The Role of Heat Shock Protein 70 in Protecting Muscle Mechanical Function & SERCA Function in Human Skeletal Muscle

by

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A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Science

in

Kinesiology

Waterloo, Ontario, Canada, 2009

Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Two studies were conducted to determine if Hsp70 is able to protect human skeletal muscle from muscle mechanical damage and alterations in SERCA activity associated with prolonged concentric exercise. In the first study, one-legged isometric knee extension exercise at 40% MVC and a duty cycle of 50% (5 sec contraction followed by 5 sec of relaxation) was used to induce a heat shock response in one leg only. Participants were followed over six recovery days to determine the time course of Hsp70 induction and decay. Results showed fiber type specific increases in Hsp70 that persisted in one leg only throughout six days of recovery. These increases in Hsp70 occurred with only transient changes in Ca^{2+} uptake and muscular force. With the exception of minor decreases in low frequency force, there were no apparent reductions in muscular force or SERCA activity by the third recovery day. Therefore an exercise protocol was established which was able to induce a heat shock response with only minor alterations in muscle mechanical function and SERCA activity. In the second study, the same isometric exercise was employed, however, on the day corresponding to recovery day 3 in the first study, participants were asked to complete a one hour cycling protocol at 70% VO_2 max. The goal was to cause similar one-legged increases in Hsp70 as the first study and to then challenge SERCA activity and muscular force in the presence of elevated Hsp70 by using cycling exercise. Results showed cycling induced reductions in maximal Ca^{2+} ATPase activity, muscular force, rates of muscle relaxation, and rates of muscle force development were attenuated by the preconditioning (isometric) exercise. These studies confirm the idea that preconditioning exercise is able to attenuate subsequent exercise induced insults to SERCA activity and muscular force, likely through an Hsp70 mediated mechanism.

Acknowledgments

I would like to thank NSERC for funding my research, Dr. Tupling for his constant encouragement and enthusiasm, Eric Bombardier for his invaluable immunohistochemical work, and the rest of the students in the Tupling lab for their various intellectual and practical assistances.

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Chapter 1: Introduction

It is well known that exercise of various modes, intensities, and durations can cause muscle fatigue (3; 13; 27; 66; 73). The mechanisms responsible for fatigue depend largely on the aforementioned characteristics of the exercise. Higher intensity shorter duration exercise causes an acute fatigue brought about by the accumulation of metabolites such as ADP, H^+, Pi, lactate and creatine(9; 26). Lower intensity longer duration exercise, however, results in chronic muscle fatigue referred to as weakness; and is generally attributed to disturbances in excitation-contraction (E-C) coupling resulting in reduced calcium (Ca^{2+}) release by the sarcoplasmic reticulum (SR), for review see (2; 8; 16; 16; 56; 70).

The SR is a membranous Ca^{2+} storage organelle that is responsible for the sequestration of Ca^{2+} for muscle contraction. During one contraction cycle, Ca^{2+} is released from the SR into the cytosol by the calcium release channel, it binds to troponin C and muscle contraction can occur. Following muscle contraction, the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) re-sequesters Ca^{2+} back into the SR and muscle relaxation ensues. This cycle is controlled carefully by the proper handling of Ca^{2+} movement between the SR and the cytosol. During an acute bout of sustained contractile activity Ca^{2+} homeostasis is compromised, excitation-contraction coupling becomes compromised, and fatigue occurs(1). The mechanisms underlying disturbances in Ca^{2+} handling are complex and have not been fully elucidated. However, there is evidence suggesting that chronically elevated intracellular Ca^{2+} ($[Ca^{2+}]_i$) from repetitive muscle contraction physically disrupts excitation-contraction coupling(40). More recently, this theory has been supported by evidence showing that calpains, Ca^{2+} activated cysteine proteases, can disrupt the mechanical link between the SR calcium release channel and the voltage gated dihydropyridine

receptors(75). Furthermore, there is a large body of evidence suggesting that reductions in force generating capacity can be linked to reductions in the activity of SERCA(15; 41; 73).

SERCA is a 110 kDa transmembrane protein responsible for the energy dependant sequestration of Ca^{2+} into the SR. In one ideal catalytic cycle, SERCA pumps 2 Ca^{2+} into the SR for every ATP consumed. SERCA is highly active during continuous muscular contractions, though it's most important function is maintaining and restoring resting $[Ca^{2+}]_i$ (48). Elevated $[Ca^{2+}]_i$ likely plays an important role in the reduced Ca^{2+} release transients observed during muscle fatigue. The mechanisms by which this occurs may be by activation of proteases or by other regulatory mechanisms. Regardless, reductions in SERCA activity could be one of the key processes in the development of muscle fatigue. SERCA has been shown to be susceptible to damage during exercise (15; 73), specifically, SERCA is susceptible to both oxidation and nitration particularly at the ATP binding site(58; 81). Given that reactive oxygen species (ROS) are produced at higher rates during exercise and that exercise is capable of causing reductions in SERCA activity (14; 73; 77), it is commonly believed that ROS are involved in exercise induced reductions in skeletal muscle force generating capacity. To further support this theory, there have been a number of studies showing that incubation of SR vesicles or homogenates with ROS or reactive nitrogen species (RNS) can also reduce SERCA activity (31; 81).

Heat shock proteins (HSPs) are a family of stress proteins that are ubiquitous among all mammals. There are several isoforms of HSPs, some examples are: ubiquitin, Hsp10, Hsp27, Hsp32, Hsp47, Hsp72, Hsp73, Hsp90, and Hsp100 (37). HSPs have several functions in the cell including protein transport, folding and repair (37). HSPs were first discovered in 1962 in *Drosophila Melanogaster* larvae exposed to heat shock (59). Since then, HSPs have become widely researched because of their possible role in the protection of cells in a variety of disease

states and in response to a variety of environmental and physiological stressors. Even though there is an abundance of HSP literature, the majority of research has been done using *in-vivo* - rodent models (38) or cell cultures (12). The number of studies looking at HSPs in humans is limited; furthermore, an even smaller number have looked at the role of HSPs in human skeletal muscle. Consequently, the functions of HSPs in human skeletal muscle are not well understood and many of the mechanisms of action of HSPs in skeletal muscle cells remain elusive.

The most commonly studied HSPs in skeletal muscle are the 70 kDa isoforms; these include the highly inducible Hsp70 and the constitutively expressed Hsc73, also known as heat shock cognate(10). A large number of studies have shown that Hsp70 can be induced by a variety of stressor such as hyperthermia (19), ischemia reperfusion (49), hypoxia (33), energy depletion (62), reactive oxygen species (ROS) (79), and acidosis (80). Given that the majority of these stressors are common disturbances during exercise, it isn't surprising that exercise can induce Hsp70 in skeletal muscle (24; 25; 32; 36; 42-44).

The characterization of exercise induced Hsp70 induction in human skeletal muscle depends on various factors. Hsp70 induction is intensity dependant, with higher intensity exercise being more effective in inducing a heat shock response(43). Eccentric exercise has been shown to be more effective in eliciting a heat shock response than concentric exercise(68) and exercise of longer duration is also more likely to induce Hsp70 protein induction(17). In addition, it has recently been shown that there are fibre type differences in Hsp70 induction(69). The characteristics of heat shock protein induction are further complicated by other factors. The heat shock response may differ between genders since estrogen has been shown to attenuate the heat shock response(78). Furthermore, Vitamin E administration has also been shown to attenuate the heat shock response(18), suggesting Hsp70 induction can be altered by antioxidant levels. While

there have been several groups who have investigated the acute effects of exercise on Hsp70 induction(17; 52; 57; 68), few groups have attempted to characterize the time course of exercise induced Hsp70 production and degradation in humans(35; 54; 69). It is clear that more investigation into the exercise induced Hsp70 response is needed before a greater understanding of Hsp70's role in human skeletal muscle can be understood. Consequently, there is quite a bit of controversy over the role of Hsp70 in human skeletal muscle during exercise. Studies in rodents suggest that Hsp70 has a protective role in skeletal muscle. In mice, overexpression of Hsp70 protects muscular function by reducing damage due to oxidative stress (7). The precise mechanisms remain to be established, though recent evidence from our laboratory suggests that the mechanisms may relate to the protection of key proteins involved in excitation-contraction coupling; in particular proteins involved in calcium handling (71). Tupling et al., have shown that when Hsp70 is incubated *in-vitro* with the fast isoform of SERCA, SERCA1a, not only is SERCA protected from thermal inactivation, but Hsp70 seems to accomplish this protection through a direct physical interaction with SERCA1a(71). In addition, it has been shown that SERCA2a has similar protection in the presences of Hsp70 (22; 70). Also, a study by Tupling et al., has shown that 16 bouts of high intensity intermittent cycling is able to attenuate reductions in SERCA function resulting from moderate intensity cycling(74). Though measurements of Hsp70 were not taken in that particular study, the authors acknowledged the possibility that Hsp70 may have played a role in the protection of SERCA. Our group has recently reported exercise induced increases in Hsp70 concurrently with reductions in SERCA activity in human skeletal muscle(71). This finding lends further support to the notion that Hsp70 responds to SERCA damage and may be involved in protecting it.

The majority of the early work investigating the role of Hsp70 in protecting SERCA stem from preconditioning studies in heart muscle in which ischemia-reperfusion(50), hyperthermia(11), and exercise(30) have all been shown to reduced infarct size during a subsequent insult. At least one major impetus for the effects of preconditioning are generally agreed to be from ROS and RNS. However, the mechanisms responsible for the protection occurring after preconditioning are not well understood. One effect of preconditioning though that has been well documented is an increase in gene expression of Hsp70, manganese SOD, and inducible nitric oxide synthase(70). Though there have been investigations into the role of preconditioning in skeletal muscle, the results are not consistent(51; 67). Regardless there have been studies that have shown that preconditioning is able to provide protection in skeletal muscle(66; 67; 74). Preconditioning is biphasic with the initial protection lasting only a few hours and a late phase of protection reappearing between 24 – 72 hours after the initial insult (4). Interestingly, if up-regulation of inducible Hsp70 is inhibited by anti-sense oligonucleotides, the second phase of protection from preconditioning can be attenuated (5). These finding illustrate the important role that Hsp70 may play in the beneficial effects of preconditioning.

To further investigate the role Hsp70 plays in protecting SERCA in human skeletal muscle, a stimulus, such as preconditioning exercise, would be required for induction of Hsp70.Once elevation of Hsp70 protein within the muscle are attained, exercise known to cause reductions in SERCA activity could be used to determine any role Hsp70 may play in protecting it. Previously, our group has been able to cause large increases in Hsp70 protein in human vastus lateralis using a 30 minute single-legged isometric knee exercise protocol at 60% maximal voluntary contraction (MVC) (69). This protocol also resulted in major reductions in SERCA activity and muscular force persisting for 144 hours (76). In order to effectively isolate the effects Hsp70 may

have on SERCA it would be vital to have a preconditioning exercise protocol with little if any other perturbations within the muscle. Also, it would be valuable to be able to cause an elevation in Hsp70 in one limb only in order to use the contralateral limb as a positive control.

To investigate the hypothesis that Hsp70 can protect exercise induced reductions in SERCA activity in human skeletal muscle, Hsp70 would have to be elevated within a muscle and SERCA activity would have to be challenged. This thesis will address these purposes in two separate studies. The purpose of the first study was to develop a preconditioning exercise protocol that was able to selectively up-regulate Hsp70 in one leg only. In addition, we wanted to follow the time course of production and breakdown of Hsp70, as well as the time required for full recovery of both SERCA activity and muscular force. To accomplish this we repeated a single-legged isometric knee extension exercise previously used by our group(69). However, an intensity of 40% MVC instead of 60% MVC was used in order to minimize the prolonged reduction in both SERCA activity and muscular force elicited with the 60% protocol. We hypothesized that Hsp70 would be elevated immediately post exercise in the exercised leg only and that it would remain elevated for at least 48 hours, and that both SERCA activity and muscular force would recover within 48 hours of the exercise.

The purpose of the second study was to determine if elevated Hsp70 could protect both muscle mechanical function and SERCA activity from exercise induced damage. To accomplish this, the exercise protocol used in the first study was repeated in order to selectively up-regulate Hsp70 in one leg only. For the purpose of the second study the leg that underwent isometric exercise was classified as the preconditioned leg. Participants then performed cycling exercise designed to reduce both SERCA activity and muscular force. We hypothesized that the cycling exercise would cause reductions in SERCA activity and in muscle mechanical properties in both legs but

that the preconditioned leg would show attenuations in exercise induced reductions in both muscular fatigue and SERCA activity.

Chapter 2: Methods

Study #1

Participant Characteristics & Experimental Design

Eight untrained but otherwise healthy male participants were recruited. Their age, height and weight were 19.8 ± 0.5 years, 179 ± 2.8 cm, and 77 ± 2.4 kg; respectively. Participants were asked to refrain from all physical activity for one week prior to testing and for the entire testing week. In addition, participants were asked to refrain from caffeine and alcohol consumption for 48 hours prior to the first test session and for the duration of the testing week. All participants were fully informed of all procedures and risks before giving written consent. Written ethics approval was given for this study by the Office of Research Ethics at the University of Waterloo. Participants were asked to perform a single-legged knee extension exercise protocol at 40% MVC; the experimental setup has been described previously(20; 69; 73). Briefly, the exercise consisted of 30 minutes of isometric leg extensions at a 50% duty cycle (5 seconds contraction, 5 seconds relaxation). All participants were able to maintain 40% MVC for the duration of the exercise protocol without any activation of the control leg; only in the last minute of exercise was there a significant 2% drop in attainable force (see Figure 1).

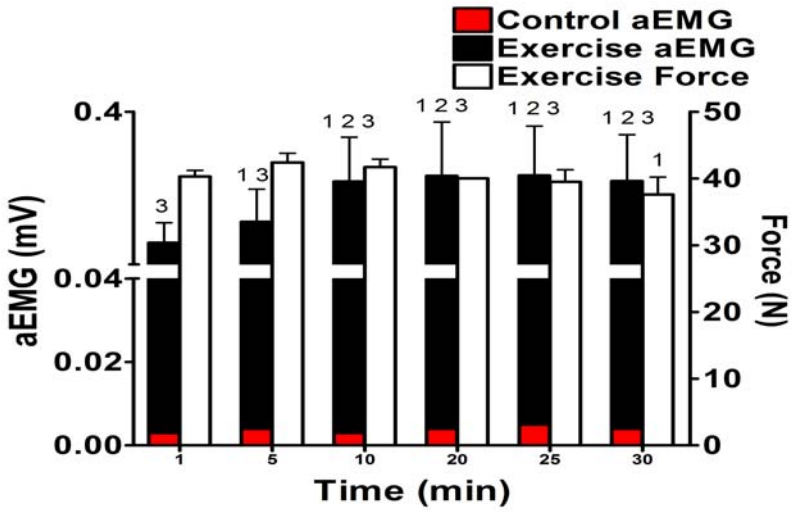

Figure 1: aEMG and Force during Isometric Exercise in Study 1. Measurements were taken every 5 min during exercise starting after the first minute of exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. 1 significantly different than 1 min, 2 significantly different than 5 min, 3 significantly different than corresponding time point in control.

The leg chosen for exercise was determined randomly without any consideration for leg dominance. Muscle electrical stimulation (described below) was performed before (PRE) and after (POST) exercise and 24 (R1), 48 (R2), 72, (R3), and 144 (R6) hours after the exercise. Muscle biopsies were taken PRE exercise in the control leg, POST exercise in the exercised leg and in both legs on R1, R2, R3, and R6. Muscle biopsy samples were used to measure maximal Ca^{2+}-ATPase activity, Ca^{2+}-uptake, and content of Hsp70, SOD1, SOD2, and catalase by western blot analysis. In addition, frozen cross sections were used to determine muscle fibre-type distribution, fibre-type specific glycogen content, and fibre-type specific Hsp70 content using immunohistochemistry.

Study #2

Participant Characteristics & Experimental Design.

Eight untrained but otherwise healthy male participants were recruited. Their age, height and weight were 24 ± 1.0 years, 178 ± 2.7 cm, and 86 ± 5.2 kg; respectively. Participants were asked to refrain from all physical activity for one week prior to testing and for the entire testing week. In addition, participants were asked to refrain from caffeine and alcohol consumption for 48 hours prior to the first test session and for the duration of the testing week. All participants were fully informed of all procedures and risks before giving written consent. Written ethics approval was given for this study by the Office of Research Ethics at the University of Waterloo. Participants were brought into the lab two weeks prior to the testing day to perform a VO_2 max test and for a familiarization of the electrical stimulation protocol. On the first testing day, participants were asked to perform a single-legged knee extension exercise protocol at 40% MVC identical to that performed in the first study. All participants were able to finish the exercise protocol showing only a small drop in force by the 30th minute. Similar to the first study, there was no measurable activation of the control leg at any point in the exercise (See Figure 2).

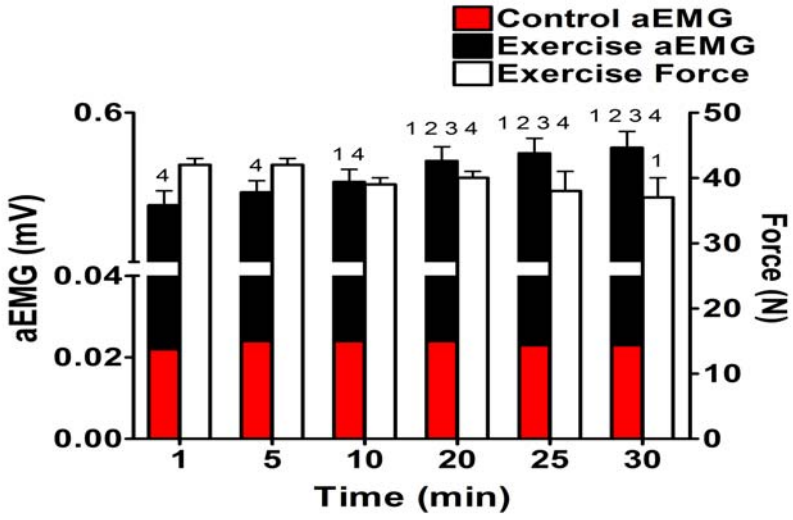

Figure 2: aEMG and Force during Isometric Exercise in Study 2. Measurements were taken every 5 min during exercise starting after the first minute of exercise in both the preconditioned (E) and control (C) leg. Values are mean +/- standard error mean. 1 significantly different than 1min, 2 significantly different than 5 min, 3 significantly different than 10 min, 4 significantly different than corresponding time point in control.

Based on the results from Study #1, participants were given 72 hours to recover and then were asked to cycle at 70% VO_2 max for sixty minutes. The mean time to fatigue was 55.6 ± 1.5 minutes. Recovery of muscle fatigue was followed in both legs for 72 hours following the cycling protocol. Muscle electrical stimulation was performed before (PRE) and after (POST) exercise and 24 (R1), 48 (R2), and 72 hours (R3) after the exercise in both legs. Muscle biopsies were taken PRE and POST exercise and on R1, R2, and R3, in both legs. Muscle biopsy samples were used to evaluate maximal Ca^{2+}-ATPase activity, Ca^{2+}-uptake, and western blot analysis of Hsp70.

Muscle Electrical Stimulation.

Both voluntary and evoked muscle contractions were performed in order to characterize changes in muscle mechanical properties of the quadriceps muscles and assess fatigue. The setup and procedures for these measurements has been used extensively in our lab(65; 66). Measurements included a supramaximal twitch, an MVC, and stimulations at 10 Hz, 20 Hz, 30 Hz, 50 Hz, and 100 Hz. In addition, the interpolated twitch technique was used to quantify any central fatigue present. The voltage used for the stimulations at various frequencies was determined prior to the testing in a familiarization session by adjusting the voltage at 100 Hz until 60% of the participant's MVC was attained. This voltage was determined independently for each leg and was kept constant for the entire study. Also, the order in which the legs received the measurements of muscle mechanical properties was randomized on all days to avoid any order effects.

Muscle Tissue sampling.

Muscle tissue samples (~50 mg) were obtained from the vastus lateralis with use of the needle biopsy technique (29). The biopsies were taken, five from each leg, from separate tissue-sampling sites under local anesthesia (2% Xylocaine). One portion of the biopsy sample was oriented under a dissecting microscope, mounted with optimal cutting temperature medium, rapidly frozen in isopentane that was pre-cooled with liquid nitrogen, and stored at -80°C. These samples were used for histochemical determination of fibre type distribution, fibre type-specific Hsp70 expression, and glycogen content. The remaining portion of the biopsy sample was diluted in a sample buffer and homogenized as described previously(73). Small aliquots of homogenate were then quick frozen in liquid nitrogen and stored at -80°C for future analysis of protein content (45), maximal SERCA activity, and Ca^{2+}-uptake. Aliquots were also used for the measurement of SOD1, SOD2, catalase and Hsp70 expression by western blot analysis.

Immunohistochemistry and Histochemistry.

Immunohistochemistry and histochemical analyses were performed on serial cross sections of tissue (8-10 mm) that were cut in a cryostat maintained at -20°C. Hsp70 immunohistochemistry was carried out according to the procedures described by Neufer et al. (55) with minor modifications. Briefly, frozen muscle sections were fixed to microscope slides in a 100% cold acetone solution for 10 min, washed (once for 5 min) in PBS (10 mM, pH 7.2), and permeabilized in 0.5% Triton X-100 in PBS for 5 min. After another wash (3 times for 5 min each) in PBS, sections were blocked with 5% horse serum solution for 30 min at 22°C in a humidified chamber. The primary monoclonal antibody specific to the inducible form of Hsp70 (SPA-810, Stressgen Biotechnologies) was applied to the sections (1:200 dilution in PBS) for 1 hour at room temperature. After the sections were washed (3 times for 5 min each) in PBS,

biotinylated horse anti-mouse immunoglobulin G (1:200 dilution in PBS; Vector Laboratories) was applied for 30 min at room temperature. The sections were rinsed in PBS (3 times for 5 min each) and then incubated for 30 min with a 1:500 dilution of an alkaline phosphatase-streptavidin conjugate (Vector Laboratories). Hsp70 antibody binding was visualized using an alkaline phosphatase secondary detection system (NovaRED substrate kit, Vector Laboratories), which produces a brown-red precipitate. To determine fibre type-specific Hsp70 expression and glycogen content, serial cross sections were stained for myosin ATPase activity using pre-incubation pH values of 4.55 and 10.3 (6) and for relative glycogen content by the periodic acid-Schiff reaction. Fibres were randomly chosen from the myosin ATPase stains (n=10 for fibre types I, IIA, and IIAX/IIX) and identified with the aid of a microscope linked to computer-based imaging analysis software (Image-Pro PLUS). No attempt was made to distinguish between a hybrid type IIAX fibre and a pure type IIX fibre; rather, intermediately stained fibres (pre-incubation pH 4.55) were classified type IIAX/IIX. After fibre type determination, corresponding Hsp70 sections were analyzed for staining intensity using the above-mentioned software. Intensity was calculated by subtracting the negative background (no primary antibody) and the background of the slide that was incubated with the primary antibody from the corresponding Hsp70-positive serial section and expressed in arbitrary linear (red-scale) units. All cross sections from a single participant were always stained and analyzed on the same day.

Western Blotting.

Western blotting was performed to determine the relative expression levels of Hsp70, SOD1, SOD2, and catalase in whole muscle homogenates prepared from muscle biopsy samples. After linearity of band density was ensured, samples were applied to polyacrylamide gels, and proteins were separated using standard SDS-PAGE protocols (39) and then transferred to polyvinylidene

difluoride membranes (Roche Diagnostics, Mannheim, Germany). After they were blocked with a 10% skim milk suspension, the membranes were incubated with a monoclonal primary antibody. Then, after the membranes were washed in Tris-buffered saline-0.1% Tween, they were treated for one hour with a secondary antibody. The membranes were washed again, and the signals were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) using a bioimaging system and densitometric analysis was performed using GeneSnap software (Syngene). All samples were run in duplicate on separate gels, and protein content was expressed relative to PRE levels. Antibody details for each specific protein blotted can be found in Table 1.

Table 1: Western blotting Antibody Information.

Protein	Loaded	1^{o} Antibody	2^{o} Antibody	Company
Hsp70	5 µg	1:1000	1:2000	Stressgen
SOD1	25 µg	1:1000	1:2000	Stressgen
SOD2	25 µg	1:1000	1:2000	Stressgen
Catalase	25 µg	1:1000	1:2000	Chemicon

Maximal Ca^{2+}-ATPase Activity.

Measurement of SERCA kinetic properties was performed using a spectrophotometric assay (63) adapted for use on a plate reader (SPECTRAmax Plus; Molecular Devices). This procedure has been describe in detail previously(14). Briefly, 40 µl of crude muscle homogenate were added to a 5 ml cocktail buffer containing ATP, lactate dehydrogenase, pyruvate kinase, and the Ca^{2+} ionophore A23187. Each sample was then aliquoted (300 µl) into 16 Eppendorf tubes and mixed with Ca^{2+} to generate 15 different Ca^{2+} concentrations, ranging between 7.6 and 4.7 pCa units. In addition, one Eppendorf contained CPA in order to determine background ATPase activity. The

Ca^{2+} ionophore A23187 was used to prevent the formation of a large Ca^{2+} gradient across the SR membrane. Samples were then loaded on a 96 well clear plate in duplicates (100 µl) and NADH was added to each well. SERCA activity (nM/mg protein/min) was determined by measuring the difference in absorbance between NADH and NAD+. All samples for a given participant were run on the same day.

Ca^{2+}-Uptake.

Oxalate-supported Ca^{2+}-uptake rates were measured using the Ca^{2+} fluorescent dye indo-1, according to published methods (60) and adapted by our laboratory (72). Briefly, 60 µl of crude muscle homogenate were added to a cuvette containing buffer, $CaCl_2$, lactate dehydrogenase, pyruvate kinase, and indo-1 reaching a final volume of 1 ml. ATP was then added to commence the reaction. Fluorescence measurements were made on a spectrofluorometer (Ratiomaster system, Photon Technology International) equipped with dual-emission monochromators. The measurements of free Ca^{2+} ($[Ca^{2+}]_f$) is based on the difference in maximal emission wavelengths between indo-1 bound and unbound to Ca^{2+}. The curve generated from the $[Ca^{2+}]_f$ vs. time was then smoothed over 21 points using the Savitsky-Golay algorithm. Linear regression was performed on values ranging at $[Ca^{2+}]_f$ of 500, 1,000, 1,500, and 2,000 nM. Differentiating the linear fit curve allowed determination of Ca^{2+} uptake rates (nM/mg protein/min). All samples for a given participant were measured in the same day.

Statistics.

Statistical measures were done using a repeated measures two-way ANOVA when comparing the control and exercised leg. When comparing difference in the 3 fibre types, repeated measures three-way ANOVA was used. To determine significance between individual data points a Newman-Keuls post-hoc analysis was used. Planned comparisons were used to determine the

differences between POST control and POST exercise in study 2. In addition, correlation coefficients were calculated to determine the relationship between the level of Hsp70 protein and the change in SERCA activity with exercise.

Chapter 3: Results

Study #1

Muscle Mechanical Properties.

Isometric knee extension exercise caused an ~ 48% reduction ($p<0.05$) in twitch peak tension of the exercise leg POST when compared to PRE, that recovered ($p<0.05$) by R1. Similarly, there was an ~ 46% reduction ($p<0.05$) in the twitch maximal rate of force development ($+dF/dt_{max}$) and an ~35% reduction ($p<0.05$) in the maximal rate of force decline ($-dF/dt_{max}$) POST when compared to PRE. Like peak tension, both $+dF/dt_{max}$ and $-dF/dt_{max}$ recovered ($p<0.05$) by R1; there were no differences ($p>0.05$) at any time point in the control leg (see Figures 3, 4, and 5).

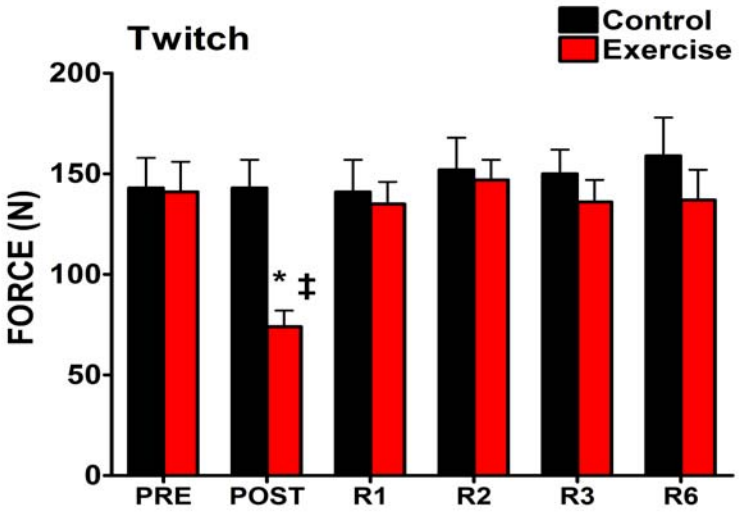

Figure 3: Peak Twitch Force in Study 1. Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE, ‡ significantly different than corresponding time point in control.

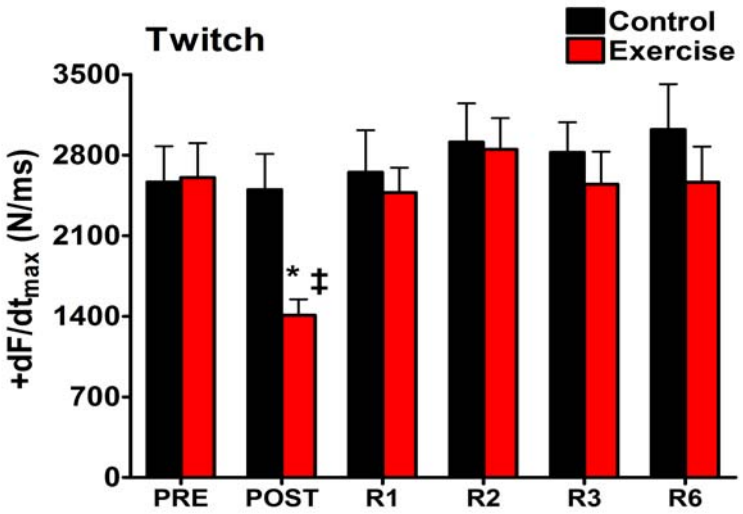

Figure 4: Twitch $+dF/dt_{max}$ in Study 1. Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE, ‡ significantly different than corresponding time point in control.

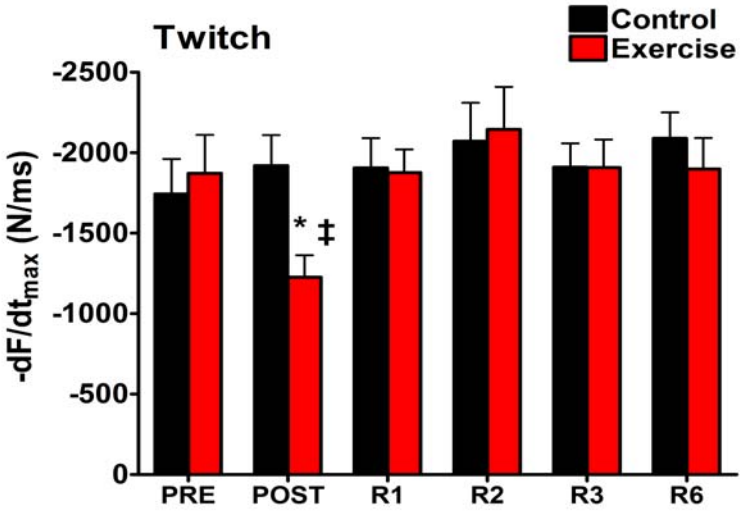

Figure 5: Twitch $-dF/dt_{max}$ in Study 1. Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE, ‡ significantly different than corresponding time point in control.

At the lowest frequencies of stimulation (10, 20 Hz), there was a reduction ($p<0.05$) in force POST when compared to PRE in the exercise leg of ~76% at 10Hz and ~65% at 20Hz that had recovered to ~43% of PRE values at 10Hz and 27% of PRE values at 20Hz ($p<0.05$) by R1 but

remained depressed by ~43% at 10Hz and ~25% at 20Hz through R6. In the control leg, there was an ~33% reduction ($p<0.05$) in force at 10 Hz POST when compared to PRE that remained depressed by ~41% ($p<0.05$) through R6; no changes were seen at 20 Hz (See Figure 6).

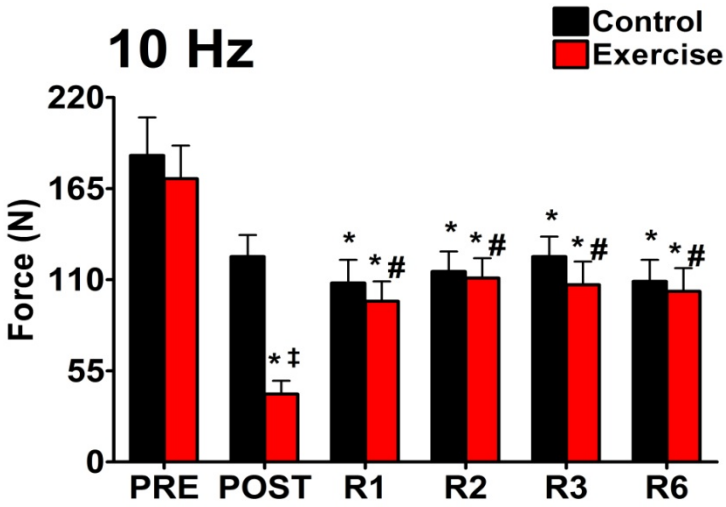

Figure 6: Force at 10Hz in Study 1. Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE, # significantly different than POST, ‡ significantly different than corresponding time point in control.

At the higher frequencies of stimulation (30, 50, 100 Hz), there was an ~54%, 44% and 38%reduction ($p<0.05$), respectively, in force POST when compared to PRE that completely recovered ($p<0.05$) by R1; there were no changes in force of the control leg at the higher frequencies of stimulation (See Figure 7).

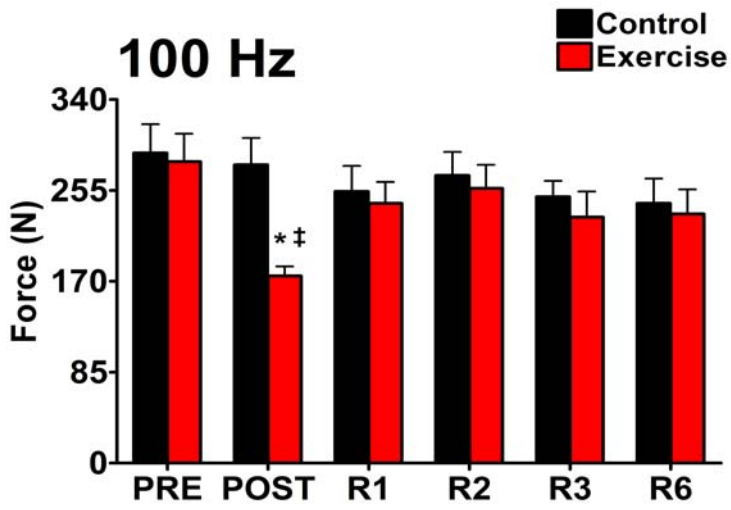

Figure 7: Force at 100Hz in Study 1. Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE, ‡ significantly different than corresponding time point in control.

At 10 Hz, there was an ~68% prolongation ($p<0.05$) in $+dF/dt_{max}$ POST when compared to PRE that recovered ($p<0.05$) to ~35% of PRE exercise values by R1 but remained prolonged ($p<0.05$) by ~35% through R6. Also, in the control leg at 10 Hz, there was an ~21% prolongation ($p<0.05$) in $+dF/dt_{max}$ POST when compared to PRE that remained altered by ~21% ($p<0.05$) through R6. At the remaining upper four frequencies of stimulation (20, 30, 50, and 100 Hz), there was an ~ 68%, 60%, 50%, and 40% prolongation ($p<0.05$), respectively, in $+dF/dt_{max}$ POST when compared to PRE that recovered ($p<0.05$) by R1; there were no changes in $+dF/dt_{max}$ of the control leg for 20, 30, 50 or 100 Hz (See Figure 8, 9).

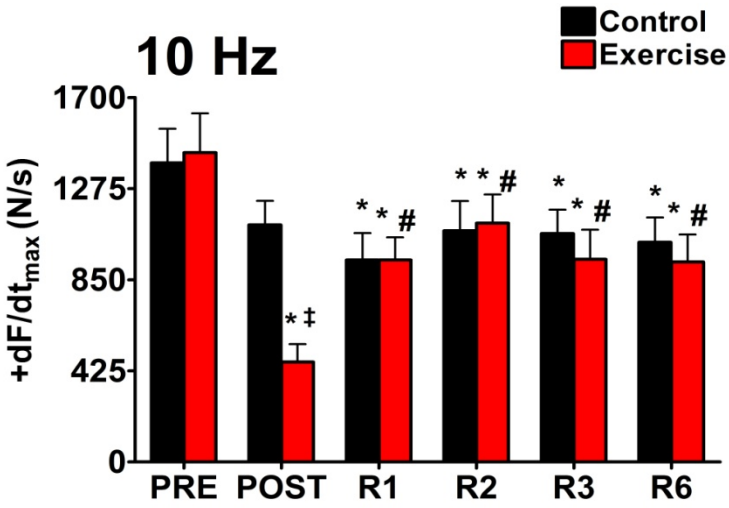

Figure 8: +dF/dt$_{max}$ at 10Hz in Study 1. Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE, # significantly different than POST, ‡ significantly different than corresponding time point in control.

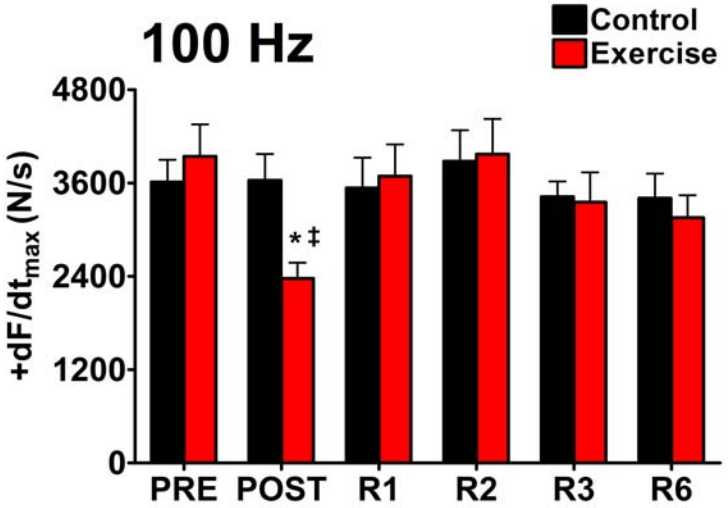

Figure 9: +dF/dt$_{max}$ at 100Hz in Study 1. Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE, ‡ significantly different than corresponding time point in control.

At 10 and 20 Hz, there was an ~ 73% and 62% prolongation ($p<0.05$), respectively, in -dF/dt$_{max}$ POST when compared to PRE in the exercise leg that recovered ($P<0.05$) to ~ 47% and 28% of PRE values by R1 but remained prolonged by similar amounts ($p<0.05$) through R6. In the

control leg, there was an ~ 38% prolongation in $-dF/dt_{max}$ at 10 Hz POST when compared to PRE that remained prolonged ($p<0.05$) by ~38% through R6. A similar trend was seen at 20 Hz though an ~22% prolongation in $-dF/dt_{max}$ was only found at R1, R3, and R6. At 30, 50, and 100 Hz, there was an ~45%, 32%, and 24% prolongation ($p<0.05$), respectively, in $-dF/dt_{max}$ POST when compared to PRE in the exercise leg that recovered ($p<0.05$) by R1; there were no changes in the control leg at any time point at 30, 50, or 100 Hz (See figures 10, 11).

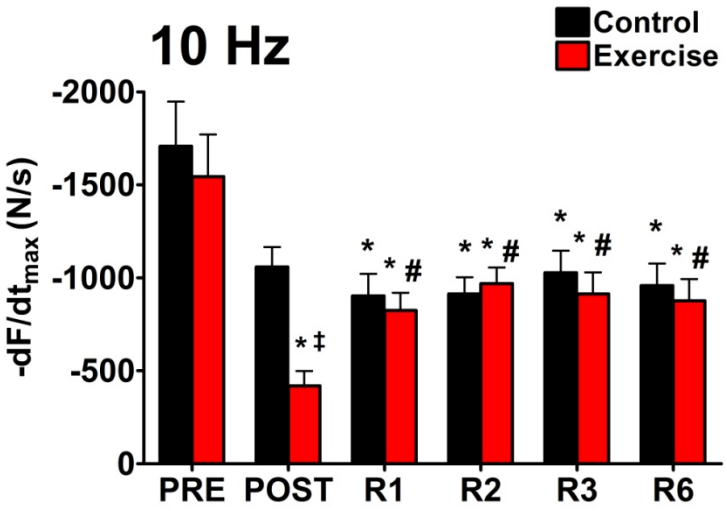

Figure 10: $-dF/dt_{max}$ at 10Hz in Study 1. Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE, # significantly different than POST, ‡ significantly different than corresponding time point in control.

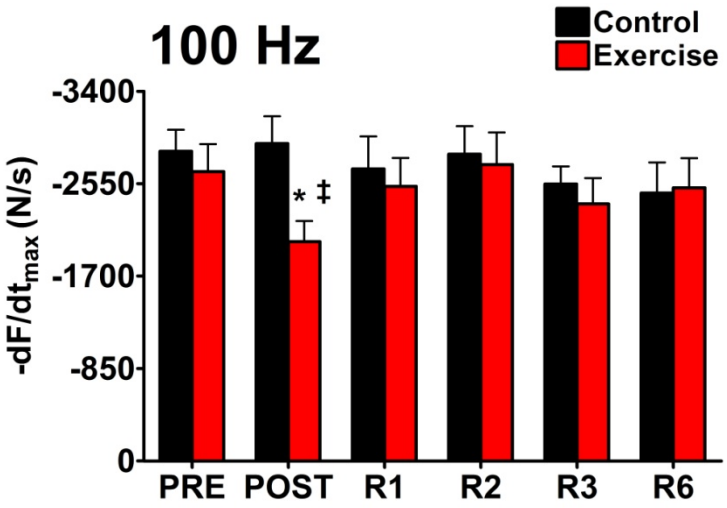

Figure 11: -dF/dt$_{max}$ at 100Hz in Study 1. Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE, ‡ significantly different than corresponding time point in control.

There was an ~ 44% reduction ($p<0.05$) in MVC force of the exercise leg POST when compared to PRE that recovered ($p<0.05$) to ~16% of PRE exercise values by R1, but remained depressed ($p<0.05$) by ~16 % through R6. Minor reductions ($p<0.05$) in MVC force of ~14% were also seen in the control leg but were only significant at R1 and R3 (See Figure 12).

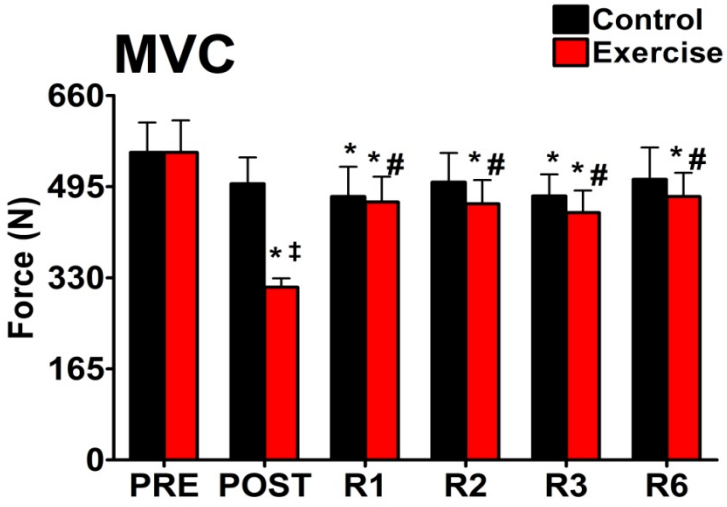

Figure 12: MVC Force in Study 1. Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the

exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE, # significantly different than POST, ‡ significantly different than corresponding time point in control.

There were no changes in motor unit activation as measured by the interpolated twitch at any point in either leg. For complete tabulated force data see appendix 1.

Maximal Ca^{2+}-ATPase Activity and Ca^{2+} Uptake.

Thirty min of isometric knee extension exercise at 40% MVC had no effect on maximal Ca^{2+}-ATPase activity at any time point in either the control or exercised leg. In addition, there were no changes in the kinetic parameters (Ca_{50} and the hill slope) of SERCA (See figure 13 and table 2).

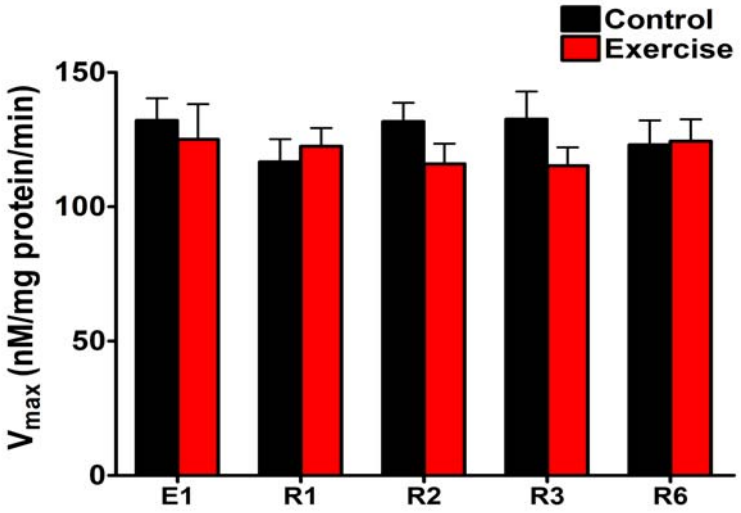

Figure 13: Maximal Ca^{2+}-ATPase Activity in Study 1. Measurements were taken before and after exercise (E1), and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean.

Table 2: Ca^{2+}-ATPase Kinetic Measures

	Time Points, n				
	E1	**R1**	**R2**	**R3**	**R6**
Ca50					
C	6.6 +/- 0.09	6.6 +/- 0.05	6.6 +/- 0.08	6.7 +/- 0.09	6.7 +/- 0.05
E	6.7 +/- 0.08	6.5 +/- 0.05	6.7 +/- 0.05	6.6 +/- 0.05	6.7 +/- 0.07
Hill Slope					
C	2.8 +/- 0.3	2.7 +/- 0.2	2.4 +/- 0.3	2.7 +/- 0.2	2.7 +/- 0.3
E	2.8 +/- 0.3	2.5 +/- 0.1	2.6 +/- 0.2	3.0 +/- 0.3	2.8 +/- 0.1

Values are mean +/- standard error mean.

Though no changes were seen in the maximal activity of the Ca^{2+}-ATPase, there was a main effect ($p<0.05$) between the control and exercise legs in the rate of Ca^{2+} uptake with the exercise leg being lower ($p<0.05$) when compared to the control leg at $[Ca^{2+}]_f$ of 500 and 1000 nM. It is important to note that on closer examination of the changes in Ca^{2+} uptake it is obvious that the main effects were driven by the large differences in uptake rates in the earlier time points and that there are no differences in uptake rates between legs by R3 (see table 3).

Table 3: Ca^{2+} Uptake Rates at Various $[Ca^{2+}]_f$

	E1	R1	R2	R3	R6
500 nM					
C	3.1 +/- 0.4	2.9 +/- 0.5	2.5 +/- 0.3	2.3 +/- 0.4	2.5 +/- 0.5
E	1.9 +/- 0.2	2.4 +/- 0.3	2.8 +/- 0.3	2.6 +/- 0.5	2.3 +/- 0.3
1000 nM					
C	6.5 +/- 0.6	6.4 +/- 0.9	4.9 +/- 0.3	5.3 +/- 0.8	5.1 +/- 0.8
E	4.5 +/- 0.5	5.1 +/- 0.7	5.9 +/- 0.8	5.3 +/- 0.8	4.9 +/- 0.6
1500 nM					
C	9.1 +/- 0.9	8.6 +/- 1.2	7.1 +/- 0.6	7.8 +/- 1.1	7.3 +/- 1.1
E	6.7 +/- 0.7	6.9 +/- 0.7	8.5 +/- 1.3	7.5 +/- 1.1	6.8 +/- 1.0
2000 nM					
C	10.5 +/- 0.9	11.1 +/- 1.5	8.8 +/- 0.8	9.1 +/- 1.0	8.9 +/- 1.4
E	8.6 +/- 0.8	9.2 +/- 0.9	10.1 +/- 1.5	9.2 +/- 1.1	8.3 +/- 1.1

Values are mean +/- standard error mean.

Immunohistochemistry and Histochemistry.

Participants were found to have on average 40.7% type I fibres, 42.2% type IIA fibres, and 17.1 % type IIAX/X fibres. Isometric knee extension exercise caused a reduction in muscle glycogen of ~58% in type IIA fibres and ~ 56% in type IIAX/X fibres POST when compared to PRE that fully recovered ($p<0.05$) by R1. There was no change in muscle glycogen in the type I fibres (see figure 14).

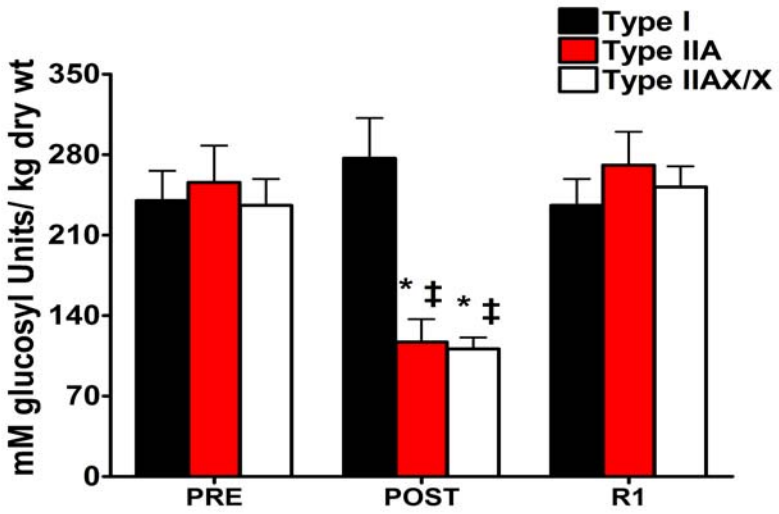

Figure 14: Fibre Type Specific Glycogen Content in Study 1. Measurements were taken before (PRE) and after (POST) exercise, and 24 hours (R1). Values are mean +/- standard error mean. * Significantly different than PRE, ‡ significantly different than corresponding time point in type I fibres.

When looking at fibre type-specific Hsp70 expression, there was a greater ($p<0.05$) basal Hsp70 expression in type I fibres than in type IIA and IIAX/X fibres, however, there were no differences between type IIA and type IIAX/X fibres. In addition, isometric knee extension exercise caused a significant increase ($p<0.05$) in Hsp70 protein in the exercise leg when compared to the control leg in type I fibres that was reduced ($p<0.05$) by R6. In addition, there was an increase ($p<0.05$) in Hsp70 expression at R1, R2 and R3 in type IIA fibres in the exercise leg when compared to the control leg, and at R1, R2, and R3 in type IIAX/X fibres. Furthermore, there was a greater ($p<0.05$) increase in Hsp70 in type I fibres when compared to type IIA and IIX fibres (see Table 15 see Figure 16).

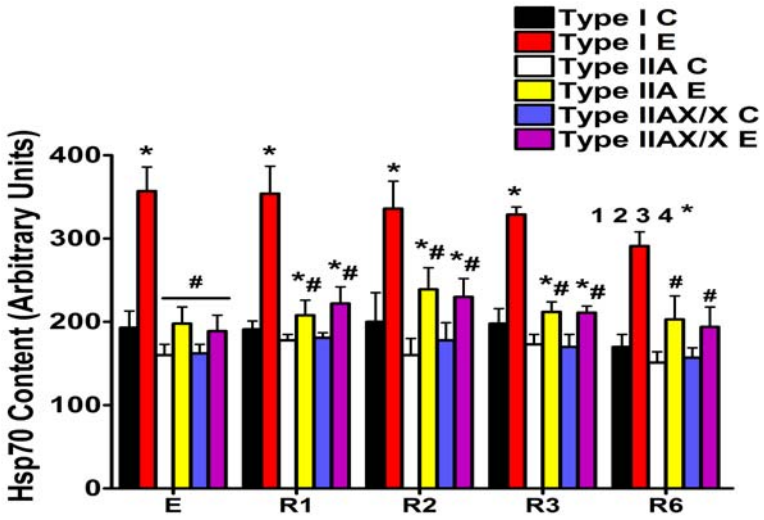

Figure 15: Fibre Type Specific Hsp70 Content in Study 1. Measurements were taken from the control leg PRE E and from the exercised leg POST E, and from both legs on R1, R2, R, and R6. Values are mean +/- standard error mean. 1 Significantly different from E1E, 2 significantly different from R1, 3 significantly different from R2, 4 significantly different than R3, * significantly different than E1 C, # significantly different than corresponding time point in type I fibres.

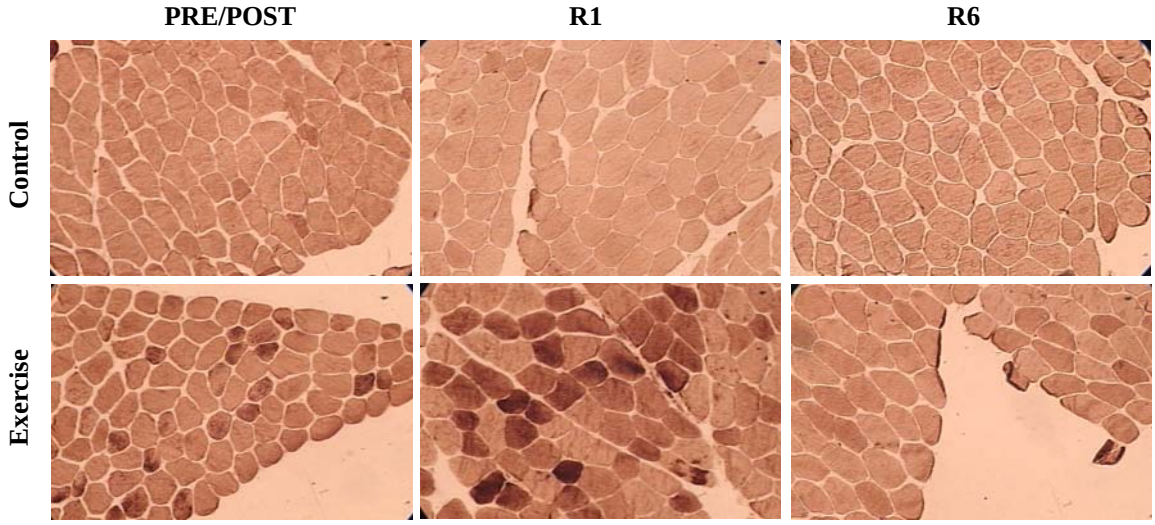

Figure 16: Representative Micrograph of Hsp70 Stains in Study 1. Representative photomicrographs (x 200) of sections from muscle biopsy samples that were taken from the control leg (top row) and exercised leg (bottom row) before and after exercise and on recovery day 1 and 6 from the same participant were subjected to immunohistochemical detection of Hsp70.

Western Blotting.

There were no differences in Hsp70 protein expression at any time point in the control leg; however, we found a strong trend (p=0.067) toward a higher Hsp70 protein content in the exercise leg when compared to the control leg (See figure 17). There were also no detectable differences in the relative protein content of catalase, SOD1, or SOD2 at any time point or in either legs (see Figures 18, 19, 20). An important observation is that Hsp70 remains elevated through to R3 and does so in the absence of any differences in twitch peak force or calcium handling properties between control and exercise legs. Therefore R3 is a crucial time point for the second study whose specific aim was to determine the role that Hsp70, when elevated, may have in protecting SERCA.

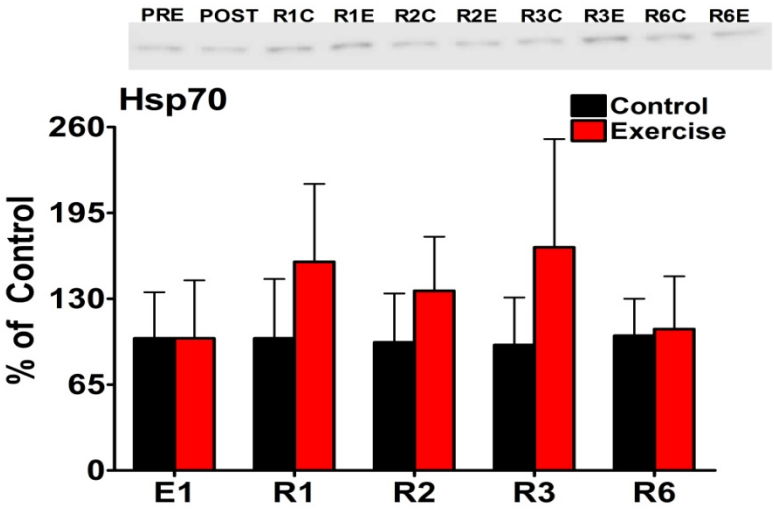

Figure 17: Hsp70 Western Blot in Study 1. Measurements were taken before (E1- C) and after (E1-E) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean.

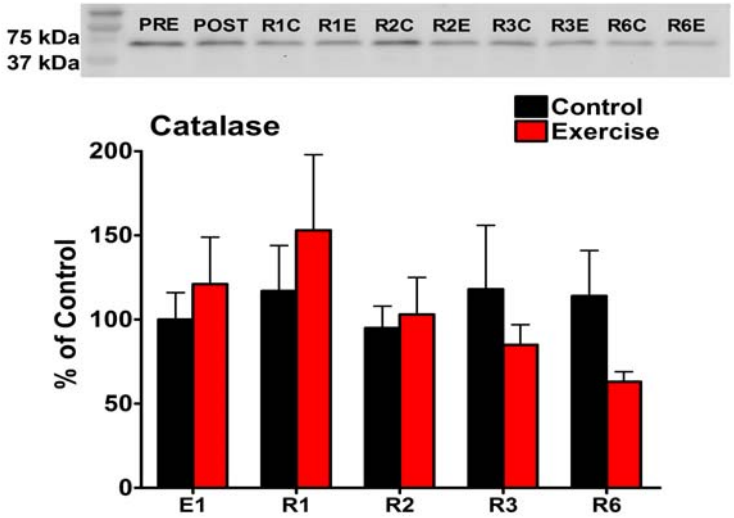

Figure 18: Catalase Western Blot in Study 1. Measurements were taken before (E1- C) and after (E1-E) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean.

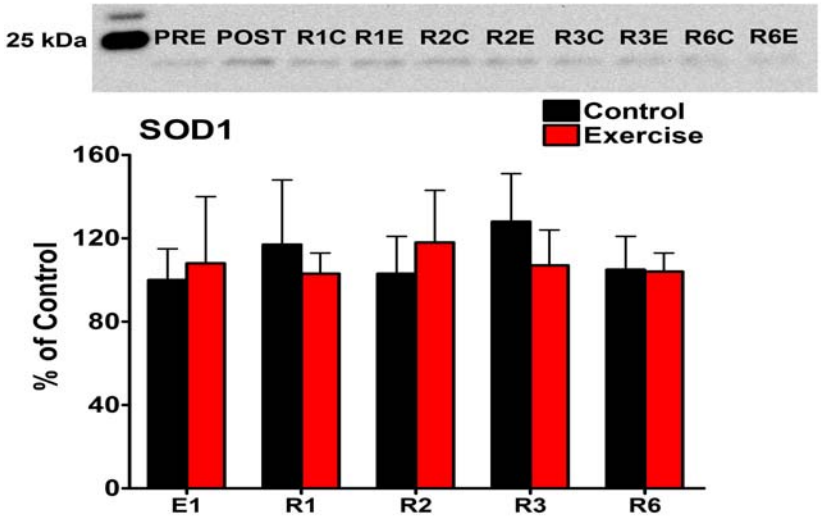

Figure 19: SOD1 Western Blot in Study 1. Measurements were taken before (E1- C) and after (E1-E) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean.

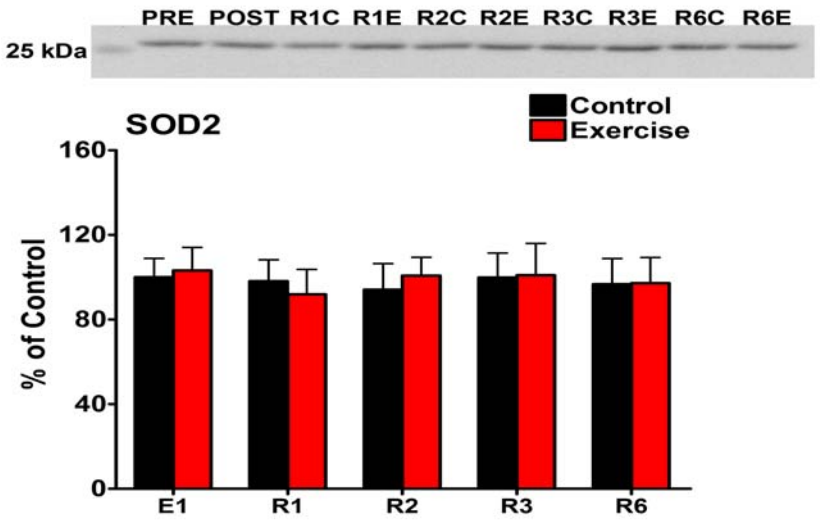

Figure 20: SOD2 Western Blot in Study 1. Measurements were taken before (E1- C) and after (E1-E) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean.

Study #2

Muscle Mechanical Properties.

Thirty minutes of isometric knee extension exercise caused an ~50% reduction ($p<0.05$) in peak twitch force in the preconditioned leg only that recovered ($p<0.05$) by PRE E2. Cycling exercise caused an ~ 43% reduction ($p<0.05$) in the control leg and an ~26% reduction ($p<0.05$) in the preconditioned leg POST E2 when compared to PRE E2; both legs fully recovered ($p<0.05$) by R1. Both twitch contraction time (~12%) and rise time (~43%) were reduced ($p<0.05$) by the isometric exercise in the preconditioned leg only; however, these parameters recovered ($p<0.05$) by PRE E2 and were not affected by the cycling exercise. The isometric exercise also caused an ~ 46% prolongation ($p<0.05$) in $+dF/dt_{max}$ in the preconditioned leg that recovered by PRE E2, in addition, the cycling exercise caused an ~ 47% prolongation ($p<0.50$) in $+dF/dt_{max}$ in the control leg only that had recovered ($p<0.05$) by R1. $-dF/dt_{max}$ was ~ 37% lower ($p<0.05$) in the

preconditioned leg when compared to the control leg POST E1; this difference was no longer apparent by PRE E2. Also, cycling exercise caused an ~ 37% prolongation ($p<0.05$) in $-dF/dt_{max}$ POST E2 when compared to PRE E2 that only occurred in the control leg. This reduction had recovered ($p<0.05$) by R1. (See figures 21, 22, and 23).

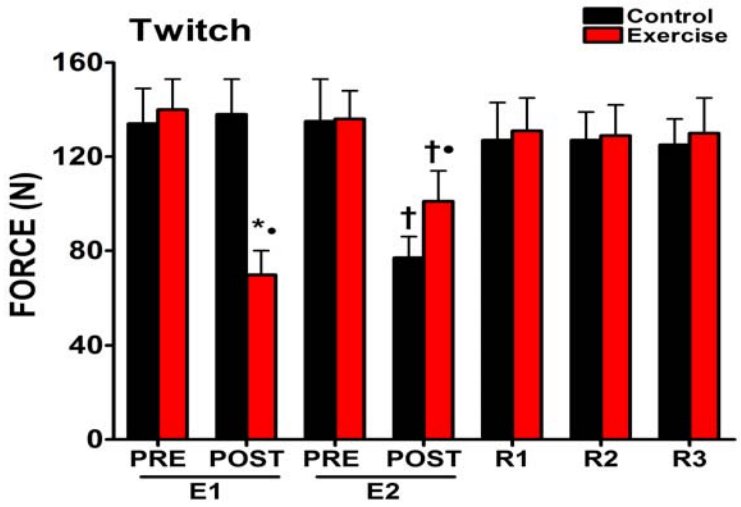

Figure 21: Peak Twitch Force in Study 2. Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE E1, † significantly different than PRE E2, • significantly different than corresponding time point in C.

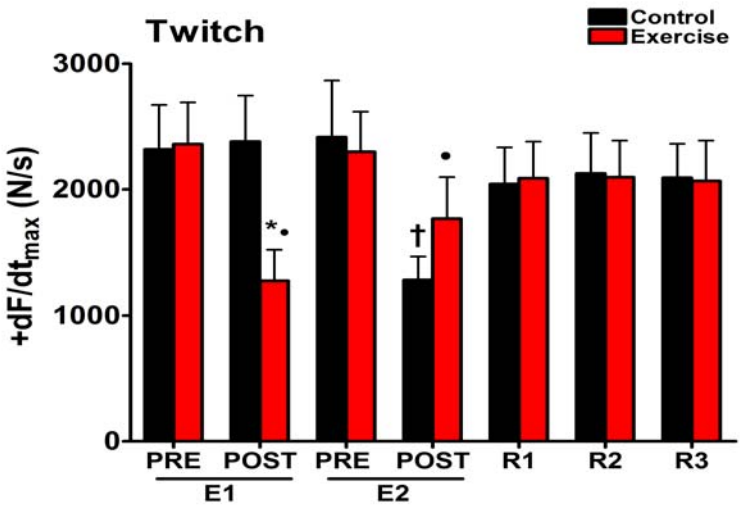

Figure 22: Twitch $+dF/dt_{max}$ in Study 2. Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE E1, † significantly different than PRE E2, • significantly different than corresponding time point in C.

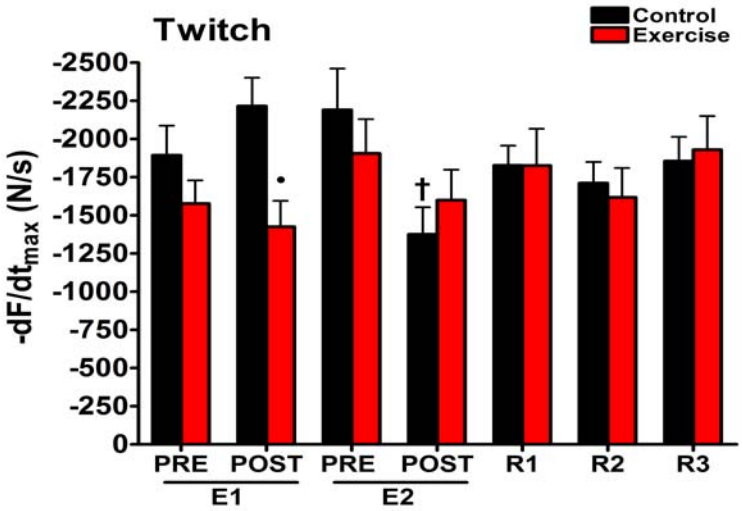

Figure 23: Twitch $-dF/dt_{max}$ in Study 2. Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean. † Significantly different than PRE E2, • significantly different than corresponding time point in C.

There was an ~42% reduction ($p<0.05$) in MVC force POST E1 when compared to PRE E1 in the preconditioned leg that was not mirrored in the control leg. The cycling exercise caused an ~21% reduction ($p<0.05$) in force in the control leg and an ~29% reduction ($p<0.05$) in the

preconditioned leg POST E2 when compared to PRE E2 that progressively recovered ($p<0.05$) by R2 (See Figure 24). There was a main effect of time ($p<0.05$) for aEMG measured during the MVC showing a reduction in aEMG POST E2 that had recovered ($p<0.05$) by R1. There were no changes in the interpolated twitch parameters at any time point in either leg (See Appendix 2).

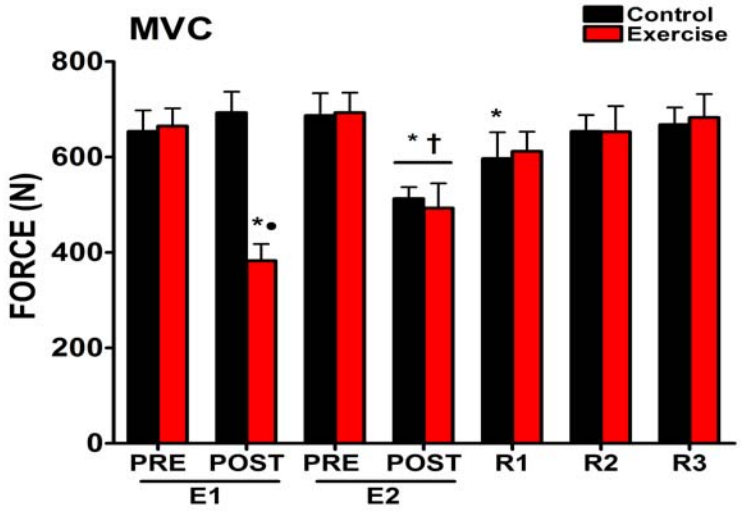

Figure 24: MVC Force in Study 2. Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE E1, † significantly different than PRE E2, • significantly different than corresponding time point in C.

At the lower frequencies of stimulation (10, and 20 Hz) the isometric exercise caused an ~ 79% reduction ($p<0.05$) in force at 10 Hz and an ~ 71% reduction ($p<0.05$) in force in the preconditioned leg with an accompanying ~17% reduction ($p<0.05$) in force at 10 Hz and an ~ 13% reduction ($p<0.05$) in force at 20 Hz in the control leg. Though neither leg recovered completely to PRE E1 levels, the preconditioned leg recovered ($p<0.05$) to ~33% of PRE E1 at 10 Hz and ~16% of PRE E1 at 20 Hz, and the control leg recovered ($p<0.05$) to ~31% of PRE E1 at 10 Hz and ~11% of PRE E1 at 20 Hz.. The cycling exercise caused an ~50% reduction ($p<0.05$) in force at 10 Hz and an ~45% reduction ($p<0.05$) in force at 20 Hz POST E2 when

compared to PRE E2 in the control leg and an ~32% reduction ($p<0.05$) in force at 10 Hz and an ~24% reduction ($p<0.05$) in force at 20 Hz POST E2 when compared to PRE E2 in the preconditioned leg. Both legs recovered ($p<0.05$) to PRE E2 force levels by R1, however, neither leg recovered to PRE E1 levels through R3 at both 10 and 20 Hz (See figure 25).

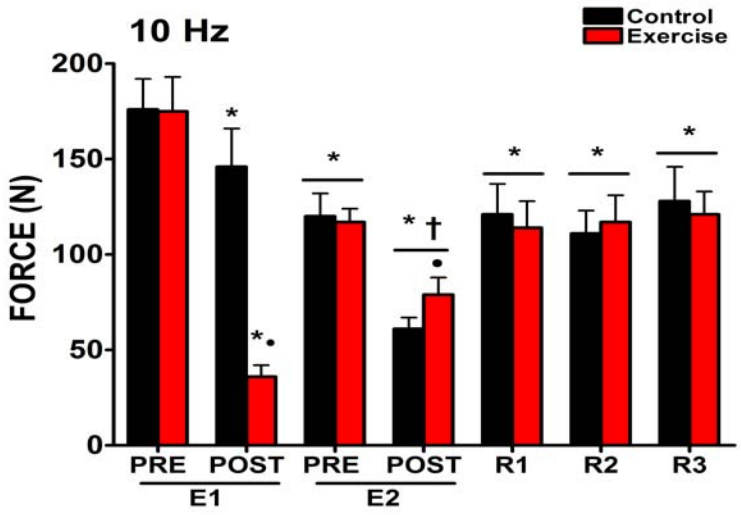

Figure 25: Force at 10Hz in Study 2. Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE E1, † significantly different than PRE E2, • significantly different than corresponding time point in C.

At the higher frequencies of stimulation (30, 50, and 100 Hz) there was an ~ 54%, 40%, and 34% reduction ($p<0.05$), respectively, in force due to the preconditioning exercise that had completely recovered ($p<0.05$) by PRE E2. The preconditioning exercise caused no changes in force in the control leg at any frequency of stimulation. The cycling exercise caused an ~19% reduction ($p<0.05$) in force at 30 Hz only that had recovered to PRE E2 levels by POST E2. For unapparent reason, there were transient reductions ($p<0.05$) in force at R2 in the control leg at all higher frequencies of stimulation and modest reductions ($p<0.05$) in force throughout recovery in the preconditioned leg at 100 Hz (see figure 26).

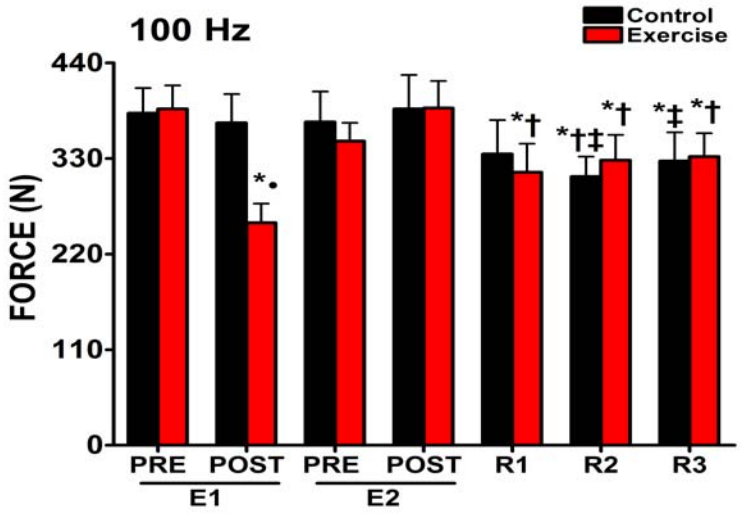

Figure 26: Force at 100Hz in Study 2. Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * Significantly different than PRE E1, † significantly different than PRE E2, ‡ significantly different than POST E2 • significantly different than corresponding time point in C.

At the lower frequencies of stimulation, there was an ~ 75% prolongation ($p<0.05$) at 10 Hz and an ~ 62% prolongation ($p<0.05$) at 20 Hz in $+dF/dt_{max}$ POST E1 when compared to PRE E1 in the preconditioned leg only. However, by PRE E2 $+dF/dt_{max}$ was prolonged ($p<0.05$) in both legs by ~35% at 10 Hz and ~20% at 20 Hz when compared to PRE E1. Cycling exercise caused an additional ~45% prolongation ($p<0.05$) in $+dF/dt_{max}$ at 10 Hz and an additional ~ 35% prolongation ($p<0.05$) in $+dF/dt_{max}$ at 20 Hz in the control leg that recovered ($p<0.05$) to PRE E2 values by R1. Both the control and the preconditioned leg remained at PRE E2 levels at both 10 and 20 Hz throughout recovery (see figure 27).

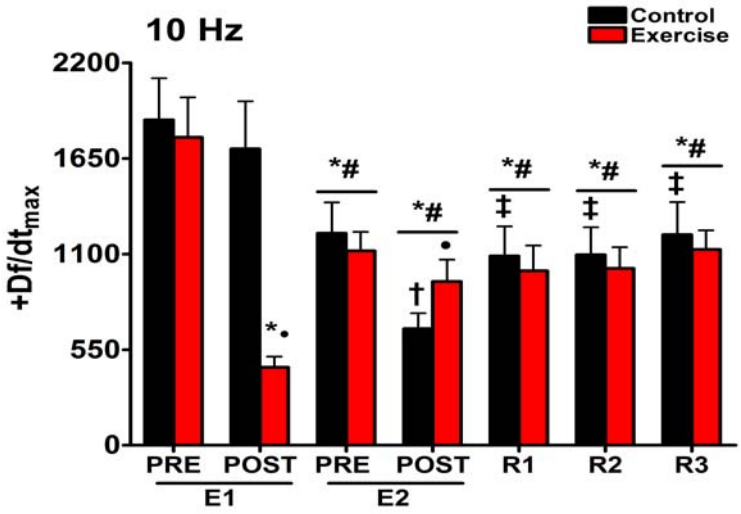

Figure 27: +dF/dt$_{max}$ at 10Hz in Study 2. Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean. # Significantly different than POST E1,* Significantly different than PRE E1, † significantly different than PRE E2, ‡ significantly different than POST E2 • significantly different than corresponding time point in C.

At the higher frequencies of stimulation (30, 50 and 100 Hz) +dF/dt$_{max}$ was prolonged ($p<0.05$) by ~ 45%, 30% and 17%, respectively, POST E1 when compared to PRE E1, but only in the preconditioned leg. +dF/dt$_{max}$ did recover ($p<0.05$) to PRE E1 values by PRE E2, however, still remained slower ($p<0.05$) when compared to the control leg. At 30 Hz, the cycling exercise caused a further ~ 19% reduction ($p<0.05$) in +dF/dt$_{max}$ in the control leg POST E2 when compared to PRE E2 that persisted ($p<0.05$) through R3; however, this reduction was not mimicked at50 and 100 Hz. At 50 and 100 Hz, +dF/dt$_{max}$ became prolonged ($p<0.05$) in both the preconditioned and control legs on R1 and did not recover ($p<0.05$) by R3 (see figure 28).

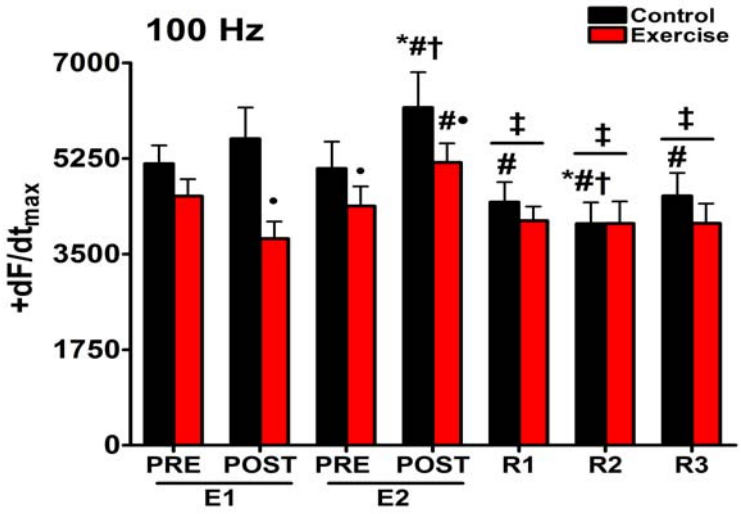

Figure 28: +dF/dt$_{max}$ at 100Hz in Study 2. Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean. # Significantly different than POST E1,* Significantly different than PRE E1, † significantly different than PRE E2, ‡ significantly different than POST E2 • significantly different than corresponding time point in C.

Isometric knee extension exercise caused a prolongation ($p<0.05$) in -dF/dt$_{max}$ at the lower frequencies of stimulation in the control leg by ~ 26% at 10 Hz and by ~ 15% at 20 Hz. In the preconditioned leg there was a much greater prolongation ($p<0.05$) in –dF/dt$_{max}$ by ~78% at 10 Hz and by ~ 71% at 20 Hz. At PRE E2, the preconditioned leg had recovered ($p<0.05$) to ~40% of PRE E1 values at 10 Hz and ~17% of PRE E1 values at 20 Hz. However, both legs still remained depressed ($p<0.05$) when compared to PRE E1 though there were no differences between the control and preconditioned leg. At POST E2, there was a further prolongation ($p<0.05$) in -dF/dt$_{max}$ of ~50% at both 10 and 20 Hz in the control leg only that recovered ($p<0.05$) by R1; however, recovery in both legs remained incomplete ($p<0.05$) through R3 (see figure 29).

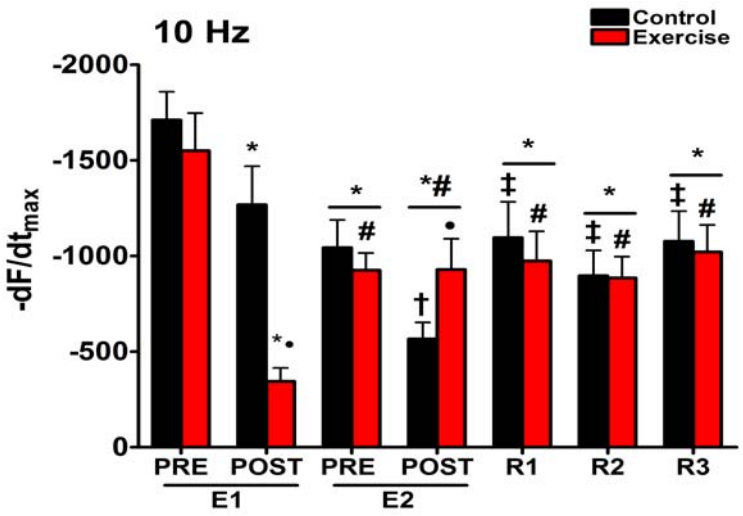

Figure 29: -dF/dt$_{max}$ at 10Hz in Study 2. Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean. # Significantly different than POST E1,* Significantly different than PRE E1, † significantly different than PRE E2, ‡ significantly different than POST E2 • significantly different than corresponding time point in C.

At 30 Hz, there was an ~49% prolongation ($p<0.05$) in -dF/dt$_{max}$ POST E1 when compared to PRE E1 in the preconditioned leg that fully recovered ($p<0.05$) by PRE E2. The cycling exercise did not cause any changes in -dF/dt$_{max}$, but there was a prolongation ($p<0.05$) of -dF/dt$_{max}$ in both legs on R1 that did not recover. At 50 and 100 Hz, there was an ~ 27% and 22% prolongation ($p<0.05$), respectively, in –dF/dt$_{max}$ that recovered by PRE E2. There was also a transient quickening ($p<0.05$) in -dF/dt$_{max}$ POST E2 when compared to PRE E2 in both legs at 50 and 100 Hz; however, at R1, similarly to 30 Hz, -dF/dt$_{max}$ became prolonged ($p<0.05$) through R3 when compared to PRE E1(see figure 30). See appendix 2 for a complete tabulated force data.

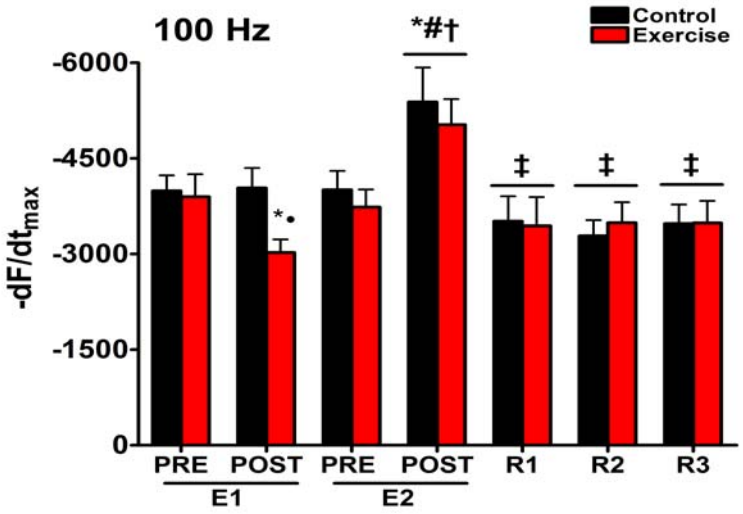

Figure 30: -dF/dt$_{max}$ at 100Hz in Study 2. Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean. # Significantly different than POST E1,* Significantly different than PRE E1, † significantly different than PRE E2, ‡ significantly different than POST E2 • significantly different than corresponding time point in C

Maximal Ca^{2+}-ATPase activity and Ca^{2+}uptake.

There was a strong trend (p=0.076) toward an interaction effect in maximal activity of the Ca^{2+}-ATPase, when the hypothesized planned comparison were performed between POST cycling exercise in the preconditioned and control legs, the control leg was found to have ~16% lower ($p<0.05$) maximal Ca^{2+}-ATPase activity than the preconditioned leg. There were no changes in the kinetic measures of the Ca^{2+}-ATPase (see figure 31 and 32). Similarly, there were no changes in the rate of SR Ca^{2+} uptake for any free Ca^{2+} concentration at any time point or in either leg (see table 4).

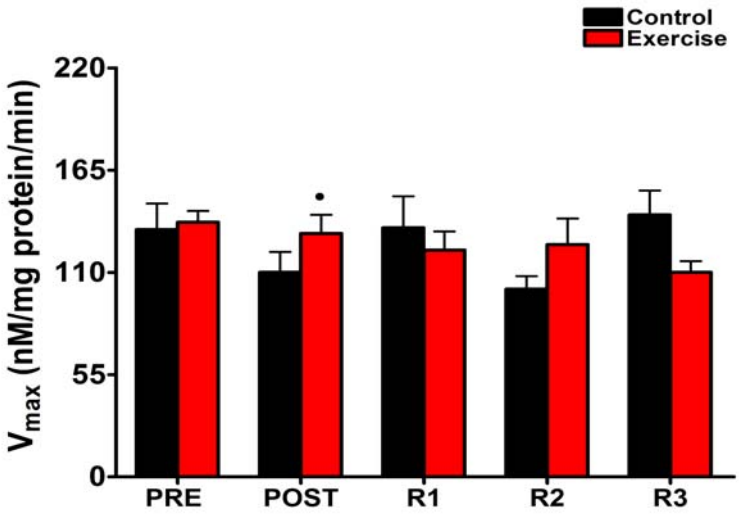

Figure 31: Maximal Ca^{2+}-ATPase Activity in Study 2. Measurements were taken before (PRE) and after (POST) cycling exercise (E2) and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean. • Significantly different than corresponding time point in C.

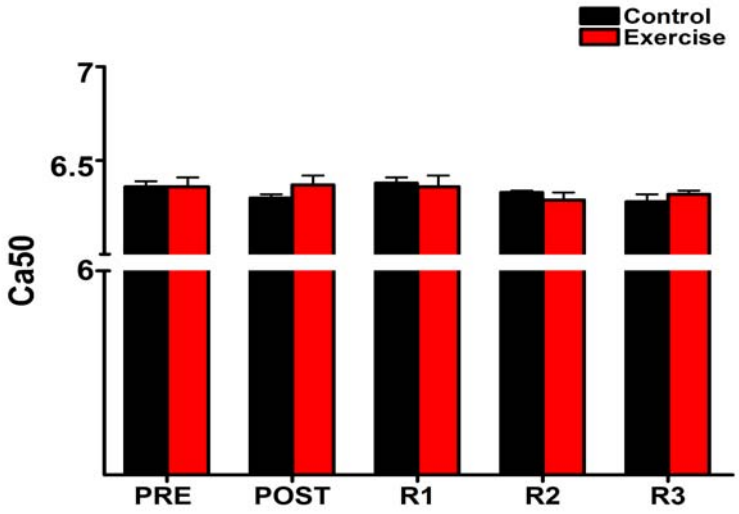

Figure 32: Ca_{50} Measurement in Study 2. Measurements were taken before (PRE) and after (POST) cycling exercise (E2) and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean. • Significantly different than corresponding time point in C.

Table 4: Ca^{2+} Uptake Measures at Various $[Ca^{2+}]_f$

	Time Points, n				
	PRE	POST	R1	R2	R3
500 nM					
C	1.8 +/- 0.4	1.0 +/- 0.1	1.8 +/- 0.2	1.0 +/- 0.1	1.5 +/- 0.3
E	1.7 +/- 0.2	1.3 +/- 0.2	1.6 +/- 0.1	1.3 +/- 0.1	1.1 +/- 0.1
1000 nM					
C	3.6 +/- 0.6	2.5 +/- 0.2	3.6 +/- 0.4	2.3 +/- 0.3	3.2 +/- 0.6
E	3.6 +/- 0.3	2.7 +/- 0.3	3.3 +/- 0.1	2.8 +/- 0.3	2.1 +/- 0.3
1500 nM					
C	4.6 +/- 0.7	3.6 +/- 0.3	4.9 +/- 0.6	3.5 +/- 0.4	4.3 +/- 0.6
E	4.7 +/- 0.3	3.6 +/- 0.3	4.3 +/- 0.2	3.7 +/- 0.3	3.0 +/- 0.2
2000 nM					
C	5.1 +/- 0.7	4.3 +/- 0.3	5.8 +/- 0.7	4.8 +/- 0.6	5.4 +/- 0.6
E	5.5 +/- 0.4	4.0 +/- 0.3	5.0 +/- 0.3	4.7 +/- 0.6	3.7 +/- 0.2

Values are mean +/- standard error mean.

Western Blotting.

We found no differences in Hsp70 protein expression at any time point in the preconditioned or control leg (see figure 32).

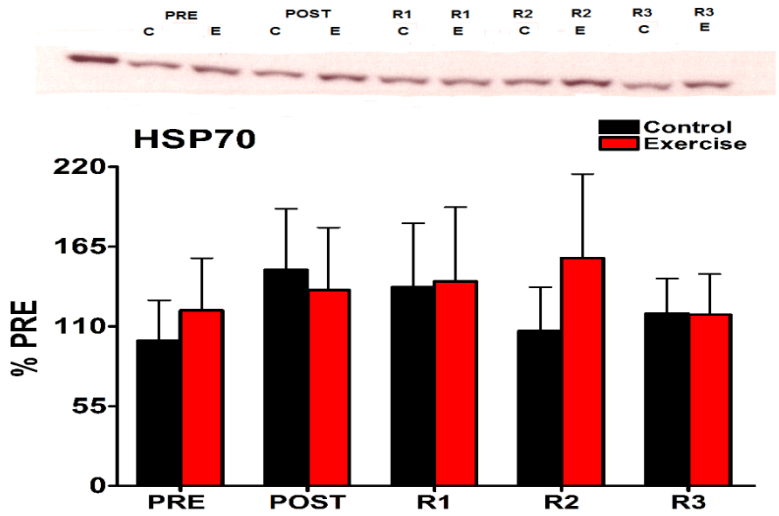

Figure 32: Hsp70 Western Blot in Study 2. Measurements were taken before (PRE) and after (POST cycling exercise, and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the preconditioned (E) and non-exercised (C) leg. Values are mean +/- standard error mean.

Chapter 4: Discussion

The purposes of these studies were two-fold: to develop a preconditioning exercise protocol in which Hsp70 could be selectively up-regulated in one leg only with minimal or no other signs of muscle fatigue, in particular damage to SERCA and reductions in muscular force, and to determine if up-regulation of Hsp70 could protect against exercise induced reductions in muscle contractile function and SERCA activity. In the first study, thirty minutes of isometric knee extension exercise at 40% MVC was able to induce a heat shock response that was apparent in all muscle fibre types, but to a much greater degree in type I muscle fibres. This increase in Hsp70 persisted throughout recovery. In addition, there was no increase in Hsp70 in the control leg at any point. There were also basal fibre type differences in Hsp70. Similar to findings in rodents (32), the more oxidative type I fibres had a higher basal Hsp70 protein content; presumably to combat a cellular environment with greater oxidative stress. One could speculate that the differences in basal Hsp70 protein content could be in part due to differences in basal protein turnover, which relates to the chaperoning function of Hsp70. Though fibre type differences in basal protein turnover are well documented in rodents(23), most recent evidence suggests that this is not the case in human skeletal muscle(53). Western blot analysis only revealed a trend toward an elevation in Hsp70 protein in the exercise leg. The inconsistency between western blotting and immunohistochemistry results from an inherent difference in sensitivity between western blotting and immunohistochemistry; western blotting displaying reduced sensitivity.

Contrary to the hypotheses, isometric exercise was unable to cause reductions in the maximal rate of the Ca^{2+}-ATPase. This was surprising since our group has previously shown that this exact protocol at 60% MVC causes reductions in SERCA activity in the order of 20 % persisting

for 6 days (76). However, isometric exercise was able to cause reductions in Ca^{2+} uptake in the exercise leg at the lower $[Ca^{2+}]_f$, though by R2 there were no differences in Ca^{2+} uptake between the control and exercise leg. This suggests that the reductions in Ca^{2+} uptake seen were the result of transient metabolic disturbances and not any major damage or alterations in SERCA characteristic of low frequency fatigue. Reductions in maximal SERCA activity were expected, however, their absence was quite serendipitous. The ability to induce Hsp70 without any damage to SERCA is far more ideal than a situation in which SERCA becomes damaged but recovers. Hsp70 has been shown to protect SERCA by protecting the nucleotide binding domain(71), therefore, if Hsp70 is elevated in the absence of changes in Ca^{2+}-ATPase activity then changes in SERCA activity from further exercise can be more strongly attributed to that particular exercise and not any structural alterations that may have resulted from the original isometric exercise.

As expected the isometric exercise protocol produced extensive muscle fatigue in the exercised leg in all parameters measured. Also, in all cases except MVC, 10 Hz, and 20 Hz, force was able to recover within 24 hours; much more quickly than hypothesized. Similarly, rates of relaxation and force development were also prolonged after the exercise but in all cases except the lower frequencies of stimulation were completely recovered by R1. Moreover, with the exception of the MVC, 10 Hz and 20 Hz there were no changes in muscular force or contraction and relaxation rates in the control leg.

Changes in force at lower frequencies of stimulation are generally attributed to alterations in SR Ca^{2+} handling specifically Ca^{2+} release due to impaired E-C coupling. Impaired SERCA function could also contribute to low frequency fatigue by a reduction in Ca^{2+} uptake into the SR resulting in a higher resting $[Ca^{2+}]_f$ and a lower SR Ca^{2+} load available for release(40). In this study, however, there were no detectable changes in SERCA activity to account for the persistent low

frequency fatigue seen in this study in both the control and exercised legs. Additionally, there were no detectable changes in central motor unit activation by the interpolated twitch technique to account for the reductions in MVC force. With the exception of peak twitch force, muscle mechanical properties are measured using submaximal voltages, therefore there is the possibility that changes in the response to the elicited voltage occurred between days. The relationship between force and $[Ca^{2+}]_f$ is sigmoidal; at lower frequencies of stimulation where $[Ca^{2+}]_f$ transients are lower, smaller changes in voltage would elicit greater changes in force. Though at higher frequencies of stimulation much smaller changes in force would occur due to variations in voltage, some would still be expected(1). Given that there were no changes in force at the higher frequencies of stimulation, it seems that voltage dependant differences in force production between days, if present, were minor. In the absence of alterations in Ca^{2+} handling, low frequency fatigue can also be caused by reduced Ca^{2+} sensitivity of troponin C and by minor mechanical damage to the contractile apparatus from isometric contractions (34). Given the absence of persistent fatigue in the peak twitch force; there was likely very little mechanical damage due to the isometric exercise. One possible explanation for the persistent low frequency fatigue in both legs during recovery is that mechanical damage from multiple biopsies may have contributed to at least some of the low frequency fatigue and reduced MVC force. This possibility has previously been reported using a similar exercise protocol (21). In this thesis, however, the isometric exercise was performed in the first study with biopsies and in the second study without biopsies and it was observed that in the absence of biopsies there was less reduction in MVC force during recovery and the reductions in low frequency force are reduced. Still, there were likely both repeated biopsy and a voltage dependant changes which contributed to the persistent low frequency fatigue occurring in both legs in this study. Given, that the

reductions in force at low frequency occur in both the exercised and control leg without alterations in the peak twitch force, a more sensitive measure of low frequency fatigue, some other processes must be occurring independently of the characteristic causes of low frequency fatigue.

Antioxidant enzymes including catalase, SOD1, and SOD2 were also measured as additional markers of how much damage may be occurring due to the exercise protocol. Exercise induces oxidative stress and if that oxidative stress is large enough there are antioxidant enzymes that are up-regulated in order to combat the stress(58; 61). No changes in antioxidant enzymes were detected, further suggesting that the perturbations occurring during and after the exercise were mild. It is important to recognize that the absence of alterations in antioxidant enzymes carries particular significance. If antioxidant enzymes were elevated in response to the isometric exercise there would be little need for an increase in Hsp70 for its proposed protective role since protein damage would presumably be reduced due to scavenged ROS and RNS. This phenomenon has been shown in rat soleus muscle(64) and can be further supported by research showing that genetic modifications in basal Hsp70 content reduces both the resting antioxidant levels as well as the oxidative damage due to muscle contractions(7).

In the second study, isometric exercise produced similar results, reductions in force in the preconditioned leg that recovered by PRE E2 at the higher frequencies, in the twitch peak force, and in the MVC, but that only partially recovered at the lower frequencies of stimulation. As previously mentioned, in the absence of biopsies isometric exercise caused no reductions in force in the twitch, MVC, and higher frequencies of stimulation; and much smaller reductions in force at the lower frequencies of stimulation in the control leg. The cycling exercise produced reductions in force in both legs in all parameters except the higher frequencies of stimulation.

Also, as hypothesized the reductions in force were attenuated in the preconditioned leg in the twitch and at the lower frequencies of stimulation. Preconditioning had no apparent effect on the recovery of force after the cycling exercise. At the lower frequencies of stimulation, including the twitch, the preconditioning exercise attenuated the prolongation of $+dF/dt_{max}$ and $-dF/dt_{max}$ after the cycling exercise. These findings in combination with the attenuations in force reduction after the cycling exercise in the preconditioned leg suggest that muscle function was in fact protected.

Western blot analysis showed no significant increases in Hsp70 at any time point; however, there were a few notable trends. There was a trend toward an increase in Hsp70 in the preconditioned leg prior to cycling exercise, this observation is important since there was also a trend at this time point in the first study (corresponds to R3 in the first study). Though this difference did not reach significance in either study, when measured by immunohistochemical analysis there were clear differences in Hsp70 between the exercise and control leg at R3 in the first study. A clear limitation to the second study is the lack of immunohistochemical analysis. Still, given that participants with similar characteristics and training status were used in both studies, it is possible to speculate that at PRE exercise in the second study there were similar differences in Hsp70 between legs. Also, though immunohistochemical confirmation was lacking, there appeared to be a transient increase in Hsp70 in both legs POST cycling exercise and on R1.

As hypothesized, there was an attenuated decrease in the maximal activity of SERCA POST exercise in the preconditioned leg. Surprisingly, there were no differences in the rate of Ca^{2+} uptake at any time point. Though the exercise modalities are different, our group has shown using a model of consecutive days of exercise a similar effect in maximal SERCA activity, that being an apparent protection in SERCA activity with prior exercise(15). The maximal rate of

SERCA activity can be affected by several factors. First, with exercise there is an increase in metabolites such as inorganic phosphate, ADP, and hydrogen ions. Studies with mechanically skinned fibres have demonstrated the deleterious effects of these metabolites on SERCA activity(3). Reactive oxygen species have also been shown by FITC binding assays to cause very specific damage to SERCA's nucleotide binding site(46). Metabolic effects, however, would result in shorter duration reductions in SERCA activity whereas oxidative damage results in irreversible damage to SERCA requiring removal of the damaged enzymes and *de novo* synthesis of SERCA proteins; this can take up to several days(47).

Most recent studies from our group have demonstrated concurrent reductions in SERCA activity and Ca^{2+} uptake(13; 73), however, in both the aforementioned studies there was a dissociation in the relative changes in Ca^{2+}-ATPase activity and in Ca^{2+} uptake; the ratio between these two parameters is termed the coupling ratio. In the first study, there were reductions in Ca^{2+} uptake and no reductions in maximal SERCA activity, whereas the opposite was true in the second study. To address this issue one must consider the possible causes for reductions in Ca^{2+} uptake. If the coupling ratio were one (i.e. both ATPase and uptake change by a similar magnitude) changes in Ca^{2+} uptake could be due to reductions in ATP hydrolysis ultimately resulting in reduced Ca^{2+} uptake. However, when the coupling ratio dissociates from one there is the possibility that ATP hydrolysis is unaltered but Ca^{2+} uptake is reduced because of Ca^{2+} leaking back into the cytosol either through a damaged membrane or through a leaky Ca^{2+} release channel. The hypothesis that Ca^{2+} release rates, due to mechanical disruption, can contribute to this effect is supported by previous work in both single fibre preparations and in humans showing that the administration of caffeine, a Ca^{2+} release channel agonist, can reverse low frequency fatigue (28; 40). Generally, maximal SERCA activity is assessed with and without the

Ca^{2+} ionophore A23187. Ionophore permeabilized the membrane and therefore when measuring maximal SERCA activity in its presence, activity is not affected by possible changes in Ca^{2+} leak across the membrane. Without ionophore reductions in SERCA activity due to Ca^{2+} leak can also be assessed. Had it been possible to do both in this study, an ionophore ratio could have been used to determine if in fact dissociations in the coupling ratio could be attributed at least in part by changes in the permeability of the membrane to Ca^{2+}.

These hypotheses for dissociated changes in maximal SERCA activity and Ca^{2+} uptake fit nicely with the observed results. In the first study, the isometric exercise protocol would be expected to cause mechanical damage, either to the SR membrane or to the mechanical link between the Ca^{2+} release channel and the DHPR. This damage would facilitate passive leakage of Ca^{2+} across the Ca^{2+} release channel or through damaged SR membranes; resulting in hindered net uptake of Ca^{2+} into the SR. Whereas in the second study, the concentric cycling exercise would be expected to cause less mechanical damage and greater oxidative damage to SERCA, resulting in reduced Ca^{2+}-ATPase activity.

As hypothesized, we found that SERCA activity was protected by preconditioning exercise designed to up-regulate Hsp70. Also, we found that force, $+dF/dt_{max}$ and $-dF/dt_{max}$ were also protected by the preconditioning exercise. The most tempting explanation for this protection is that Hsp70 has bound to the nucleotide binding site of SERCA thus protecting it from oxidative damage associated with the cycling exercise. The result of this protection would be maintenance of resting $[Ca^{2+}]_f$, maintenance of the Ca^{2+} pumping ability of SERCA (which can be related to attenuations in $-dF/dt_{max}$) maintenance of SR Ca^{2+} load and therefore Ca^{2+} releasing ability (which can be related to attenuation in $+dF/dt_{max}$) and an overall protection in muscular force.

However, when correlation coefficients were calculated between Hsp70 protein content and the changes in SERCA activity, only a weak negative relationship was found ($r = -0.20$).

In conclusion, these studies were able to show that with preconditioning exercise designed to up-regulate Hsp70, reduction in SERCA activity and muscle mechanical properties due to subsequent aerobic exercise could be attenuated. It must be pointed out, however, that the dissociated coupling ratio as well as the persistent low frequency fatigue during recovery in the absence of changes in Ca^{2+} handling properties were unexpected and deserves further investigation.

Chapter 5: Limitations

There are several limitations which unfortunately have complicated the interpretations of the results seen in these two studies. In the first study, the persistent low frequency fatigue, though believed to be unimportant since its magnitude was similar in both legs, may have been a confounding factor in the second study. In the second study, time considerations did not allow for the inclusion of several important analyses including: immunohistochemistry for Hsp70, western blots for SERCA isoforms and antioxidant enzymes, and tissue limitations did not allow for a determination of the interaction of Hsp70 and SERCA by co-immunoprecipitation. These results would have been valuable additions for the interpretation of the results included. In addition, the results presented do not definitively point to Hsp70 as the most important contributor to the effects of preconditioning; it would be interesting to determine if any differences in metabolic perturbations or enzymes occurred. Finally, it would have been helpful in the second study to have biopsies in both legs before the isometric exercise; this would have allowed assurance that there were no differences in Hsp70 or SERCA activity between legs or subjects prior to the exercise.

Chapter 6: Future Direction

The most important information to come from these studies is the confirmation that preconditioning attenuates reductions in muscular force and SERCA activity. Future studies should further investigate the physical interaction of Hsp70 and SERCA in human skeletal muscle. Secondary to Hsp70, it is still unclear for what reasons muscular force remains depressed in the absence of changes in SERCA activity with certain exercise protocols. Though some hypotheses for this phenomenon have been presented, a better understanding of the exact mechanisms would be an important contribution to the exercise physiology literature.

Appendix 1: Study #1 Tabulated Force Data

Table 1. Twitch Characteristics

	Time Points, n					
	PRE	POST	R1	R2	R3	R6
Pt, N						
C	143 +/- 15	143 +/- 14	141 +/- 16	152 +/- 16	150 +/- 12	159 +/- 19
E	141 +/- 15	74 +/- 8 *‡	135 +/- 11	147 +/- 10	136 +/- 11	137 +/- 15
CT, ms						
C	97 +/- 2	100 +/- 4	100 +/- 4	89 +/- 3	93 +/- 3	94 +/- 4
E	91 +/- 4	91 +/- 3	92 +/- 3	89 +/- 2	93 +/- 3	92 +/- 4
1/2RT, ms						
C	62 +/- 8	54 +/- 6	55 +/- 6	60 +/- 8	58 +/- 8	53 +/- 6
E	63 +/- 9	44 +/- 2	63 +/- 9	60 +/- 9	67 +/- 11	60 +/- 9
RT, ms						
C	55 +/- 1	57 +/- 2	53 +/- 1	52 +/- 1	52 +/- 1	52 +/- 2
E	54 +/- 1	53 +/- 1	54 +/- 1	53 +/- 1	54 +/- 2	53 +/- 1
+dF/dtmax, N/s						
C	2566 +/- 310	2500 +/- 310	2651 +/- 364	2912 +/- 337	2823 +/- 262	3022 +/- 394
E	2606 +/- 297	1409 +/- 139 *‡	2475 +/- 215	2850 +/- 271	2547 +/- 282	2565 +/- 308
-dF/dtmax, N/s						
C	-1743 +/- 217	-1918 +/- 191	-1905 +/- 184	-2070 +/- 239	-1909 +/- 148	-2088 +/- 161
E	-1871 +/- 239	-1226 +/- 136 *‡	-1876 +/- 144	-2144 +/- 264	-1907 +/- 174	-1898 +/- 193

Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * significantly different than PRE, # significantly different than POST, ‡ significantly different than corresponding time point in control.

Table 2. Force at different frequencies of stimulation

	Time Points, n											
	PRE	**POST**		**R1**		**R2**		**R3**		**R6**		
10Hz, N												
C	185 +/- 23	124 +/- 13	*	108 +/- 14	*	115 +/- 12	*	124 +/- 12	*	109 +/- 13	*	
E	171 +/- 20	41 +/- 8	*‡	97 +/- 12	*#	111 +/- 12	*#	107 +/- 14	*#	103 +/- 14	*#	
20Hz, N												
C	261 +/- 25	232 +/- 21		207 +/- 20	*	223 +/- 18		219 +/- 14		207 +/- 22		
E	253 +/- 24	89 +/- 13	*‡	185 +/- 15	*#	207 +/- 19	*#	192 +/- 21	*#	191 +/- 20	*#	
30Hz, N												
C	281 +/- 26	265 +/- 24		244 +/- 23		258 +/- 21		243 +/- 14		234 +/- 23		
E	276 +/- 26	126 +/- 13	*‡	225 +/- 19	#	243 +/- 23	#	218 +/- 24	#	221 +/- 22	#	
50Hz, N												
C	289 +/- 27	280 +/- 26		257 +/- 24		271 +/- 22		251 +/- 16		245 +/- 24		
E	287 +/- 26	160 +/- 12	*‡	242 +/- 20	#	261 +/- 24	#	232 +/- 25	#	234 +/- 24	#	
100Hz, N												
C	290 +/- 27	279 +/- 25		254 +/- 24		269 +/- 22		249 +/- 15		243 +/- 23		
E	282 +/- 26	175 +/- 9	*‡	243 +/- 20	#	257 +/- 22	#	230 +/- 24	#	233 +/- 23	#	

Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * significantly different than PRE, # significantly different than POST, ‡ significantly different than corresponding time point in control.

Table 3. +dF/dtmax at different frequencies of stimulation

	Time Points, n											
	PRE	**POST**		**R1**		**R2**		**R3**		**R6**	
10Hz, N/s												
C	1395 +/- 159	1106 +/- 112	*	942 +/- 126	*	1078 +/- 139	*	1065 +/- 112	*	1025 +/- 115	*
E	1443 +/- 183	466 +/- 84	*‡	942 +/- 106	*#	1114 +/- 134	*#	945 +/- 138	*#	933 +/- 128	*#
20Hz, N/s												
C	2290 +/- 229	2035 +/- 189		1842 +/- 192		2129 +/- 197		1896 +/- 116		1940 +/- 176	
E	2347 +/- 242	751 +/- 145	*‡	1797 +/- 161	#	2153 +/- 243	#	1901 +/- 236	#	1760 +/- 182	#
30Hz, N/s												
C	2864 +/- 271	2758 +/- 258		2586 +/- 280		2896 +/- 280		2610 +/- 170		2557 +/- 252	
E	3028 +/- 295	1221 +/- 176	*‡	2544 +/- 248	#	2958 +/- 327	#	2437 +/- 273	#	2359 +/- 235	#
50Hz, N/s												
C	3429 +/- 347	3326 +/- 324		3126 +/- 347		3421 +/- 316		3028 +/- 221		3097 +/- 292	
E	3647 +/- 350	1831 +/- 211	*‡	3178 +/- 330	#	3586 +/- 409	#	2929 +/- 329	#	2842 +/- 256	#
100Hz, N/s												
C	3615 +/- 284	3634 +/- 340		3536 +/- 391		3879 +/- 400		3425 +/- 197		3405 +/- 317	
E	3943 +/- 410	2373 +/- 202	*‡	3689 +/- 407	#	3972 +/- 452	#	3353 +/- 384	#	3155 +/- 288	#

Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * significantly different than PRE, # significantly different than POST, ‡ significantly different than corresponding time point in control.

Table 4. -dF/dtmax at different frequencies of stimulation

	Time Points, n											
	PRE	**POST**		**R1**		**R2**		**R3**		**R6**	
10Hz, N/s												
C	-1707 +/- 240	-1058 +/- 107	*	-902 +/- 119	*	-913 +/- 89	*	-1027 +/- 118	*	-958 +/- 118	*
E	-1544 +/- 226	-418 +/- 80	*‡	-824 +/- 95	*#	-968 +/- 87	*#	-913 +/- 116	*#	-876 +/- 117	*#
20Hz, N/s												
C	-2967 +/- 296	-2674 +/- 220		-2321 +/- 263	*	-2484 +/- 239		-2330 +/- 176	*	-2289 +/- 267	*
E	-2881 +/- 303	-1093 +/- 195	*‡	-2082 +/- 163	*#	-2314 +/- 221	*#	-2089 +/- 225	*#	-2142 +/- 207	*#
30Hz, N/s												
C	-3130 +/- 280	-3027 +/- 253		-2722 +/- 292		-2908 +/- 262		-2683 +/- 185		-2588 +/- 279	
E	-3063 +/- 296	-1698 +/- 209	*‡	-2513 +/- 211	#	-2719 +/- 256	#	-2364 +/- 249	#	-2464 +/- 244	#
50Hz, N/s												
C	-3115 +/- 270	-3111 +/- 277		-2793 +/- 292		-2951 +/- 266		-2655 +/- 186		-2607 +/- 275	
E	-3116 +/- 274	-2126 +/- 194	*‡	-2634 +/- 248		-2835 +/- 297		-2444 +/- 245		-2532 +/- 263	
100Hz, N/s												
C	-2847 +/- 198	-2918 +/- 252		-2686 +/- 299		-2821 +/- 257		-2547 +/- 161		-2464 +/- 281	
E	-2661 +/- 252	-2017 +/- 189	*‡	-2525 +/- 261		-2725 +/- 296		-2364 +/- 238		-2512 +/- 272	

Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * significantly different than PRE, # significantly different than POST, ‡ significantly different than corresponding time point in control.

Table 5. MVC Force, AEMG, and IT % Activation

	Time Points, n					
	PRE	POST	R1	R2	R3	R6
MVC Po, N						
C	557 +/- 54	500 +/- 48	477 +/- 54 *	503 +/- 53	478 +/- 39 *	508 +/- 58
E	557 +/- 58	313 +/- 16 *‡	467 +/- 46 *#	464 +/- 43 *#	448 +/- 40 *#	477 +/- 43 *#
AEMG, mV						
C	0.49 +/- 0.09	0.38 +/- 0.09	0.48 +/- 0.11	0.58 +/- 0.16	0.59 +/- 0.20	0.54 +/- 0.11
E	0.49 +/- 0.13	0.30 +/- 0.10	0.55 +/- 0.14	0.59 +/- 0.15	0.38 +/- 0.09	0.44 +/- 0.08
IT, %						
C	99 +/- 0.2	99 +/- 0.2	98 +/- 0.6	98 +/- 0.4	99 +/- 0.2	99 +/- 0.2
E	99 +/- 0.2	98 +/- 0.6	98 +/- 0.6	98 +/- 1.2	99 +/- 0.7	99 +/- 0.5

Measurements were taken before (PRE) and after (POST) exercise, and 24 (R1), 48 (R2), 72 (R3), and 144(R6) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * significantly different than PRE, # significantly different than POST, ‡ significantly different than corresponding time point in control.

Table 6. Average EMG and Force During Exercise

	Time Points, n					
	1min	5min	10min	20min	25min	30min
aEMG, mV						
C	0.003 +/- 0.002	0.004 +/- 0.001	0.003 +/- 0.001	0.004 +/- 0.001	0.005 +/- 0.002	0.004 +/- 0.002
E	0.14 +/- 0.039 3	0.18 +/- 0.064 13	0.26 +/- 0.087 123	0.27 +/- 0.106 123	0.27 +/- 0.097 123	0.26 +/- 0.091 123
Force, % MVC						
E	40.3 +/- 0.9	42.4 +/- 1.4	41.7 +/- 1.2	40.0 +/- 0.9	39.5 +/- 1.8	37.6 +/- 2.6 1

Measurements were taken every 5 min during exercise starting after the first minute of exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. 1 significantly different than 1 min, 2 significantly different than 5 min, 3 significantly different than corresponding time point in control.

Appendix 2: Study 2 Tabulated Force Data

Table 1. Twitch Characteristics

	Time Points, n						
	Pre E1	Post E1	Pre E2	Post E2	R1	R2	R3
Pt, N							
C	134 +/- 15	138 +/- 15	135 +/- 18	77 +/- 9 *#†	127 +/- 16 ‡	127 +/- 12 ‡	125 +/- 11 ‡
E	140 +/- 13	70 +/- 10 *•	136 +/- 12 #	101 +/- 13 *#†•	131 +/- 14 #‡	129 +/- 13 #‡	130 +/- 15 #‡
CT, ms							
C	97 +/- 4	106 +/- 7	105 +/- 8	103 +/- 5	113 +/- 12	101 +/- 5	98 +/- 5
E	93 +/- 5	82 +/- 8 •	99 +/- 5	109 +/- 10 #	103 +/- 7 #	104 +/- 6 #	108 +/- 8 #
1/2RT, ms							
C	48 +/- 4	44 +/- 3	44 +/- 3	40 +/- 2	48 +/- 3	50 +/- 4	48 +/- 4
E	61 +/- 5 •	35 +/- 1 *	54 +/- 6 #	40 +/- 3 *†	54 +/- 7 #‡	57 +/- 4 #‡	51 +/- 5 #
RT, ms							
C	58 +/- 3	61 +/- 3	59 +/- 3	61 +/- 3	62 +/- 4	61 +/- 3	60 +/- 3
E	56 +/- 3	52 +/- 5 *•	62 +/- 4 #	61 +/- 3 #	62 +/- 4 #	60 +/- 2 #	63 +/- 4 #
+dF/dtmax, N/s							
C	2318 +/- 354	2380 +/- 367	2415 +/- 452	1285 +/- 181 *#†	2042 +/- 293 ‡	2127 +/- 322 ‡	2092 +/- 271 ‡
E	2360 +/- 333	1278 +/- 242 *•	2299 +/- 319 #	1767 +/- 331 *#•	2088 +/- 293 #	2096 +/- 293 #	2067 +/- 321 #
-dF/dtmax, N/s							
C	-1893 +/- 193	-2215 +/- 185	-2190 +/- 270	-1375 +/- 177 #†	-1826 +/- 130	-1710 +/- 138	-1854 +/- 159
E	-1576 +/- 152	-1424 +/- 170 •	-1905 +/- 224	-1598 +/- 200	-1825 +/- 241	-1617 +/- 192	-1928 +/- 221

Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * significantly different than PRE E1, # significantly different than POST E1, † significantly different than PRE E2 ‡ significantly different than POST E2, • significantly different than corresponding time point in C.

Table 2. Force at different frequencies of stimulation

	Time Points, n												
	Pre E1	**Post E1**		**Pre E2**		**Post E2**		**R1**		**R2**		**R3**	
10Hz, N													
C	176 +/- 16	146 +/- 20	*	120 +/- 12	*#	61 +/- 6	*#†	121 +/- 16	*#‡	111 +/- 12	*#‡	128 +/- 18	*#‡
E	175 +/- 18	36 +/- 6	*•	117 +/- 7	*#	79 +/- 9	*#†•	114 +/- 14	*#‡	117 +/- 14	*#‡	121 +/- 12	*#‡
20Hz, N													
C	321 +/- 29	279 +/- 28	*	286 +/- 32	*	156 +/- 18	*#†	260 +/- 33	#‡	233 +/- 18	#‡	256 +/- 27	#‡
E	317 +/- 26	93 +/- 11	*•	267 +/- 16	*#	204 +/- 20	*#†•	240 +/- 23	*#‡	251 +/- 24	*#‡	262 +/- 22	*#‡
30Hz, N													
C	363 +/- 33	340 +/- 33		346 +/- 33		280 +/- 33	*#†	311 +/- 38		282 +/- 21	*†	307 +/- 34	
E	346 +/- 24	159 +/- 18	*•	326 +/- 24	#	308 +/- 28	#	294 +/- 33	#	305 +/- 28	#	314 +/- 25	#
50Hz, N													
C	363 +/- 30	365 +/- 33		368 +/- 34		371 +/- 39		334 +/- 40		303 +/- 23	*#†‡	327 +/- 34	
E	367 +/- 25	222 +/- 21	*•	346 +/- 23	#	370 +/- 30	#	313 +/- 35	#	328 +/- 31	#	335 +/- 27	#
100Hz, N													
C	382 +/- 29	371 +/- 33		372 +/- 35		387 +/- 39		335 +/- 39		309 +/- 23	*#†‡	327 +/- 33	*‡
E	387 +/- 27	256 +/- 22	*•	350 +/- 21	#	388 +/- 31	#	314 +/- 33	*#‡	328 +/- 29	*#‡	332 +/- 27	*#‡

Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * significantly different than PRE E1, # significantly different than POST E1, † significantly different than PRE E2 ‡ significantly different than POST E2, • significantly different than corresponding time point in C.

Table 3. +dF/dtmax at different frequencies of stimulation

	Time Points, n													
	Pre E1	**Post E1**		**Pre E2**		**Post E2**		**R1**		**R2**		**R3**	
10Hz, N/s														
C	1871 +/- 240	1705 +/- 274		1219 +/- 178	*#	670 +/- 90	*#†	1089 +/- 170	*#‡	1096 +/- 158	*#‡	1211 +/- 188	*#‡
E	1771 +/- 230	448 +/- 62	*•	1118 +/- 109	*#	942 +/- 126	*#•	1003 +/- 146	*#	1017 +/- 122	*#	1126 +/- 110	*#
20Hz, N/s														
C	3221 +/- 234	2911 +/- 283		2557 +/- 323	*	1616 +/- 198	*#†	2086 +/- 180	*#‡	2078 +/- 198	*#‡	2262 +/- 233	*#‡
E	2780 +/- 281	1058 +/- 134	*•	2231 +/- 223	*#	2102 +/- 217	*#	1984 +/- 105	*#	2038 +/- 205	*#	2084 +/- 189	*#
30Hz, N/s														
C	4055 +/- 358	4214 +/- 404		3866 +/- 371		3139 +/- 384	*#†	3119 +/- 276	*#†	2895 +/- 260	*#†	3256 +/- 317	*#†
E	3483 +/- 299	1902 +/- 195	*•	3050 +/- 286	•	3151 +/- 288		2918 +/- 174	#	2896 +/- 281	#	2921 +/- 255	#
50Hz, N/s														
C	4726 +/- 329	5111 +/- 540		4642 +/- 431		4938 +/- 576		3935 +/- 315	*#‡	3516 +/- 321	*#†‡	4067 +/- 366	#‡
E	4141 +/- 342	2888 +/- 273	*•	3833 +/- 331	•	4308 +/- 331	•	3543 +/- 193	‡	3617 +/- 361	‡	3477 +/- 313	#‡
100Hz, N/s														
C	5155 +/- 334	5612 +/- 570		5065 +/- 493		5337 +/- 423		4451 +/- 366	#‡	4056 +/- 389	*#†‡	4564 +/- 418	#‡
E	4561 +/- 308	3781 +/- 313	•	4379 +/- 359	•	5176 +/- 351		4111 +/- 259	‡	4061 +/- 402	‡	4063 +/- 360	‡

Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * significantly different than PRE E1, # significantly different than POST E1, † significantly different than PRE E2 ‡ significantly different than POST E2, • significantly different than corresponding time point in C.

Table 4. -dF/dtmax at different frequencies of stimulation

	Time Points, n						
	Pre E1	Post E1	Pre E2	Post E2	R1	R2	R3
10Hz, N/s							
C	-1710 +/- 148	-1267 +/- 202 *	-1043 +/- 145 *	-565 +/- 87 *#†	-1095 +/- 187 *‡	-896 +/- 132 *‡	-1076 +/- 158 *‡
E	-1550 +/- 196	-344 +/- 70 *•	-925 +/- 90 *#	-928 +/- 162 *#•	-973 +/- 155 *#	-884 +/- 112 *#	-1020 +/- 142 *#
20Hz, N/s							
C	-3895 +/- 316	-3325 +/- 289 *	-3460 +/- 393	-1714 +/- 282 *#†	-3024 +/- 413 *‡	-2621 +/- 256 *#†‡	-3052 +/- 404 *‡
E	-3744 +/- 254	-1099 +/- 124 *•	-3118 +/- 230 *#	-2708 +/- 394 *#•	-2789 +/- 316 *#	-2815 +/- 274 *#	-2971 +/- 293 *#
30Hz, N/s							
C	-4346 +/- 349	-4049 +/- 336	-4114 +/- 398	-4014 +/- 531	-3595 +/- 481 *	-3230 +/- 277 *#†‡	-3529 +/- 399 *
E	-4124 +/- 254	-2104 +/- 217 *•	-3807 +/- 290	-4454 +/- 468	-3385 +/- 413 *	-3468 +/- 325 *	-3534 +/- 293 *
50Hz, N/s							
C	-4228 +/- 328	-4130 +/- 315	-4160 +/- 374	-5442 +/- 533 *#†	-3737 +/- 514 ‡	-3404 +/- 287 *‡	-3601 +/- 358 ‡
E	-4128 +/- 275	-3004 +/- 241 *•	-3852 +/- 265	-5254 +/- 432 *#†	-3465 +/- 427 ‡	-3640 +/- 327 ‡	-3585 +/- 280 ‡
100Hz, N/s							
C	-3989 +/- 243	-4035 +/- 313	-4004 +/- 297	-5382 +/- 540 *#†	-3511 +/- 392 ‡	-3282 +/- 247 ‡	-3473 +/- 301 ‡
E	-3896 +/- 354	-3021 +/- 204 *•	-3735 +/- 274	-5026 +/- 402 *#†	-3438 +/- 452 ‡	-3489 +/- 321 ‡	-3484 +/- 346 ‡

Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * significantly different than PRE E1, # significantly different than POST E1, † significantly different than PRE E2 ‡ significantly different than POST E2, • significantly different than corresponding time point in C.

Table 5. MVC Force, AEMG, and IT % Activation

	Time Points, n						
	Pre E1	**Post E1**	**Pre E2**	**Post E2**	**R1**	**R2**	**R3**
MVC Po, N							
C	654 +/- 44	693 +/- 44	687 +/- 47	513 +/- 24 *#†	597 +/- 55 *‡	654 +/- 34 ‡	668 +/- 36 ‡
E	665 +/- 37	383 +/- 35 *•	693 +/- 42 #	493 +/- 52 *#†	612 +/- 41 #‡	653 +/- 54 #‡	683 +/- 49 #‡
aEMG, mV							
C	0.78 +/- 0.09	0.66 +/- 0.11	0.87 +/- 0.08	0.36 +/- 0.08	0.77 +/- 0.09	0.76 +/- 0.14	0.80 +/- 0.11
E	0.72 +/- 0.09	0.55 +/- 0.06	0.86 +/- 0.15	0.38 +/- 0.07	0.62 +/- 0.12	0.74 +/- 0.14	0.75 +/- 0.10
IT, %							
C	99 +/- 0.4	99 +/- 0.1	99 +/- 0.4	99 +/- 0.3	99 +/- 0.2	99 +/- 0.2	99 +/- 0.1
E	99 +/- 0.2	99 +/- 0.1	99 +/- 0.2	99 +/- 0.4	99 +/- 0.1	99 +/- 0.1	99 +/- 0.1

Measurements were taken before (PRE) and after (POST) isometric exercise (E1) and cycling exercise (E2), and 24 (R1), 48 (R2), and 72 (R3) hours after the exercise in both the exercised (E) and non-exercised (C) leg. Values are mean +/- standard error mean. * significantly different than PRE E1, # significantly different than POST E1, † significantly different than PRE E2 ‡ significantly different than POST E2, • significantly different than corresponding time point in C. Main effect of time ($p<0.05$) for aEMG, for aEMG Post E2 < PRE E2, R1, R2, & R3.

References

1. **Allen DG, Kabara AA and Westerblad H**. Muscle Fatigue: the role of intracellular calcium stores. *Can J Appl Physiol* 27: 83-96, 2002.

2. **Allen DG, Lamb GD and Westerblad H**. Skeletal Muscle Fatigue: Cellular Mechanisms. *Physiol Rev* 88: 287-332, 2008.

3. **Allen DG, Lannergren J and Westerblad H**. Muscle cell function during prolonged activity: Cellular mechanisms of fatigue. *Exp Physiol* 80: 497-527, 1995.

4. **Bolli R**. The Late Phase of Preconditioning. *Circ Res* 87: 972-983, 2000.

5. **Bolli R**. Preconditioning: A Paradigm Shift in the Biology of Myocardial Ischemia. *Am J Physiol Heart Circ Physiol* 292: H19-H27, 2007.

6. **Brooke MH and Keiser KK**. Muscle fiber types: how many and what kind? *Arch Neurol* 23: 369-379, 1970.

7. **Broome CS, Kayani AC, Palomero J, Dillmann WH, Mestril R, Jackson MJ and McArdle A**. Effects of lifelong overexpression of Hsp70 in skeletal muscle on age-related oxidative stress and adaptation after nondamaging contractile activity. *FASEB* 20: E855-E860, 2006.

8. **Cairns SP and Lindinger MI**. Do Multiple Ionic Interactions Contribute to Skeletal Muscle Fatigue? *J Physiol* 586: 4039-4054, 2008.

9. **Cooke R, Franks K, Luciani GB and Pate E**. The inhibition of rabbit skeletal muscle contraction by hydrogen ions and phosphate. *J Physiol* 395: 77-79, 1988.

10. **Craig AE**. The Heat Shock Response. *Crit Rev Biochem* 18: 239-280, 1985.

11. **Currie RW, Karmazyn M, Kloc M and Mailer K**. Heat-shock Response is Associated with Enhanced Postischemic Ventricular Recovery. *Circ Res* 63: 543-549, 1988.

12. **Dokladny K, Wharton W, Lobb R, Ma TY and Moseley PL**. Induction of physiological thermotolerance in MDCK monolayers: contribution of heat shock protein. *Cell Stress & Chaperons* 11: 268-275, 2006.

13. **Duhamel TA, Green HJ, Perco JG and Ouyang J**. Metabolic and Sarcoplasmic Reticulum Ca^{2+} Cycling Responses in Human Muscle 4 Days Following Prolonged Exercise. *Can J Physiol Pharmacol* 83: 643-655, 2005.

14. **Duhamel TA, Green HJ, Stewart RD, Foley KP, Smith IC and Ouyang J**. Muscle metabolic, SR Ca(2+) -cycling responses to prolonged cycling, with and without glucose supplementation. *J Appl Physiol* 103: 1986-1998, 2007.

15. **Duhamel TA, Stewart RD, Tupling AR, Ouyang J and Green HJ**. Muscle sarcoplasmic reticulum calcium regulation in humans during consecutive days of exercise and recovery. *J Appl Physiol* 103: 1212-1220, 2007.

16. **Edwards RHT, Hill DK, Jones DA and Merton PA**. Fatigue of long duration in human skeletal muscle after exercise. *J Physiol* 182: 92-109, 1977.

17. **Febbraio M and Koukoulas I**. Hsp72 Gene Expression Progressively Increases in Human Skeletal Muscle During Prolonged Exhaustive Exercise. *J Appl Physiol* 89: 1055-1060, 2000.

18. **Fischer CP, Hiscock N, Basu S, Vessby B, Kallner A, Sjoberg L, Febbraio M and Pedersen BK**. Vitamin E Isoform-Specific Inhibition of Exercise-Induced Heat Shock Protein 72 Expression in Humans. *J Appl Physiol* 100: 1679-1687, 2006.

19. **Flanagan SW, Ryan AJ, Gisolfi CV and Moseley PL**. Tissue-Specific HSP70 Response in Animals Undergoing Heat Stress. *Am J Physiol Regul Integr Comp Physiol* 268: R28-R32, 1995.

20. **Fowles CTM, Green HJ, Schertzer JD and Tupling AR**. Reduced activity of muscle $Na^{+/K+\text{-ATPase after prolonged running in rats}}$. *J Appl Physiol* 93: 1703-1708, 2002.

21. **Fowles CTM, Green HJ, Tupling AR, O'Brien S and Roy BD**. Human neuromuscular fatigue is associated with altered Na^{+}/K+-ATPase activity following isometric exercise. *J Appl Physiol* 92: 1585-1593, 2002.

22. **Fu M and Tupling AR**. Hsp70 Provides Functional Protection to the Cardiac Ca^{2+} Pump (SERCA2a) Against Heat Stress. *Applied Physiology, Nutrition, and Metabolism* 32: S32, 2007.

23. **Garlick PJ, Maltin CJ, Baillie AG, Delday MI and Grubb DA**. Fibre-type Composition of Nine Rat Muscles. II. Relation to Protein Turnover. *Am J Physiol Endocrinol Metab* 257: E828-E832, 1989.

24. **Gjovaag TF, Vikne H and Dahl HA**. Effects of Eccentric or Concentric Weight Training on the Expression of Heat Shock Proteins in M. Biceps Brachii of Very Well Trained Males. *Eur J Appl Physiol* 96: 355-362, 2006.

25. **Gonzalez B, Hernando R and Manso R**. Stress Proteins of 70 kDa in Chronically Exercised Skeletal Muscle. *Eur J Appl Physiol* 440: 42-49, 2000.

26. **Green HJ**. Mechanisms of muscle fatigue in intense exercise. *J Sports Sci* 15: 247-256, 1997.

27. **Green HJ**. Cation pumps in skeletal muscle: potential role in muscle fatigue. *Acta Physiol Scand* 162: 201-213, 1998.

28. **Green HJ, Symon S and Daley R**. Attenuation of Human Quadriceps Fatigue with Caffeine (abstract). *Canadian Journal of Applied Sports Science* 15: 11S, 1990.

29. **Green HJ, Tupling AR, Roy BD, O`Toole D, Burnett M and Grant S**. Adaptations in Skeletal Muscle Exercise Metabolism to a Sustained Session of Heavy Intermittent Exercise. *Am J Physiol Heart Circ Physiol* 278: E118-E126, 2000.

30. **Guo Y, Wu WJ, Zhu XP, Li Q, Tang XL and Bolli R**. Exercise-induced Late Preconditioning is Triggered By Generation of Nitric Oxide. *J Mol Cell Cardiol* 33: A41, 2001.

31. **Gutierrez-Martin Y, Martin-Romero FJ, Inesta-Vaquera FA, Gutierrez-Merino C and Henao F**. Modulation of Sarcoplasmic Reticulum Ca^{2+}-ATPase by Chronic and Acute Exposure to Peroxynitrite. *Eur J Biochem* 271: 2647-2657, 2004.

32. **Hernando R and Manso R**. Muscle Fibre Stress in response to Exercise: Synthesis, Accumulation and Isoform Transition of 70-kDa Heat-Shock Proteins. *FASEB* 243: 460-467, 1997.

33. **Iwaki K, Chi SH, Dillmann WH and Mestril R**. Induction of HSP70 in Cultured Rat Neonatal Cardiomyocytes By Hypoxia and Metabolic Stress. *Circulation* 87: 2023-2032, 1993.

34. **Jones DA, Newham DJ and Torgan C**. Mechanical infuences on long-lasting human muscle fatigue and delayed-onset pain. *J Physiol* 412: 415-427, 1989.

35. **Khassaf M, Child RB, McArdle A, Brodie DA, Esanu C and Jackson MJ**. Time Course of Responses of Human Skeletal Muscle to Oxidative Stress Induced by Non Damaging Exercise. *J Appl Physiol* 90: 1031-1035, 2001.

36. **Kim K, Kim M and Lee D**. The Effect of Exercise in Cool, Control, and Hot Environments on Cardioprotective Hsp70 Induction. *J Physiol Anthropol Appl Hum Sci* 23: 225-230, 2006.

37. **Kregel KC**. Heat Shock Proteins: Modifying factors in Physiological Stress Responses and Acquired Thermotolerance. *J Appl Physiol* 92: 2177-2186, 2002.

38. **Kuboki S, Schuster R, Blanchard J, Pritts TA, Wong HR and Lentsch AB**. Role of Heat Shock Protein 70 in Hepatic Ischemia/Reperfusion Injury in Mice. *Am J Physiol Gastrointest Liver Physiol* 292: G1141-G1149, 2007.

39. **Laemmli UK**. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685, 1970.

40. **Lamb GD**. Excitation-contraction coupling and fatigue mechanisms in skeletal muscle: Studies with mechanically skinned fibres. *J Mus Res Cell Motil* 23: 81-91, 2002.

41. **Leberer E, Hartner KT and Pette D**. Reversible Inhibition of Sarcoplasmic Reticulum Ca^{2+}-ATPase by Altered Neuromuscular Activity in Rabbit Fast-Twitch Muscle. *FEBS* 162: 555-561, 1987.

42. **Liu Y, Lormes W, Baur C, Opitz-Gress A, Altenburg D, Lehmann M and Steinacker AM**. Human Skeletal Muscle Hsp70 Response to Physical Training Depends on Exercise Intensity. *Int J Sports Med* 21: 351-355, 2000.

43. **Liu Y, Lormes W, Wang L, Reissnecker S and Steinacker AM**. Different Skeletal Muscle Hsp70 Response to High-Intensity Strength Training and Low-Intensity Endurance Training. *Eur J Appl Physiol* 91: 330-335, 2004.

44. **Liu Y, Mayr S, Opitz-Gress A, Zeller C, Lormes W, Baur C, Lehmann M and Steinacker AM**. Human Skeletal Muscle Hsp70 Response to Training in Highly Trained Rowers. *J Appl Physiol* 86: 101-104, 1999.

45. **Lowry OH, Rosebrough NJ, Farr AL and Randall RJ**. Protein Measurement with Folin Phenol Reagent. *JBC* 193: 265-275, 1951.

46. **Luckin KA, Favero TG and Klug GA**. Prolonged Exercise Induces Structural Changes in SR Ca^{2+}-ATPase of Rat. *Biochem Med Metab Biol* 46: 391-405, 1991.

47. **MacLennan DH, Abu-Abed M and Kang C**. Structure-function relationships in Ca^{2+} cycling proteins. *J Mol Cell Cardiol* 34: 897-918, 2002.

48. **MacLennan DH, Rice WJ and Green NM**. The mechanism of Ca2+ transport by sarco(endo)plasmic reticulum Ca2+-ATPases. *JBC* 272: 28815-28818, 1997.

49. **Marber MS, Mestril R, Chi SH, Sayen R, Yellon YM and Dillmann WH**. Overexpression of the rat inducible 70-kDa stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J Clin Invest* 95: 1446-1456, 1995.

50. **Marber MS, Mestril R, Chi SH, Sayen R, Yellom YM and Dillmann WH**. Overexpression of the Rat Inducible 70-kDa Stress Protein in a Transgenic Mouse Increases thed Resistance of the Heart to Ischemic Injury. *J Clin Invest* 95: 1446-1456, 1995.

51. **McHugh MP, Connolly DAJ, Eston RJ and Gleim GW**. Exercise-induced Muscle Damage and Potential Mechanisms for the Repeated Bout Effect. *Sports Med* 27: 157-170, 1999.

52. **Milne KJ and Noble EG**. Exercise-Induced Elevations of Hsp70 is Intensity Dependant. *J Appl Physiol* 93: 561-568, 2002.

53. **Mittendorfer B, Andersen JL, Plombgaard P, Saltin B, Babraj JA, Smith K and Rennie MJ**. Protein Synthesis Rates in Human Muscles: Neither Anatomical Location Nor Fibre-type Composition are Major Determinants. *J Physiol* 563: 203-211, 2005.

54. **Morton JP, MacLaren DPM, Cable NT, Bongers T, Griffiths RD, Campbell IT, Evans L, Kayani A, McArdle A and Drust B**. Time Course and Differential Responses of the Major Heat Shock Protein Families in Human Skeletal Muscle Following Nondamaging Treadmill Exercise. *J Appl Physiol* 101: 176-182, 2006.

55. **Neufer PD, Ordway GA, Hand GA, Shelton JM, Richardson JM, Benjamin IJ and Williams RS**. Continuous contractile activity induces fiber type specific expression of HSP70 in skeletal muscle. *Am J Physiol Cell Physiol* [271]: [C1828-C1837], 1996.

56. **Powers SK and Jackson MJ**. Exercise-induced Oxidative Stress: Cellular Mechanisms and Impact on Muscle Force Production. *Physiol Rev* 88: 1243-1276, 2008.

57. **Puntschart A, Vogt M, Widmer HR, Happeler H and Billeter R**. Hsp70 Expression in Human Skeletal Muscle After Exercise. *Acta Physiol Scand* 157: 411-417, 1996.

58. **Reid MB and Durham WJ**. Generation of reactive oxygen and nitrogen species in contracting skeletal muscle: Potential impact on aging. *Ann NY Acad Sci* 959: 108-116, 2002.

59. **Ritossa F**. A New Puffing Pattern Induced By Temperature Shock and DNP in *Drosophila*. *Experientia* 18: 571-573, 1962.

60. **Ruell PA, Booth J, McKenna MJ and Sutton JR**. Measurement of sarcoplasmic reticulum function in mammalian skeletal muscle. Technical aspects. *Anal Biochem* 228: 194-201, 1995.

61. **Salo DC, Donovan CM and Davies KJA**. Hsp70 and Other Possible Heat Shock or Oxidative Stress Proteins are Induced in Skeletal Muscle, Heart, and Liver During Exercise. *Free Radic Biol Med* 11: 239-246, 1991.

62. **Sciandra JJ and Subjeck JR**. The Effect of Glucose on Protein Synthesis and Thermosensitivity in Chinese Hamster Ovary Cells. *JBC* 258: 12091-12093, 1983.

63. **Simonides WS and van Hardeveld C**. An assay for sarcoplasmic reticulum Ca^{2+}-ATPase activity in muscle homogenates. *Anal Biochem* 191: 321-331, 1990.

64. **Smolka MB, Zoppi CC, Alves AA, Silveira LR, Marangoni S, Pereira-Da-Silva L, Novello JC and Macedo DV**. Hsp72 as a Complementary Protection Against Oxidative Stress Induced by Exercise in the Soleus Muscle of Rats. *Am J Physiol Regul Integr Comp Physiol* 279: R1539-R1545, 2000.

65. **Stewart RD, Duhamel TA, Foley KP, Ouyang J, Smith IC and Green HJ**. Protection of muscle membrane excitability during prolonged cycle exercise with glucose supplementation. *J Appl Physiol* 103: 331-339, 2007.

66. **Stewart RD, Duhamel TA, Rich S, Tupling AR and Green HJ**. Effects of consecutive days of exercise and recovery on muscle mechanical function. *Med Sports Sci* 40: 316-325, 2008.

67. **Thompson HS, Clarkson P and Scordilis SP**. The Repeated Bout Effect and Heat Shock Proteins: Intramuscular Hsp72 and Hsp70 Expression Following Two Bouts of Eccentric Exercise in Humans. *Acta Physiol Scand* 174: 47-56, 2002.

68. **Thompson HS, Scordilis SP, Clarkson P and Lohrer WA**. A Single Bout of Eccentric Exercise Increases Hsp27 and Hsc/Hsp70 in Human Skeletal Muscle. *Acta Physiol Scand* 171: 187-193, 2001.

69. **Tupling AR, Bombardier E, Stewart RD, Vigna C and Aqui AE**. Muscle Fiber-Type Specific Responses of Hsp70 Protein Expression in Human Quadriceps Following Acute Isometric Exercise. *J Appl Physiol* 103: 2105-2111, 2007.

70. **Tupling AR, Bombardier E, Vigna C, Quadrilatero J and Fu M**. Interactions Between Hsp70 and the SR Ca^{2+} Pump: A Potential Mechanism for Protection in Heart and Skeletal Muscle. *Applied Physiology, Nutrition, and Metabolism* 33: 1023-1032, 2008.

71. **Tupling AR, Gramolini AO, Duhamel TA, Kondo H, Asahi M, Tsuchiya SC, Borrelli MJ, Lepock JR, Otsu K, Hori M, MacLennan DH and Green HJ**. Hsp70 Binds to the Fast-Twitch Skeletal Muscle Sarco(endo)plasmic Reticulum Ca^{2+}-ATPase (SERCA1a) and Prevents Thermal Inactivation. *JBC* 279: 52382-52389, 2004.

72. **Tupling AR and Green HJ**. Silver ions induce Ca^{2+} release from the SR in vitro by acting on both the Ca^{2+} release channel and the Ca^{2+} pump. *J Appl Physiol* 92: 1603-1610, 2002.

73. **Tupling AR, Green HJ, Roy BD, Grant S, Burnett M and Ranney D**. Post Contractile Force Depression in Humans is Associated With an Impairment in SR Ca^{2+} Pump Function. *Am J Physiol Regul Integr Comp Physiol* 278: R87-R94, 2000.

74. **Tupling AR, Green HJ, Roy BD, Grant S and Ouyang J**. Paradoxical Effects of Prior Activity on Human Sarcoplasmic Reticulum Ca^{2+}-ATPase Responses to Exercise. *J Appl Physiol* 95: 138-144, 2003.

75. **Verburg E, Murphy RM, Stephenson DG and Lamb GD**. Disruption of excitation-contraction coupling and titin by endogenous Ca2+-activated proteases in toad muscle fibres. *J Physiol* 564: 775-790, 2005.

76. **Vigna C, Stewart RD, Aqui AE, Duhamel TA, Ng P and Tupling AR**. Delayed Recovery of Force and SR Ca^{2+} Pump Function in Human Quadriceps Following Intense Isometric Exercise. *Applied Physiology, Nutrition, and Metabolism* 31 Supplement: S86, 2006.

77. **Vigna C, Stewart RD, Aqui AE, Duhamel TA, Ng P and Tupling AR**. Delayed Recovery of force and SR Ca2+ Pump Function in Human Quadriceps Following Intense Isometric Exercise. *Applied Physiology, Nutrition, and Metabolism* 2008.

78. **Voss MR, Stallone JN, Li M, Cornelussen RM, Kneufermann P and Knowlton AA**. Gender differences in the expression of heat shock proteins: the effect of estrogen. *Am J Physiol Heart Circ Physiol* 285: H687-H692, 2003.

79. **Wallen ES, Buettner GR and Moseley PL**. Oxidants Differentially Regulate the Heat Shock Response. *Int J Hyperthermia* 13: 517-524, 1997.

80. **Weitzel G, Pilatus U and Rensing L**. Similar Dose Response of Heat Shock Protein Synthesis and Intracellular pH Change in Yeast. *Exp Cell Res* 159: 252-256, 1985.

81. **Xu KY, Zweier JL and Becker LC**. Hydroxyl radical inhibits sarcoplasmic reticulum Ca2+-ATPase function by direct attack on the ATP binding site. *Circ Res* 80: 76-81, 1997.