Chapter 3:

Methodology

Experimental designs.

Study 1. The objective of this study was to determine which muscles undergo changes in MHC isoform expression in response to CHF and to determine if these changes are specific to the severity of the disease. This study incorporated the use of the rat myocardial infarction model to study the changes in MHC isoform composition of the plantar flexor muscles during CHF. Thirteen rats underwent surgical ligation of the coronary artery, while 7 rats were subjected to all surgical procedures except the ligation and served as sham-operated control animals (SHAM). After 4 weeks, which has been shown to be a sufficient amount of time for the animals to develop CHF using the coronary ligation model (Musch and Terrell 1992), the animals were euthanized. A portion of this study was done in collaboration with Dr. Tim Musch, who performed the CHF surgeries and collected all of the myocardial function data at Kansas State University. The CHF animals were subsequently divided into moderate CHF and severe CHF based on myocardial performance data. The muscles of the plantar flexor group (i.e soleus, plantaris, red gastrocnemius (RG) and white gastrocnemius (WG) were removed and frozen. The MHC isoform composition was then determined for each individual muscle using SDS-PAGE procedures. Correlations were then preformed to determine relationships between peripheral muscle MHC isoform composition and the severity of CHF.

Study 2. This study examined a possible intervention that would prevent or attenuate the skeletal muscle changes, which occur as a result of CHF. This study used the same myocardial infarction model in combination with a skeletal muscle functional overload (FO) model. This FO model (also called synergist ablation or compensatory hypertrophy) has been used to study functional and structural changes, which occur to the muscle during hypertrophy. This model causes skeletal muscle hypertrophy by removal of a synergist muscle (*ex.* gastrocnemius) thereby causing the remaining musculature (*i.e.* the soleus and plantaris) to be placed in an overloaded state (Roy et al. 1997, Sugiura et al. 1993). The effects, which are imposed by the FO model (i.e., a fast to slow phenotypic shift) on the remaining musculature, are in direct contrast to the reported effects of CHF on skeletal muscle (i.e. a slow to fast phenotypic shift) (Roy et al. 1997). So, this study examined changes in skeletal muscle structure and function after imposition of CHF and FO.

Twenty-three adult female rats were subjected to surgically induced myocardial infarctions and 13 additional rats were subjected to SHAM surgeries. The animals were then allowed to recover for three weeks and 16 CHF and 8 SHAM animals were subjected to unilateral FO surgeries. A unilateral FO model was chosen because the myocardial infarction model results in variable severity of CHF. Therefore, this design allows for each animal to have its own non-FO limb and FO limb or an internal control. The remaining animals served as control animals to ensure that the non-ablated leg of the FO animals was indicative of a non-operated control limb. The animals remained in their cages for 9 weeks, which has been shown to be sufficient time to allow for significant muscle hypertrophy. The animals were then euthanized, their heart, lungs, soleus, and

plantaris were removed and prepared for the latter experiments. These experiments included examining the entire muscle for changes in MHC isoform composition and SERCA expression, changes in Ca^{2+} uptake rates, and changes in individual fiber MHC and SERCA isoform protein content.

The animals were infected by and recovered from an unexpected exposure to sialodacryoademitis (SDA) virus. This virus can cause the animal to lose weight due to reduction in appetite. However, at no time after the infection did the animals demonstrate loss of body weight. Also, the virus infected all of the animals, so it is unlikely that the virus had a significant impact on the results of the study.

Detailed Methodology.

Animal care and surgery: Female Sprague-Dawley (~200 gm) rats were used for all experiments. All animals were individually housed in the Virginia Tech Animal Care Facility, where they were monitored daily for any complications. Each animal was provided rat chow (Tekland 7001) and water *ad libitum.* The Virginia Tech Animal Care Committee approved all procedures prior to the initiation of the study.

Animal Model. For all experiments, the rat coronary ligation model to induce CHF was used. Female Sprague-Dawley (~200 gm) rats were anesthetized with 2% halothane and 3 L/min of O_2 using a gas anesthesia machine. The rats were then placed on a rodent ventilator (Harvard Apparatus). A left lateral thoracotomy was performed, the pericardium was incised and the heart was isolated. The left coronary artery was

ligated with 6-0 Tri-cron™ suture, while the sham animals underwent the same procedures except the artery was not ligated. The heart was checked for damage other than the effects of ligation, and gently placed back into the chest. The intercostal muscles and the incision in the intercostal space was closed in a single layer using 3.0 silk suture in a single uninterrupted pattern. The animal was given a prophylactic dose of 90 kU dose of penicillin G i.m.. The animal was also given a 0.3 ml dose of lidocaine HCl i.p. to help alleviate pain and reduce the number of arrhythmias associated with the surgeries. The animals were checked daily for the next week to assess and respond to any post-surgical complications. There was a 58% survival rate immediately after the surgical operation, which is higher than other investigations. Another 16% died within the first 24 h after the surgery. No other deaths occurred after the first postoperative day.

For study #2, the ablation surgeries were preformed approximately 3 weeks post-infarction. The FO surgeries were performed according to the methodology of Sugiura et al. (1993). The animals were anesthetized with 1.5% halothane and 3L/min O_2 using a gas anesthesia machine. Then under aseptic conditions, an incision was made through the skin on the posterior aspect of the animal's lower leg. The lower portion of the hamstrings were subsequently separated to allow access to gastrocnemius. Then the distal tendon of the gastrocnemius was transected and approximately half of the muscle was isolated and removed from the surrounding tissue. The skin and hamstrings were sutured closed with 3-0 silk suture. The animals were then returned to their cages for nine weeks, which has been shown to be ample time to allow for adaptation of both the soleus and plantaris (Sugiura et al. 1993). The animals were observed daily for sudden changes in health and/or monitoring of body weight.

Tissue processing. Nine weeks post-ablation the animals were anesthetized with an i.p. injection of sodium pentobarbital (60 mg/kg). The plantaris muscles of both limbs were then subsequently removed, blotted dry, and weighed. The muscle was then cut in half, with one half used for SR function and SERCA measurements and the other half placed in melting isopentane cooled by liquid nitrogen. The first half of the muscle was homogenized in a buffer consisting of 20 mM HEPES (pH 7.4), 250 mM sucrose, 0.2% sodium azide, and 0.2 mM phenylmethylsulfonylfluoride (PMSF) in a ratio of 10:1 (vol:weight) (Ward et al. 1998). The homogenate was then centrifuged for 10 min. at 1600g at 4°C. The supernatant was removed and stored at –80°C. A portion of this supernatant was used for SERCA isoform determination and the other portion of the supernatant was used for SR Ca^{2+} uptake measurements (see below). Protein concentrations were determined via the Bradford methodology (Bradford, M. M. 1976). The frozen portion of the muscle was stored at –80° C for immunohistochemistry. The heart and lungs were removed trimmed free of connective tissue, weighed and placed into 10% buffered formalin. The animals were euthanized with an additional overdose injection (65 mg/kg, i.p. injection) of sodium pentobarbital.

Determination of Infarct Size and Ventricular Mass. Determination of the changes in heart mass and infarct size were determined according to the methods of Pfeffer et al. (1978). The right and left ventricles were carefully dissected, separated, and weighed. They were then placed back into the 10% buffered formalin. The left ventricle was cut into three transverse sections and processed for histological studies. Sections 10μm thick

were cut, mounted, and stained with Masson's trichrome. The sections were then projected onto a monitor where the percent of the left ventricle circumference containing the infarcted region was determined. This was accomplished by magnifying the slide with a camera (Nikon) and projecting the picture onto a video monitor. The segment of infarcted ventricle was then calculated using a protractor. The fraction of the left ventricle that was infarcted vs. the amount non-infarcted was then calculated from these measurements.

Western Blots for SERCA. The methods were performed according to the modified methodology of Kandarian et al. (1994). For the blotting, the proteins were first electrophoretically separated on 7.5% SDS-polyacrylamide gels according to the methods of Laemmli (1970). Proteins were then transferred to nitrocellulose membranes with transfer buffer (20% methanol) by subjecting them to a high-intensity electrical field for 2.5 hrs at 5°C (Towbin et al.1979). The blots were then blocked for 1 h with 5% non-fat dry milk and incubated overnight with the primary antibody against slow/cardiac Ca^{2+} pump (2A7-A1) (1:500) or the fast skeletal Ca^{2+} pump (IIH11) (1:2500) at 5° C.
The blots were incubated in secondary antibody, an anti-mouse IgG conjugated to alkaline phosphatase (Sigma) (1:30,000). Color development was performed using bromochloroindolyl phosphatase-nitro blue tetrazolium as a substrate for alkaline phosphatase. The bands were scanned using a video densitometer (Alpha Innotech Image Analysis System). Each blot also contained a homogenized EDL sample, known to only express SERCA 1a, or a homogenized heart sample, known to only express SERCA 2a

(Wu and Lytton, 1993). This allowed each plantaris sample to be normalized by the heart or EDL sample, providing semi-quantification of protein content.

SR homogenate Ca^{2+} transient measurements. The SR Ca^{2+} uptake measurements were measured using a homogenate fraction according to the methods of Ward et al. (1998). These methods have been shown to be reproducible and reliable when compared to isolated SR vesicles (Ward et al. 1998). Briefly, 250 μg of homogenate protein was added to 1 ml of buffer containing, 100 mM KCl, 20 mM HEPES, 5.0 mM MgCl, 2.5 mM ATP and 5.0 mM oxalate (pH 7.0). Uptake was initiated by the addition of 1.0 μl of 40 mM CaCl and continued until no change in extravesicular free Ca^{2+} is observed. This addition resulted in a free $[Ca^{2+}]$ of 1.6-1.8 μM. Temperature was maintained at 37°C and the buffer constantly stirred with a micro stir bar. Extravesicular free Ca^{2+} was measured using the fluorescent Ca^{2+} indicator fura-2 and a JASCO fluorometer. Excitation light was filtered at 340 (F_{340}) and 380 nm (F_{380}) and emission fluorescence was detected at 500 nm. The data were continuously recorded and stored via micro-computer for subsequent analysis. Fluorescence ratios were used to calculate free $[Ca^{2+}]$ in the incubation medium according to the methods of Grynkiewicz et al. (1985), using the following equation: $[Ca^{2+}]_{free} = K_d \times \beta \times (R-R_{min})/(R-R_{max})$. The K_d of Fura-2 was 70 nM (Spangenburg and Williams, unpublished observations). R_{min} and R_{max} are the R values measured in the uptake buffer with 10 mM EGTA or with 1 mM Ca^{2+} added, respectively. β is the ratio F_{350} recorded in the EGTA and Ca^{2+} supplemented buffers. The rates of Ca^{2+} uptake were calculated from the steepest portion of the negative slope of the free $[Ca^{2+}]$ versus time curve and then normalized to the protein concentration.

Whole muscle MHC expression. These techniques were performed according to Talmadge and Roy (1993). SDS-PAGE was used to separate isolated myofibrils, which were obtained a myofibrillar pellet. This pellet was obtained after the 1600g spin for the SR homogenate isolation and resuspended for MHC analysis. The protein concentration was determined by the Bradford protocol. The samples were then placed in sample buffer (0.25 ml 2-β-mercaptoethanol, 10% SDS, 30% glycerol, 0.5 M Tris-HCl, pH 6.8, and 0.5% bromophenol blue) at a final concentration of 0.125 mg/ml and boiled for 2 minutes. The MHC isoforms were separated by use of a glycerol based SDS-PAGE gel composed of a 4% stacking gel and 8% separating) gel. Approximately 2.5-3.0 μg of protein was loaded per lane. The gels were run for 25 h at 6.0 mA (4°C). The gel was then stained with Coomassie blue overnight, destained and scanned using a densitometer (Alpha Innotech Image Analysis System) to determine the relative proportions of MHC isoforms in each muscle.

Immunohistochemistry and individual fiber size. Serial sections from the deep region of the plantaris were cut in a cryostat (Microm HM 505 N) maintained at –20°C. The 10 μm thick sections were placed on gelatin coated slides. The immunohistochemistry was preformed according to the techniques of Talmadge et al. (1999). The sections were exposed to mouse monoclonal antibodies specific to the various isoforms of MHC and SERCA of the rat (see Table 1 for corresponding antibody and protein isoform). The sections were incubated in the primary antibody for 1.5 hrs at 37°C. The avidin-biotin procedure was used to amplify the antigen-antibody complex (Vectastain ABC kits,

Vector Laboratories). The slides were exposed to the secondary antibody for 1 hr at room temperature and then subsequently washed repeatedly. The sections were then incubated in the ABC reagent for 1 hr, washed again, and then exposed to a DAB reaction mixture. After staining, ~100 random fibers from each muscle were followed through each section using video images generated off a microscope (Nikon e400) and the Scion image program on a microcomputer. A fiber, which showed a positive reaction, was considered to be positive for that particular primary antibody. Any mention of specific fiber types from here onward is referring to individual muscle fibers classified by their MHC expression. Individual muscle fibers positive for different isoforms of SERCA, will be labeled as SERCA isoform positive fibers.

Fiber size or cross sectional area (CSA) was measured according to the techniques of Grossman et al. (1998). Briefly, the fibers corresponding to the previously 100 chosen fibers were manually outlined in a blank section using an image processing system (Scion image). The calibrated system allows for determination of the CSA of individual fibers. The data was subsequently stored on microcomputer for later analysis.

Statistics. All statistics were preformed on the Sigma STAT package. For study 1, a two-way analysis of variance (ANOVA) with repeated measures was used to determined differences in each dependent variable between groups (SHAM, moderate CHF, and severe CHF) and MHC isoforms (I, IIa, IIx, IIb). For study 2, a two-way analysis of variance (ANOVA) with repeated measures were used to determine differences in each dependent variable between groups (SHAM, CHF) and treatment (FO, non-FO). Repeated measures were preformed across treatments. Differences were considered statistically significant at the $p<0.05$ level.

Table 1. Monoclonal antibodies used with immunohistochemistry for the identification of specific muscle fiber phenotypes from the deep medial region of plantaris.

Protein	Slow	Fast	71	35	F3	F1.652	VE12	IID8
MHC I	+	-	-	+	-	-	-	-
MHC IIa	-	+	+	+	-	-	-	-
MHC IIx	-	+	-	-	-	-	-	-
MHC IIb	-	+	-	+	+	-	-	-
Embryonic. MHC	-	-	+	+	-	+	-	-
SERCA 1	-	-	-	-	-	-	+	-
SERCA 2	-	-	-	-	-	-	-	+

* Each monoclonal antibody bound to specific MHC or SERCA protein isoforms: (+) positive, (-) negative