2014)	1.387	1.052
(2015;2016)	1.811	1.538
Mean	1.614	1.323
SEM	0.091	0.175
t-test		0.192

Table 14: CaM Raw data. Optical densities for the Western blots of individual SHHF and control gastrocnemius and diaphragm samples using the 6D4 clone antibody. (Refer to Figure 7 for presentation of data)

Appendix B:

Calcium Uptake and Release Data

Calcium Uptake, Release Rates and ATPase Activities

Previous calcium uptake and release rate measurements (E. Spangenburg and M. Stockunas, unpublished data) revealed changes in both rates of congestive heart failure rats as opposed to the control animals. In the gastrocnemius, the net calcium uptake rate was 25.5% more in the SHHF rats as compared to the controls. The net calcium release rates, however, were 35.9% less in the SHHF rats when compared to the controls. In the diaphragm, the net calcium release rate was 20.6% less for the SHHF rats as compared to the control. Yet with the net calcium uptake rate, 33.8% less was seen in the SHHF animals, showing a difference from the gastrocnemius in which the rate increased.

The direction of the change in the net calcium uptake rates was different in the gastrocnemius (increase) when compared to the diaphragm (decrease). Yet with both of the muscles, a reduction in ATPase activity was seen. In the gastrocnemius of the SHHF animals, a 37.2% reduction in ATPase activity was observed. In the diaphragm of the SHHF animals the reduction of ATPase activity was 34.1%.

Gastrocnemius

	Uptake Rate (mmol/mg/min)		*Release Rate (mmol/mg/min)*		*ATPase Activity (mmol/mg/min)*	
	Control	*SHHF*	*Control*	*SHHF*	*Control*	*SHHF*
Mean	2.718	3.403	2.833	1.815	3.122	1.960
SEM	0.076	0.091	0.144	0.194	0.328	0.156
p-value		0.023		0.008		0.010

Diaphragm

	Uptake Rate (mmol/mg/min)		*Release Rate (mmol/mg/min)*		*ATPase Activity (mmol/mg/min)*	
	Control	*SHHF*	*Control*	*SHHF*	*Control*	*SHHF*
Mean	3.128	2.072	2.184	1.731	3.030	1.997
SEM	0.123	0.121	0.111	0.049	0.112	0.046
p-value		0.000		0.000		0.000

Table 15: Summary of calcium uptake rates, release rates and ATPase activities for the SHHF and Control gastrocnemius and diaphragm samples. (E. Spangenburg and M. Stockunas, unpublished data)

Curriculum Vitae

Emily Elizabeth Allen was born on March 19,1978 in Charlottesville, Virginia. After graduating from Liberty High School in Bedford, Virginia in 1996, she began her college career at Virginia Polytechnic Institute and State University. In May of 2000, she graduated summa cum laude with a Bachelor of Science degree in science of food, nutrition and exercise from the department of Human Nutrition, Foods and Exercise. She spent the next two years at Virginia Tech working to obtain a Master of Science specializing in muscle physiology and biochemistry. After graduation, she plans to move to Richmond and enter the work force.