2008

CELLULAR LOCALIZATION AND EXPRESSION OF HSP70 AND HSF1 IN RAT SKELETAL MUSCLES FOLLOWING EXERCISE AND HEAT SHOCK

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CELLULAR LOCALIZATION AND EXPRESSION OF HSP70 AND HSF1 IN RAT SKELETAL MUSCLES FOLLOWING EXERCISE AND HEAT SHOCK

Spine Title: (Cellular localization of Hsp70 and HSF1 in skeletal muscle)

Thesis Format: (Integrated Article)

by

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Graduate Program in Kinesiology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

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ABSTRACT

The objective of the present study was to examine the stress-specific and muscle-specific differences in the localization of Hsp70 and HSF1 following two stressful conditions, exercise and heat shock. Two fast muscles, the plantaris (Plnt) and the white vastus (WV) muscles were harvested from adult male Sprague-Dawley rats either 30 min and 24 hr post-exercise (EX) (1 hour treadmill run, 30m/min, 2% grade) or post-heat shock (HS) (41.5-42.0 °C for 15 min). Western blots demonstrated an ~2 fold and an ~3.5 fold increase in Hsp70 in the Plnt and an ~5.5 fold and an ~9 fold increase in the WV 24 hr post-EX and 24 hr post-HS, respectively ($P<0.05$). Both stressors induced Hsp70 in the endothelium and smooth muscle compartments but unlike heat shock, the exercise response was characterized by fiber type-specific differences that varied between muscles. At the transcriptional level, increases in phosphorylated HSF1 (pHSF1) did not exhibit similar fiber specific expression supporting a role for additional downstream regulation of this response.

Keywords: fiber type, hyperthermia, immunofluorescence, vascular smooth muscle, vascular endothelium, transcription factor

CO-AUTHORSHIP

E.G. Noble
Involved in the design of the experiment

ACKNOWLEDGEMENTS

The past two years have been an unbelievable learning experience. I feel that the help and support of many people have not only contributed to the completion of my Masters degree but have also prepared me for the next stages of my life. The time I spent in the lab has been a great learning experience for me and I am thankful for the opportunity to have met and worked with many wonderful people.

First and foremost, I would like to thank Dr. Earl Noble for your guidance throughout this project. I am thankful for your encouragement, ideas, and countless hours of work, which enabled me to complete this thesis. You have been an incredible teacher and advisor both inside and outside the lab.

As a part of the Exercise Biochemistry Lab, I have been able to work with many unforgettable individuals. I would like to thank Dr. Kevin Milne, who always took the time to answer my questions. Without your help and expertise I would have been lost. To the boys in the lab, Steve Wolff and Bruce Frier, you have offered me a tremendous amount of support and friendship throughout my time in the lab. Thank you all for your encouragement and I wish you the best in your future endeavors. Of course, many thanks to Tom Dzialoszinski, Marcus Van Aaren and Mike Sawchuk, your continued support throughout my degree will always be greatly appreciated.

To my parents, family and friends, I can only hope to offer you the same unconditional support you have given me. Dana, you know me best, thank you for your unwavering support and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

Con	Control
DAPI	4', 6-diamidino-2-phenylindole
EC	Endothelial cells
EX	Exercise
HS	Heat shock
HSF1	Heat shock transcription factor 1
Hsp	Heat shock protein
Ig	Immunoglobulin
LAM	Laminin
MHC	Myosin heavy chain
PBS	Phosphate buffered saline
pHSF1	Phosphorylated heat shock transcrition factor 1
Plnt	Plantaris muscle
SDS-PAGE	Sodium dodecyl suplphate – polyacrylamide get electrophoresis
SE	Standard error
SM	Smooth muscle
TBS	Tris buffered saline
vWF	Von Willebrand factor
WV	White portion of the vastus lateralis muscle

CHAPTER 1

1.1 Introduction

To combat protein-related homeostatic disruption, cells respond by synthesizing a family of highly conserved, cytoprotective proteins, called heat shock proteins (Hsps). In 1962, Ritossa (38) first identified the cellular stress response as a heat-induced reversible change in the puffing pattern of chromosomes of the fruit fly *Drosophila buschii*. The puffs were identified as sites of active transcription and it was concluded that 'heat shock' had led to activation of specific genes, increased RNA translation, and subsequent synthesis of Hsps (38). As research into the cellular stress response advanced, heat stress was shown to induce a number of proteins with different molecular weights, ranging from 15-110 kilodaltons. Further investigations revealed that cellular stresses other than heat, such as hypoxia, glucose deprivation, decreased pH, and amino acid analogues also caused synthesis of Hsps (extensively reviewed in (48)). The term heat shock protein and stress protein (SP) are used interchangeably and refer to families of heat shock proteins (HSP/SP) categorized by molecular weight and/or individual protein isoforms (Hsp) within each family.

The most prominent HSPs are small Hsps, Hsp60, HSP70 and HSP90. Of these, the 70-kilodalton family is the best characterized. HSP70 proteins have been identified in many organisms and their primary structures are highly conserved across all species investigated (16). Furthermore, HSP70 proteins are expressed in a variety of tissues, including heart (34; 39), liver (39), skeletal muscle (20), and brain (6).

1.2 HSP70 family

Members of the 70-kilodalton family are among the most highly induced HSPs in response to a wide variety of stresses. The HSP70 family is composed of several isoforms including the constitutively expressed isoform, Hsp73, and the highly inducible member, Hsp70. Although these two proteins exhibit high sequence homology (95%), similar biochemical properties, and are thought to carry out similar functions, they differ in their expression (49). Specifically, Hsp73 is constitutively expressed in the cytoplasm of cells under normal conditions and generally does not exhibit high levels of induction following stress, whereas Hsp70 is highly stress inducible (19). As such, it has been proposed that both Hsp73 and Hsp70 act during non-stressed conditions, and upon stress more Hsp70 is produced to help regain homeostasis.

HSP70 proteins are involved in a number of functions within cells. Notably, they aid in proper protein formation during translation, translocate proteins within the cell, and bind and refold denatured or aggregated proteins (1; 2). Generally, all of these functions aid in the maintenance of cellular protein homeostasis.

Expression of inducible Hsp70 is regulated primarily at the transcriptional level by pre-existing heat shock transcription factors, of which HSF1 is the most important in regulating the response to stress. HSF1 interacts with a heat shock element (HSE) located on the promoter region of the Hsp70 gene to induce transcription (22). Under normal conditions, HSF1 is a monomeric protein, which upon activation undergoes a multistep cascade of events including, nuclear localization, oligomerization, HSE DNA binding and transcriptional activation (40). Reports have indicated that HSF1-HSE binding alone is not sufficient to induce transcriptional activity of HSF1, and that

additional regulation is required for HSF1 transcriptional activation. It has been demonstrated that anti-inflammatory drugs can induce HSF1 to bind to HSE DNA without the occurrence of transcription (10). Furthermore, it is believed that HSF1 undergoes hyperphosphorylation on specific serine residues following acquisition of HSE DNA binding to initiate activity (3). Specifically, Holmberg et al (8) demonstrated that phosphorylation of site ser230 on HSF1 is essential for activation and largely contributes to the stress-induced transcriptional activity of HSF1, and hence the induction of Hsp70.

1.3 Protective role of Hsp70

While acting as a molecular chaperone and performing functions associated with protein synthesis, repair, and transport, Hsp70 has been shown to behave as a protective agent. Initial studies demonstrated that cells in culture, having produced Hsp70 proteins following exposure to elevated temperatures, could survive a subsequent exposure to a normally lethal heat shock, with little or no damage (reviewed in (17)). Since then, most work on the protective role of Hsp70 in whole organisms has been conducted in the heart tissue. Karmazyn et al. (11) observed enhanced postischemic ventricular recovery of rat heart following a bout of heat shock (which also raises the levels of Hsp70 in the heart). Similarly, studies employing transgenic mice to overexpress Hsp70 revealed enhanced myocardial function following a period of ischemia compared to wild type animals (25; 37). As well, increased expression of Hsp70 via gene transfection was deemed responsible for enhanced myocardial tolerance to ischemic injury (43).

While it is apparent that Hsp70 has significant physiological implications in the heart, studies examining skeletal muscles have found conflicting evidence regarding the

protective role of Hsp70. Lepore et al. (14) found that after 2 hours of ischemia followed by 24 hours of reperfusion, gastrocnemius muscle viability was 86% in rats previously subjected to a mild heat stress compared with only 11% for control animals. If true, this finding could be applied to help decrease muscle damage following surgery. Moreover, research by Naito et al. (28), showed that Hsp70 induced by heat stress, significantly attenuated the amount of atrophy seen in the postural soleus muscle of rats after eight days of hindlimb unweighting compared with control animals. Hsp70 could therefore help attenuate muscle atrophy resulting from periods of limb immobilization and/or maintain muscle mass during periods of weightlessness, such as in space. More recently, McArdle et al. (26) demonstrated a dramatic improvement in the capacity to develop maximum tetanic force in the extensor digitorum longus muscles of old transgenic mice with high levels of Hsp70 compared to old wild-type mice 3 days after a severe lengthening contraction protocol. This finding is especially pertinent considering the current trend to an aging population. Other observations, however, suggest that Hsp70 may not play a protective role in skeletal muscle. Lille et al. (15) found no differences in gracililis muscle viability between control and heat stressed rats, either 24 or 48 hours after 4 hours of ischemia followed by 3 hour reperfusion, despite the approximate ten-fold increase in Hsp70 levels in the muscle of heat shocked animals. Similarily, Nosek et al. (30) observed no differences in total protein content and the maximum isometric force generating capabilities in the extensor digitorum longus, soleus, and diaphragm muscles of transgenic mice overexpressing Hsp70 compared with control animals. These results were similar to those which had been reported by Thomas and Noble (45), in which plantaris muscles from rats subjected to heat shock (41.5°C for 15 min) either 1 or 4 days

prior to a fatiguing protocol at 40Hz (5 minutes and 20 seconds), showed a detriment in contractile force during low frequency stimulation similar to non-heat shocked control rats at all time points measured after the fatiguing protocol. From these studies, it is clear that the protective role of Hsp70 in skeletal muscle needs to be further clarified.

1.4 Hsp70 in response to exercise

While heat shock is the classic means by which Hsp70 is elevated, it is unreasonable to suggest that subjecting individuals to a heat of ~42°C is a viable means to induce this protein in a clinical setting. Fortunately, as noted earlier, in addition to elevated temperatures, stresses such as hypoxia, increased intracellular calcium, decreased pH, and decreased energy stores also elicit production of Hsp70 (12). Interestingly, these known inducers of stress proteins are often characteristic of intensely exercised skeletal muscle (4). Therefore, because of the potential clinical benefits of Hsp70, and because exercise is a more practical means to induce stress proteins than heat shock, Hsp induction with exercise has become an area of active research. However, as with heat shock, the potential role of exercised-induced Hsp70 is less understood in skeletal than cardiac muscle. Hence, it is of great interest to further characterize the degree and nature of the response in skeletal muscle. Locke et al. (20) were among the first investigators to demonstrate that rats produce Hsp70 in skeletal muscle following a bout of treadmill running. However, the observation that post-exercise body temperatures (41°C) were similar to those often seen following heat shock, raised the question of whether the response was solely the result of hyperthermia, as suggested by some (7), or whether metabolic stresses contributed as well.

To address this issue, Skidmore et al. (41) exercised rats at different ambient temperatures to control for changes in core body temperature during exercise. Following an exercise bout in a cold room the gastrocnemius and soleus muscles displayed enhanced Hsp70 expression compared to control animals, despite minimal increases in body temperature. Although it was determined that increased temperature was not the sole factor responsible for exercise-associated increases in Hsp70, the exercise group did experience elevated temperatures and displayed even greater levels of Hsp70 than their cold room exercised counterparts. Therefore, while metabolic factors alone may induce the stress response, exercise-induced hyperthermia may still be an important component of Hsp70 production.

1.5 Skeletal muscle expression of Hsp70

Skeletal muscle is a heterogeneous system with regard to the constitutional proteins and energy metabolism of its various components (36). One of the most important functions of skeletal muscle is to facilitate movement of the body, which can cause a variety of cellular changes (42), including Hsp induction (5; 18; 19). Skeletal muscle is composed of individual muscle fibers, which in turn are comprised of contractile proteins. One of the major contractile proteins is the myosin heavy chain (MHC), which exists as several isoforms. MHC I for example, is present in slow-twitch fiber types which have a high oxidative capacity, while MHC IIx and IIb are found in fast-twitch fiber types which have a high glycolytic capacity (an intermediate isoform of MHC is MHC IIa) (36). As a result of their individual metabolic and contractile characteristics, muscles fibers are recruited in a specific order with increasing exercise

intensity. Type I fibres are recruited first, followed by IIa fibres, then IIx, and finally IIb fibres.

It has been known for some time that under normal, unstressed conditions, levels of Hsp70 are variable across muscles with different fibre type compositions (29). For example, the rodent soleus, which is rich in slow oxidative (SO) fibres (87% SO and 13% FOG), expresses high levels of Hsp70 at rest, whereas muscles comprised primarily of IIx/IIb fibres, such as the white portion of the vastus (0% SO, 3% FOG, 97% FG) contain nearly undetectable constitutive levels of Hsp70 (21). In muscles of mixed fiber compostition, such as the plantaris, Hsp70 content at rest is roughly proportional to the percentage of type I and IIa fibers (32). This phenomenon may be linked to the proportion of MHC I and IIa in a given muscle and/or the continual stresses due to postural functions often associated with these muscles.

Hsp70 expression also differs between slow and fast muscles following exercise. Milne et al. (27) observed that in the slow twitch soleus, Hsp70 levels were greatest following treadmill running at 18m/min, remained significantly elevated up to 27m/min, and declined thereafter. Conversely, in the fast contracting white portion of the vastus, Hsp70 concentrations were significantly increased at 27m/min and greatest at 33m/min. This demonstrates that the exercise induced increase in Hsp70 exhibits an intensity-dependent threshold, whereby the response in skeletal muscle is potentially related to recruitment patterns. Interestingly, the magnitudes of the stress response differed in the soleus and white portion of the vastus, in that, the soleus showed a 1.5 fold increase in Hsp70 compared to a 6 fold increase in the white vastus. Similar observations have been shown in muscles following heat shock. Oishi et al. (33) observed that in the deep region

of the gastrocnemius containing slow fibers, Hsp70 levels increased 2 fold and were greatest 8 hours after heat shock. Conversely, in the superficial regions of the gastrocnemius containing fast fibers, Hsp70 levels increased 10 fold were greatest 36 hours following heat shock. These results suggest fast muscle fibers are more responsive than slow fibers following heat stress. The deep gastrocnemius constitutively expresses high Hsp70 levels, and therefore may be more protected during stress than the superficial gastrocnemius (23).

1.6 Fiber type expression of Hsp70

As well as differences between fast and slow muscles, Hsp70 expression also differs in a fiber type specific manner within muscles of mixed fiber composition. At rest, immunohistochemical analysis of sedentary rabbit tibialis anterior muscle revealed that Hsp70 is constitutively expressed in type I and IIa fibers, exclusively (29). Following continuous low-frequency motor nerve stimulation, expression of Hsp70 remained restricted to type I and IIa fibers during the first 3 days of stimulation, and only after 21 days of continuous stimulation was Hsp70 expression present in a majority of type II fibers. Likewise, O'Neill et al. (31) observed constitutive expression of Hsp70 restricted to type I and IIa fibers in rat plantaris muscles subjected to compensatory overload. This fiber specific pattern of Hsp70 expression was maintained for 28 days. More recently, Tupling et al. (46) found that following acute isometric exercise in human quadriceps muscle, peak Hsp70 expression increased in type I fibers by 87% but was unchanged in type II fibers. Conversely, studies have shown increased Hsp70 levels following exercise in muscles such as the white portion of the vastus and gastrocenemius,

which contain significant proportions of type IIx/IIb fibers (27; 35). Essentially devoid of MHC I, Hsp70 content in these muscles reached levels comparable to those in muscles high in MHC I. However, because whole muscle homogenates were used to measure Hsp70 levels, these studies did not specifically investigate the potential for a fiber specific response within the exercised muscle. It therefore remains unclear as to whether the exercised-induced stress response in these muscles is general throughout the entire muscle, perhaps as a consequence of elevated temperature, or whether Hsp70 production is restricted to specific fibers as a consequence of recruitment.

1.7 Vascular expression of Hsp70

Vessels supply blood and nutrients to all tissues and organs and are comprised of endothelial cells and in larger vessels, underlying layers of smooth muscle cells. These cells, when subjected to stressful conditions, have been shown to induce Hsp70 (9; 24; 47). Specifically, mechanical stresses, such as shear stress and stretch stress have been shown to induce Hsp70 in myocardial endothelial cells. Wang et al. (47) observed dramatic increases in Hsp70 expression in cultured bovine aortic endothelial cells following 6 hours of laminar shear stress. Furthermore, Hsp70 induction has been shown to be associated with stress fiber formation in rat arterial endothelial cells occurring as a result of stretching of the vasculature (24). As well as mechanical stress, heat stress has also been observed to increase Hsp70 in the vascular cells of the heart. Using arterial whole-mounts, Leger et al. (13) demonstrated robust increases in Hsp70 that were exclusively localized to the vasculature 24 hours after a 15-minute bout of heat shock.

In regards to skeletal muscle, Tarricone et al. (44) observed increases in Hsp70 in smooth muscle vascular cells of rat muscles following both chronic exercise training and chronic hypoxia. Immunocytochemistry revealed Hsp70 in both the arterial and venous smooth muscle following exercise training, and in venous smooth muscle following hypoxia. Hsp70 was not examined in the endothelium. Aside from this study, Hsp70 expression and response in skeletal muscle vasculature is limited and therefore requires further study. Hsp70 induced in endothelial and smooth muscle cells in response to stresses associated with exercise, such as shear/stretch stress and increases in temperature, may play a role in protecting the skeletal muscle vasculature, and in addition may aid in the protection of the muscle itself from injuries such as ischemia (9).

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CHAPTER 2

2.1 Introduction

Hsp70, the inducible isoform of the 70kDa family of heat shock proteins, significantly increases in cardiac and skeletal muscle following a variety of environmental and intracellular stresses (20). Heat shock is the traditional and often used method to induce the expression of Hsp70. While studies in the heart have characterized the localization of Hsp70 following heat shock (1; 16), the cellular distribution in skeletal muscle has yet to be fully examined. As such, the role of Hsp70 in skeletal muscle seems less clear than in the myocardium (21; 33). In the heart, studies have demonstrated the induction of Hsp70 following heat shock occurred solely in the vasculature (1; 16). In fact, the cardiomyocytes remain resistant to the induction of Hsp70, indicating that the mechanism of protection occurs primarily through the vessels (1). The heart acts as a functional syncytium, whereby all fibers are recruited with each beat, as opposed to skeletal muscle which contracts as individual motor units. This may result in a differential sensitivity to the stress activity from that observed in the myocardium.

In skeletal muscle the effects of differential recruitment can be observed in differences in heterogeneity of muscle fibers in specific skeletal muscles and indeed within muscles. For example, frequently recruited muscles like the soleus have high proportions of high oxidative type I and IIa fibers, whereas less frequently recruited portions of muscle, such as the white portion of the vastus lateralis have the low oxidative type IIx and IIb fibres. Importantly, rat hindlimb muscle Hsp70 is highly abundant in more frequently recruited muscles, such as the soleus, compared to those that are less frequently recruited (11; 21; 25). This suggests that many of the factors associated with

exercise, including increased temperature, ischemia (18; 32), decreased pH (40) and decreased energy stores (8) which are known to induce Hsp70, may do so in a fiber specific pattern unlike in the myocardium. However, the fiber specific localization of Hsp70 has not been extensively examined and of those studies that have done so, few have examined the effect of exercise on the heat shock response in the vasculature. Therefore, it remains unclear as to whether the exercised-induced stress response in these muscles produces a response similar to that observed in the heart following heat shock, primarily as consequence of the increase in temperature, or whether Hsp70 production can be restricted to specific fibers, perhaps as a consequence of recruitment.

The present investigation will examine the expression of Hsp70 in skeletal muscle in response to a moderate to high intensity exercise bout and an acute exposure to whole-body heat shock. Two fast muscles, the plantaris (Plnt) and the white portion of the vastus lateralis (WV) were chosen for analysis. These muscles exhibit different recruitment patterns (25) and are comprised of different fiber types (2), with the Plnt containing all fiber types and the WV containing only IIx and IIb fibers. It is hypothesized that following heat shock, Hsp70 will increase significantly throughout the vasculature, similar to the response observed in the heart. In contrast, following exercise, Hsp70 is hypothesized to accumulate in a fiber specific manner as a result of recruitment and/or muscle fiber type responsiveness. As the Hsp70 distribution may further be characterized through the activation and localization of its transcription factor, HSF1, it is hypothesized that an increase in the active phosphorylated HSF1 (pHSF1) will only occur within Hsp70-positive cells. An examination of these responses may provide insight into the way in which Hsp70 is accumulated and provides protection in skeletal muscle.

2.2 Materials and Methods

Ethics approval

Ethics approval for the involvement of rats was obtained through the Research Ethics Board of the University of Western Ontario (Appendix A).

Animals

The University of Western Ontario Council on Animal Care approved the use and treatment of laboratory animals according to the guidelines of the Canadian Council on Animal Care. Thirty-four adult male Sprague-Dawley rats were obtained from Charles River Laboratories and housed in standard rat cages (n=2/cage) prior to the experimental protocols. The animal room was maintained at constant temperature (22°C) and humidity (50%) with a 12:12-hour light-dark cycle. Food and water was given *ad libitum*.

Experimental procedures

After being housed for a minimum of 5 days, animals were randomly assigned to control (Con; n=8), exercise (EX; n=16) and heat shock (HS; n=10) groups. Following two 10-minute familiarization sessions 5 and 3 days prior to the full exercise bout, EX rats were run on a motorized treadmill for 1 hour at 30m/min, 2% grade. Pre and post-exercise temperatures were recorded using a thermistor probe inserted 5cm into the rectum. Rats in the HS group were given an intraperitoneal (IP) injection of pentobarbital sodium (35mg/kg) and placed on a temperature-controlled heating pad until body temperature, monitored with a rectal thermometer, reached 41.5°C. Core body temperature was maintained between 41.5°C and 42°C for an additional 15 minutes. Following treatment animals were given water and monitored during recovery.

Tissue collection

All animals were anaesthetized via an IP injection of pentobarbital sodium (65mg/kg), and the plantaris and white portions of the vastus muscles were harvested at 30 minutes or 24 hours post-EX (n=8/group) and post-HS (n=5/groups) (Figure 1). A sample from each muscle was frozen in liquid nitrogen and stored at –70°C. An additional sample was mounted on cork with OCT medium and frozen in isopentane cooled in liquid nitrogen.

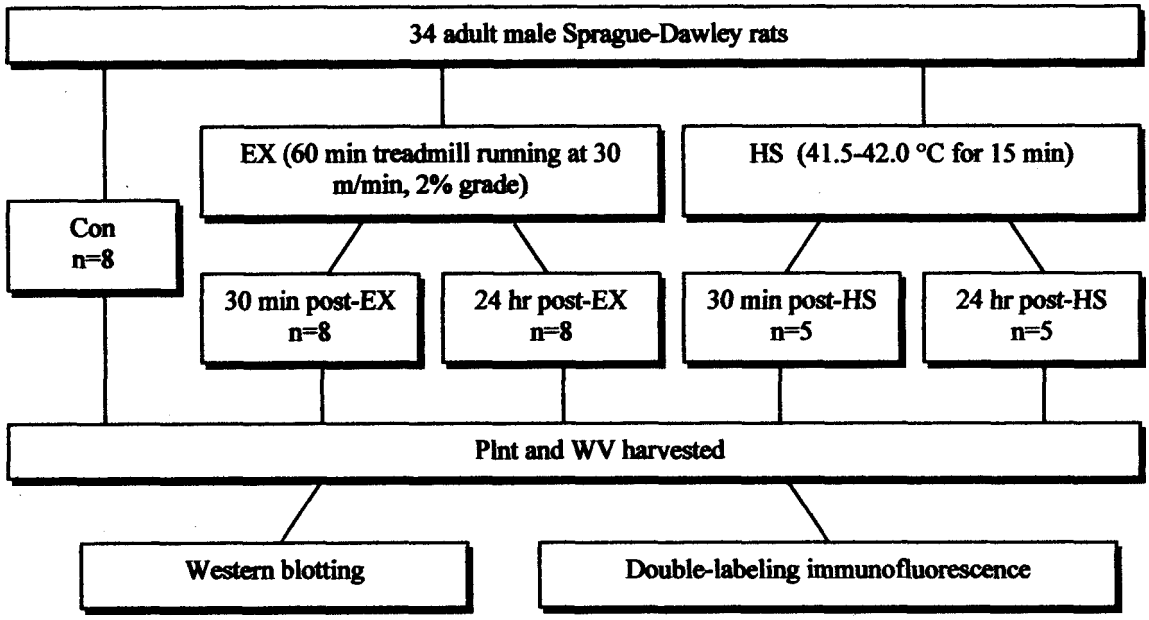


Figure 1. Outline of experimental design: Con=animals not subjected to any of the experimental conditions; EX=exercise; HS=heat shock; Plnt=plantaris muscle; WV=white portion of the vastus muscle. See text for details.

Data Collection

Western blotting

SDS-PAGE: Approximately 100mg of tissue was cut from the midbelly of frozen muscle samples and homogenized in a 1:10 ratio of homogenizing buffer (600mM NaCl and

15mM Tris base adjusted to pH ~7.5, 0.5% proteinase inhibitor, 10% Nonidet P40). During homogenization, samples were kept on ice and then centrifuged at 5,000 *g* for 10 min at 4°C, and the supernatant was stored at –70°C until time of total protein determination and electrophoresis. Total protein concentration was determined with the use of the Bradford protein assay (4). Homogenates were mixed with an equal volume of sample buffer (0.5 M Tris base, 13% glycerol, 0.05% SDS, 13% 2-beta-mercaptoethanol, and bromophenol blue). Equal amounts of protein (Plnt=50μg/well; WV=100μg/well) from each sample were loaded into the wells and then separated according to their molecular weights by running the gel for 2 hours at a constant voltage (110V) in running buffer (25mM Tris base, 200mM glycine, and 0.1% SDS, pH ~8.3). A standard sample known to express Hsp70 at high levels (male exercised heart, 50μg) and a broad band molecular weight standard (Bio-Rad kaleidoscope prestained standard) were run concurrently on each gel for determination of Hsp70 and pHSF1. The gels consisted of a 4% acrylamide stacking gel overlaying a 10% acrylamide separating gel.

Immunoblotting: Proteins were electrophoretically transferred to nitrocellulose membranes for a total of 200 volt-hours in transfer buffer (10% running buffer, 20% methanol in ddH_20). The membranes were blocked in 5% dry milk in Tris buffered saline (TBS) (80mM Tris Base, 0.5 M NaCl) for 1 hour and then washed (3x5 min) in TTBS (0.05% Tween-20 in TBS). Membranes were then incubated with a primary antibody specific to Hsp70 (anti-Hsp70, monoclonal antibody, StressGen SPA-810) or pHSF1 (anti-pHSF1, phospho-ser230, Santa Cruz sc30443-R) diluted to 1:3000 in TTBS with 2% milk blotto powder overnight. The following day, membranes were washed in TTBS (3x5 min) and incubated with the appropriate secondary antibodies (goat anti-

mouse and goat anti-rabbit horseradish peroxidase conjugated for Hsp70 and pHSF1, respectively) diluted to 1:9000 in TTBS with 2% milk for 2 hours. After a final set of washes in TTBS and TBS, blots were developed using chemiluminescent detection (Amersham) and exposed to Kodak BioMax Light film. Blots were scanned, and Scion Image analysis software was used for denositometric quantification.

Confocal microscopy

Transverse sections (15um thick) of skeletal muscle cut using a Leica microtome and cryostat were adhered to glass slides (VWR) and stored at –20 °C. Prior to staining, sections were air dried for 30 min, and fixed in 80% acetone/20% methanol for 15 min at 4°C. Slides were washed in phosphate buffer saline (PBS) for 3x5 min and then incubated for 5 minutes in 0.05% TritonX-100/PBS. Slides were washed as before, and blocked for 1 hour in 10% goat serum/PBS solution. Sections were incubated with two suitably diluted primary antibodies in a 1% goat serum/PBS solution overnight at 4°C (Table 1). Double-labeling was performed using the following antibodies: Hsp70 (StressGen, SPA-810); pHSF1 (Santa Cruz, sc30443-R); HSF1 (StressGen, SPA-901); a basement membrane marker, polyclonal rabbit anti-laminin (Sigma, L9393); an endothelial cell marker, polyclonal rabbit anti-human Von Willebrand factor (Dako, A0082); a smooth muscle marker, rabbit pAB to alpha smooth muscle actin (Abcam, 5694-100); and myosin heavy chain (MHC) isoform antibodies, MHC I (BA-D5), IIa (SC-71), and IIx/IIb (212F). The next day, slides were washed in PBS (3x5 min) and incubated with two secondary antibodies (Alexa Fluor 488 conjugated goat anti-mouse immunoglobin G and Alexa Fluor 594 conjugated goat anti-rabbit immunoglobulin G) diluted to 1:400 in PBS for 1 hour. After washes in PBS, sections were mounted with a

fluorescent mounting medium (Invitrogen) containing 1% 4', 6-diamidino-2-phenylindole (DAPI), a fluorescent nuclear stain. One section per slide was incubated in the absence of primary antibody to serve as a negative control and ensure specificity of the antibodies. Images were captured in a dark room using confocal microscopy (Zeiss LSM 410). All images were captured at the same microscope settings (brightness and contrast). Plates were compiled from confocal images with Adobe Photoshop software.

Table 1. List of antibodies used for double-labeling immunofluorescence: MHC=myosin heavy chain isoform; EC=endothelial cells; HSF1=heat shock transcription factor 1; IgG=immunoglobulin G; LAM=laminin; pHSF1=phosphorylated heat shock transcription factor 1; SM=smooth muscle; vWF= Von WilleBrand factor. See text for details.

Monoclonal 1°Antibody:	*Target*	*Dilution*	*2°Antibody*	*Dilution*
SPA-810	Hsp70	1:100	Alexa Fluor 488 Goat Anti-Mouse IgG	1:400
BA-D5	MHC I	1:400		1:400
SC-71	MHC IIa	1:100		1:400
212F	MHC IIx/IIb	1:200		1:400

Polyclonal 1°Antibody:	Target	Dilution	*2°Antibody*	*Dilution*
ab5694-100	SM	1:200	Alexa Fluor 594 Goat Anti-Rabbit IgG	1:400
sc30443-R	pHSF1	1:100		1:400
L9393	LAM	1:200		1:400
A0082	vWF (EC)	1:200		1:400
SPA-901	HSF1	1:100		1:400

Fiber counting

Approximately 100 fibers from each sample were randomly chosen, imaged and examined by hand to determine the percentage of Hsp70-positive fibers in the Plnt and WV. Staining was categorized into 4 grades as follows: *grade 1*, minimal staining; *grade 2*, weak staining; *grade 3*, modest staining; *grade 4*, intense staining. Fibers were

deemed positive if classified as either *grade 3* or *grade 4*. The percentage of Hsp70-positive fibers was initially calculated per animal then determined per group (n=8).

Statistical measures

Body weight, temperature, and protein data as measured by Western blotting were analyzed using a one-way analysis of variance (ANOVA). Tukey's post-hoc test was used to determine differences between groups. Percentages of Hsp70-positive fibers between Con and EX muscles were compared by an unpaired t-test. All statistical analyses were performed using sigma stat 2.03 software. All data is expressed as mean ± SE. Differences were considered significant at $P<0.05$.

2.3 Results

Animal Weight and Temperature

Animal weight and temperature measurements are presented in Table 2. Weights of Con, EX and HS groups were not significantly different ($P>0.05$). Resting rectal temperatures were not different between Con, EX and HS groups ($P>0.05$), but post-EX and post-HS temperatures were significantly greater than resting temperatures ($P<0.05$). Post-HS temperatures were significantly greater than post-EX temperatures ($P<0.05$).

Hsp70 protein content in exercise and heat shock muscles

In the Plnt, Western blots revealed no significant increases in Hsp70 levels 30 min post-EX and post-HS compared to Con levels. By 24 hr there was a ~2 fold increase in Hsp70 post-EX and a ~3.5 fold increase in Hsp70 levels post-HS ($P<0.05$) (Figure 2A). Similarly, in the WV, blots revealed no significant increases in Hsp70 levels 30 min post-EX and post-HS compared to Con levels. By 24 hr there was an ~5.5 fold increase in

Hsp70 levels post-EX and an ~9 fold increase in Hsp70 levels post-HS ($P<0.05$) (Figure 2B). In both the Plnt and WV, Hsp70 levels 24 hr post-HS was significantly greater than Hsp70 levels 24 hrs post-EX ($P<0.05$) (Figure 2A, 2B). Representative blots for Hsp70 from the Plnt and WV are shown in Figure 2C.

Cellular distribution of Hsp70 in exercised muscles compared to heat shocked muscles

Vasculature: The Plnt showed increases in Hsp70 within the microvasculature following both EX and HS (Plate 1). Hsp70 co-localization with the endothelial cell (EC) marker, Von Willebrand factor (vWF), was observed marginally in Con Plnt, however, Hsp70 staining intensity and co-localization with vWF increased post-EX and post-HS, particularly after 24 hr. Similar results were observed in WV (Plate 2). In addition, Hsp70 was co-localized with smooth muscle (SM), which is indicative of larger vessels, 24 hr post-EX and 24 hr post-HS (Plate 3). The accumulation of Hsp70 throughout the vasculature appeared to reach the greatest level at 24 hr post-HS.

Myofibers: The Plnt showed Hsp70 constitutively expressed in a fiber specific manner. This non-uniform staining pattern was maintained 30 min and 24 hr post-EX (Plate 1). The WV showed negligible Hsp70 expression in Con. The appearance of Hsp70 accumulation in myofibers was not apparent 30 min post-EX or 30 min post-HS. At 24 hr, however, Hsp70 accumulation in myofibers was apparent following both EX and HS (Plate 2). This paralleled the Western blotting data presented in Figure 1. Interestingly, as with the Plnt, Hsp70 distribution in EX WV muscles appeared in a fiber specific manner, whereas Hsp70 in HS muscles appeared evenly distributed. In addition, Hsp70 in HS muscles appeared to be localized to the periphery of the fibers and in conjunction with the vWF.

Characterization of the cellular distribution of Hsp70 following exercise

Clear Hsp70-positive fiber type differences were observed between the Plnt and WV. Serial sections in the Plnt revealed Hsp70 constitutively expressed exclusively in type I and IIa fibers. This fiber type-specific distribution remained the same in the Plnt 24 hr post-EX (Plate 4). Type IIx/IIb fibers were negative for Hsp70 pre- and post-EX in the Plnt. In the WV, only type IIx/IIb fibers were detected. Immunofluorescence detected negligible amounts of Hsp70 in Con WV fibers, however, Hsp70 accumulated non-uniformly throughout its type IIx/IIb fibers 24 hr post-EX (Plate 5). It should be noted that identification of pure IIb and pure IIx fibers was not possible due to the availability of antibodies, hence, the term IIx/IIb is used to denote either pure IIb fibers or hybrid fibers, expressing both IIx and IIb MHC.

Through fiber counting, the percentage of Hsp70-positive fibers in the Plnt and WV were obtained pre- and post-EX. Hsp70 in Con Plnt was detected in approximately half of the fibers (56.3% ± 6.8) (Figure 3A). The percentage of Hsp70-positive fibers did not significantly change 24 post-EX (59.3% ± 8.8) ($P<0.05$). Hsp70 in Con WV was barely detectable in its fibers, however, following exercise Hsp70-positive fibers significantly increased (36.2% ± 14.2) ($P>0.05$). (Figure 3B).

Expression and cellular distribution of pHSF1 from exercised muscles cannot explain fiber specific Hsp70

Western blots revealed that Plnt pHSF1 levels increased 30 post-EX ($P<0.05$) and 24 hr post-EX ($P<0.05$) compared to Con (Figure 4A). Similar increases were seen in the WV. Blots revealed that WV pHSF1 levels increased significantly 30 min post-EX ($P<0.05$) and 24 hr post-EX ($P<0.05$) compared to Con (Figure 4B). Overall, pHSF1 increased to a greater degree in WV than Plnt, paralleling exercise-induced changes in

Hsp70 content 24 hr later (Figure 2A, 2B). Representative blots for pHSF1 from the Plnt and WV are shown in Figure 4C.

Confocal microscopy revealed the Con Plnt expressed pHSF1 in most nuclei (Plate 6). Increased nuclear co-localization and staining intensity of pHSF1 was observed 30 min and 24 hr post-EX. In particular, staining intensities 30 min post-EX were greater than that observed in Con. Co-localization of pHSF1 and Hsp70 revealed nuclear staining of pHSF1 in fibers inducing Hsp70 following EX. However, pHSF1 was also found in nuclei of fibers not demonstrating increases in Hsp70. Similar results were found in the WV (Plate 7). Of the nuclei that were positive for pHSF1 in the Con WV, staining was minimal compared to the Plnt. Increases in nuclear pHSF1 were observed in the WV 30 min and 24 hr post-EX.

Cellular distribution of pHSF1 differs from HSF1

Comparisons were shown demonstrating differing localization patterns between pHSF1 and HSF1 (Plate 8). From control and 30 min post-EX (the time point in which pHSF1 expression was greatest after EX) rats, pHSF1 is primarily localized to the nuclei, whereas HSF1 (phosphorylated and non-phosphorylated) was observed abundantly in the cytoplasm in addition to the nuclei. Additionally, localization of pHSF1 and HSF1 post-EX was similar to post-HS observations in both the Plnt and WV (data not shown).

Table 2. Rat body weights and rectal temperatures

	Con	EX		HS	
		30 min	24 hr	30 min	24 hr
Body Weight (g)	338.7 ± 3.9	345.5 ± 6.0	344.2 ± 4.9	349.3 ± 5.1	339.1 ± 5.7
Rectal Temperature (°C)					
Resting	37.4 ± 0.1	37.8 ± 0.2	37.8 ± 0.2	37.4 ± 0.2	37.8 ± 0.2
Post-EX		40.5 ± 0.5*	40.5 ± 0.3*		
Post-HS				41.9 ± 0.1*†	42.0 ± 0.1*†

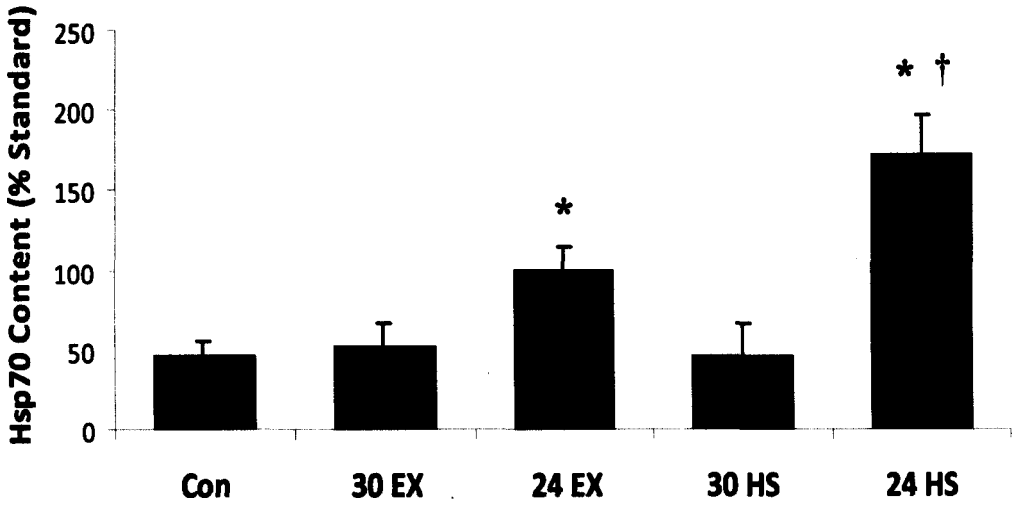
Rats were sacrificed 30 min and 24 hr post-exercise (EX) and post-heat shock (HS). Values are mean ± SE of 8 rats/EX group and 5 rats/HS group.

*Significantly greater than control (Con) and resting temperatures, $P<0.05$.

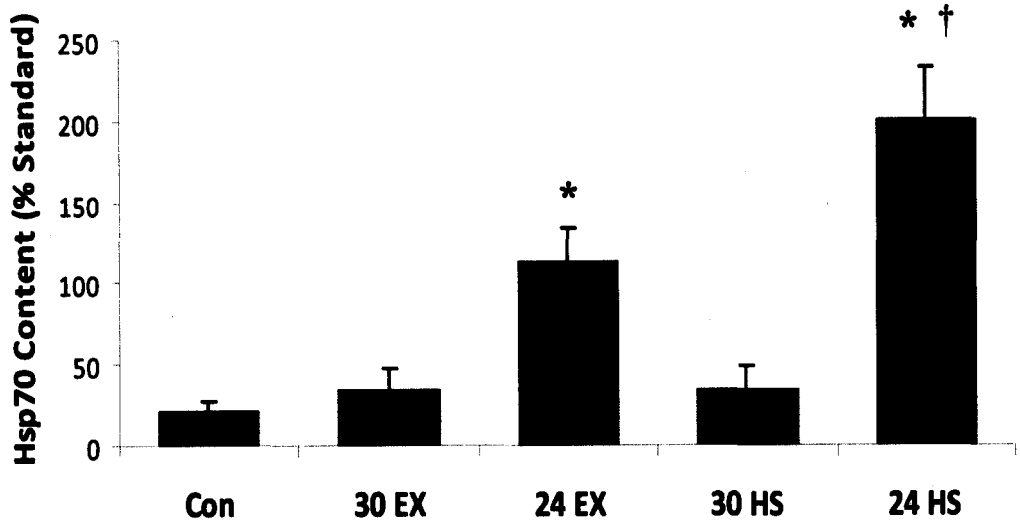
†Significantly greater than post-EX temperatures, $P<0.05$.

Figure 2. Hsp70 levels in hindlimb muscles of control (Con), exercised (EX) and heat-shocked (HS) rats. **A.** Hsp70 expression in the plantaris (Plnt) as measured by Western blotting. Values are mean ± SE. *Significantly greater than Con, 30 min post-EX and 30 min post-HS, $P<0.05$. †Significantly greater than 24 hr post-EX, $P<0.05$. **B.** Hsp70 expression in the white portion of the vastus lateralis (WV) as measured by Western blotting. Values are mean ± SE. *Significantly greater than Con, 30 min post-EX and 30 min post-HS, $P<0.05$. †Significantly greater than 24 hr post-EX, $P<0.05$. **C.** Representative blots for Hsp70 from the Plnt and WV.

A. Plnt


Hsp70 Content (% Standard)
250
200
150
100
50
0
* †
*
Con
30 EX
24 EX
30 HS
24 HS

B. WV


Hsp70 Content (% Standard)
250
200
150
100
50
0
* †
*
Con
30 EX
24 EX
30 HS
24 HS

C.

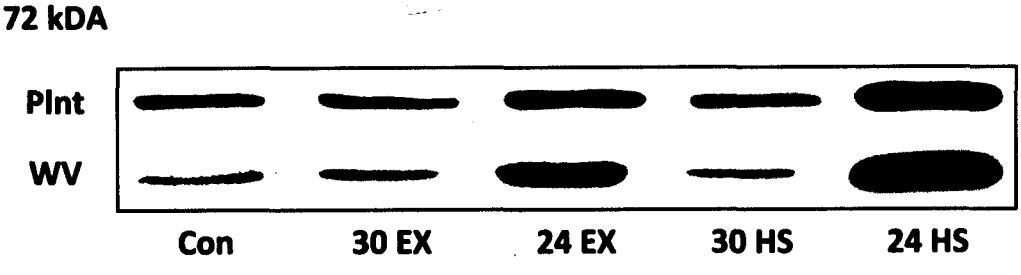

72 kDA
Plnt
WV
Con
30 EX
24 EX
30 HS
24 HS

Plate 1. Cross sections from the plantaris (Plnt) captured by confocal microscopy double-labeled with antibodies for Hsp70 (green) and Von Willebrand factor (vWF) (red) of control (Con), exercised (EX) and heat-shocked (HS) rats. Hsp70 appears only in selected muscle fibers pre- and post-EX and to a greater extent than fibers observed 24 hr post-HS. Relative to Con, 24 hr post-EX and 24 hr post-HS muscles demonstrate increases in Hsp70 co-localizing (yellow) with vWF. Inserts, indicated by arrows, are high magnification images of vessels staining for Hsp70 and vWF. Arrowhead indicates a large vessel demonstrating a robust increase in Hsp70. Bar=50μm (all images, except inserts). Width of inserts=25μm.

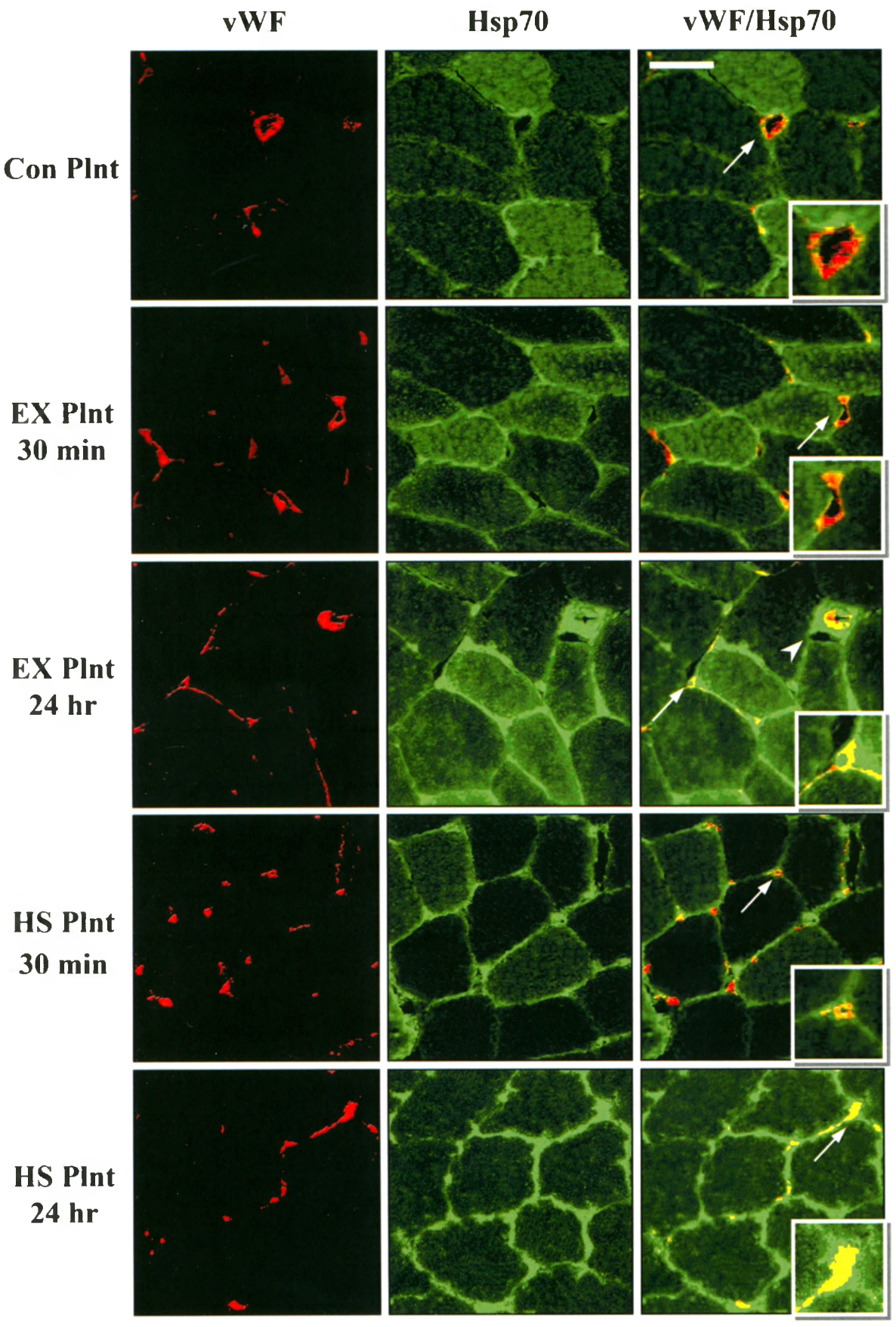

vWF
Hsp70
vWF/Hsp70
Con Plnt
EX Plnt
30 min
EX Plnt
24 hr
HS Plnt
30 min
HS Plnt
24 hr

Plate 2. Cross sections from the white portion of the vastus lateralis (WV) captured by confocal microscopy double-labeled with antibodies for Hsp70 (green) and von Willebrand factor (vWF) (red) of control (Con), exercised (EX) and heat-shocked (HS) rats. Hsp70 increases only in selected fibers 24 hr post-EX and to a greater extent than fibers observed 24 hr post-HS. Relative to Con, 24 hr post-EX and 24 post-HS muscles demonstrate increases in Hsp70 co-localizing (yellow) with vWF. Inserts, indicated by arrows, are high magnification images of vessels staining for Hsp70 and vWF. Bar=50μm (all images, except inserts). Width of inserts=25μm.

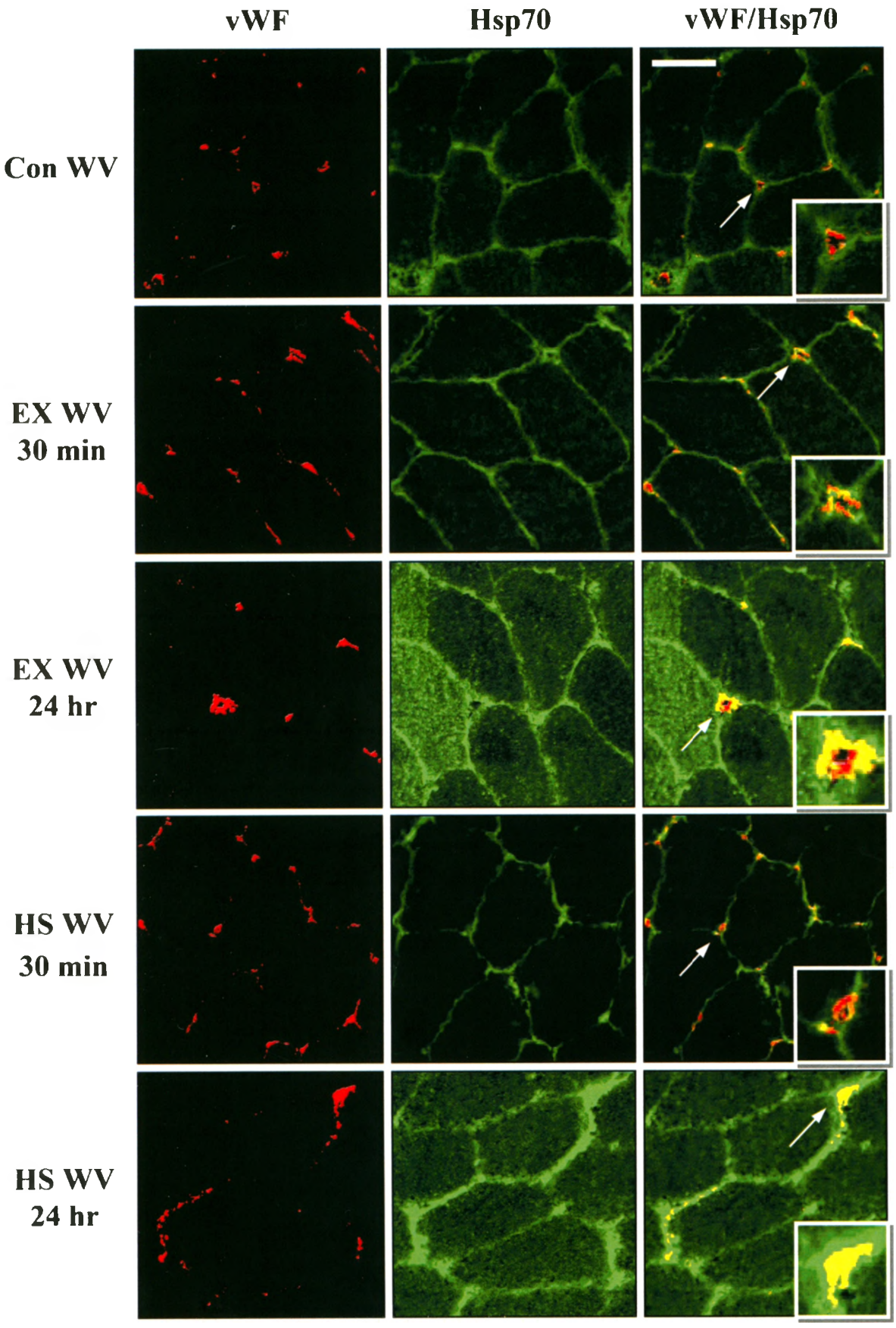

vWF
Hsp70
vWF/Hsp70
Con WV
EX WV
30 min
EX WV
24 hr
HS WV
30 min
HS WV
24 hr

Plate 3. Hsp70 localization in larger vessels, indicated by smooth muscle (SM), from the white portion of the vastus lateralis (WV) of exercised (EX) and heat-shocked (HS) rats. SM (red) shows co-localization (yellow) with Hsp70 (green) 24 hr post-EX and 24 hr post-HS, the time points in which Hsp70 expression is greatest. Inserts, indicated by arrows, are high magnification images of vessels staining for Hsp70 and SM. Bar=50μm (all images, except inserts). Width of inserts=40μm.

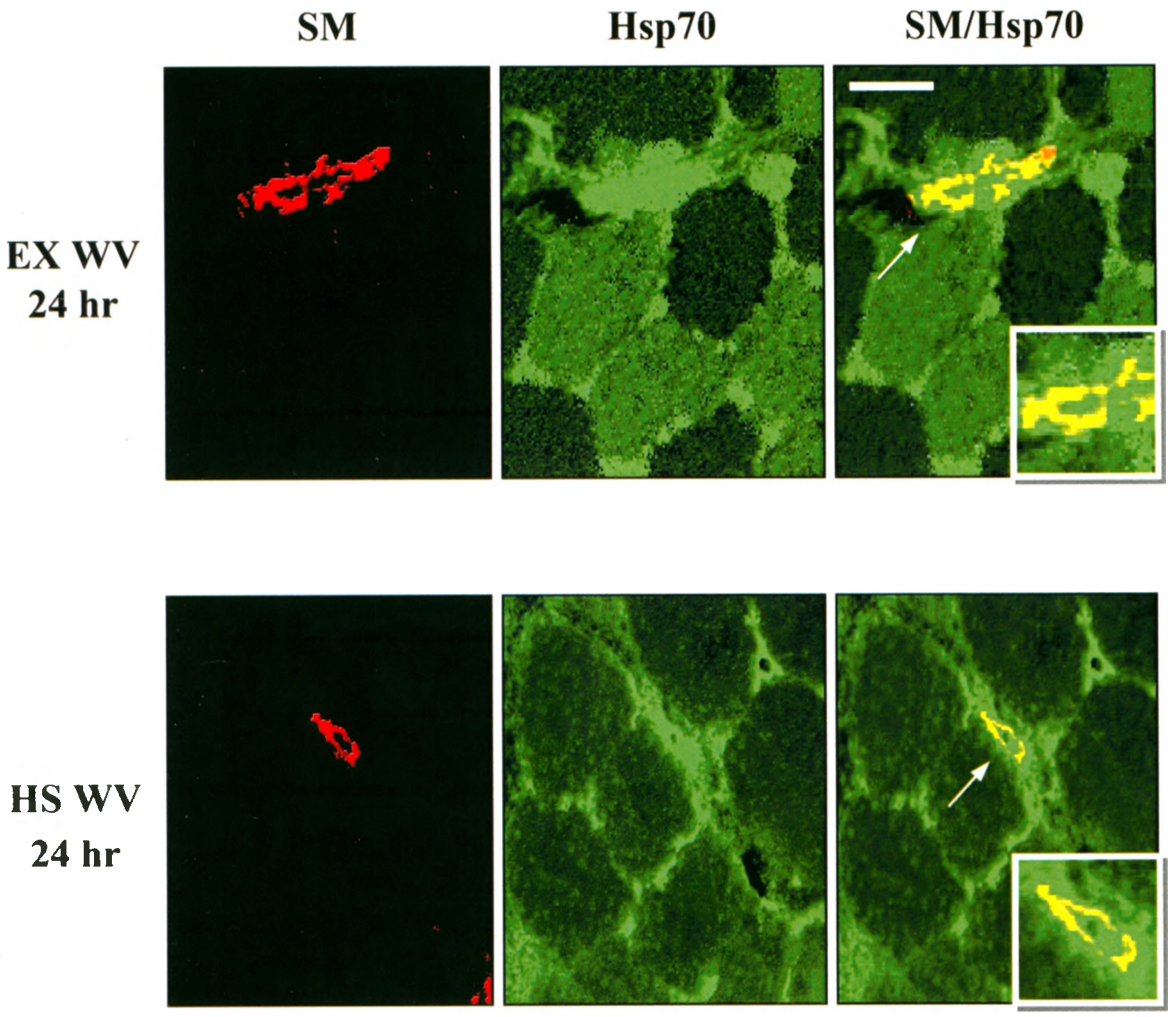

SM
Hsp70
SM/Hsp70
EX WV
24 hr
HS WV
24 hr

Plate 4. Fiber type-specific accumulation of Hsp70 captured by confocal microscopy from cross sections of the plantaris (Plnt) of control (Con) and exercised (EX) rats. Serial sections were stained with antibodies for type I (BA-D5), IIa (SC-71) and IIx/IIb (212F) myosin heavy chain (MHC) isoforms and Hsp70. Double-labeling with laminin (red) was used to outline fibers stained with MHC isoforms. Bar=50μm (all images).

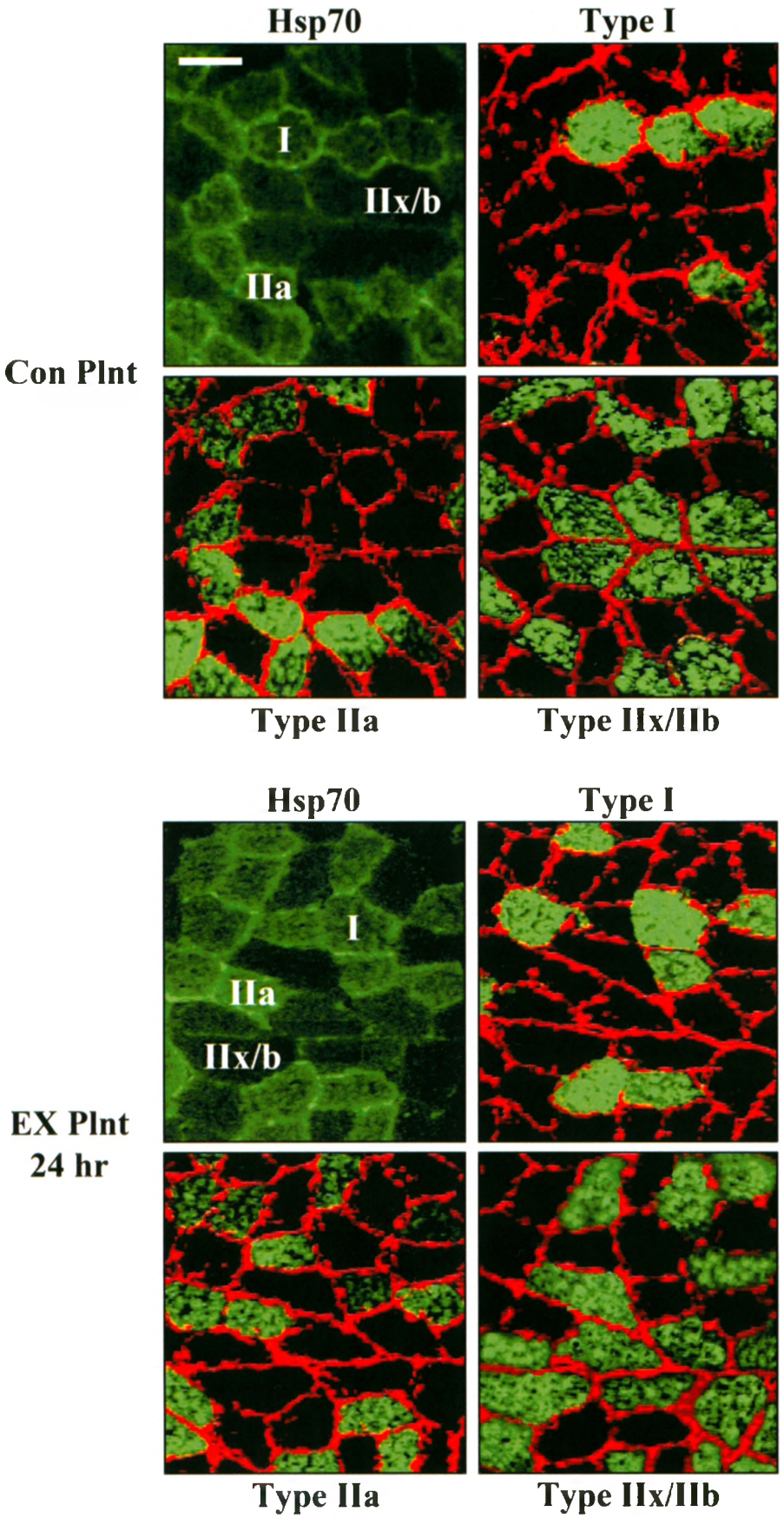

Hsp70
Type I
I
IIx/b
IIa
Con Plnt
Type IIa
Type IIx/IIb
Hsp70
Type I
I
IIa
IIx/b
EX Plnt
24 hr
Type IIa
Type IIx/IIb

Plate 5. Fiber type-specific accumulation of Hsp70 captured by confocal microscopy from cross sections of the white portion of the vastus lateralis (WV) of control (Con) and exercised (EX) rats. Serial sections were stained with antibodies for type IIa (SC-71) and IIx/IIb (212F) myosin heavy chain (MHC) isoforms and Hsp70. Double-labeling with laminin (red) was used to outline fibers stained with MHC isoforms. Bar=50μm (all images).

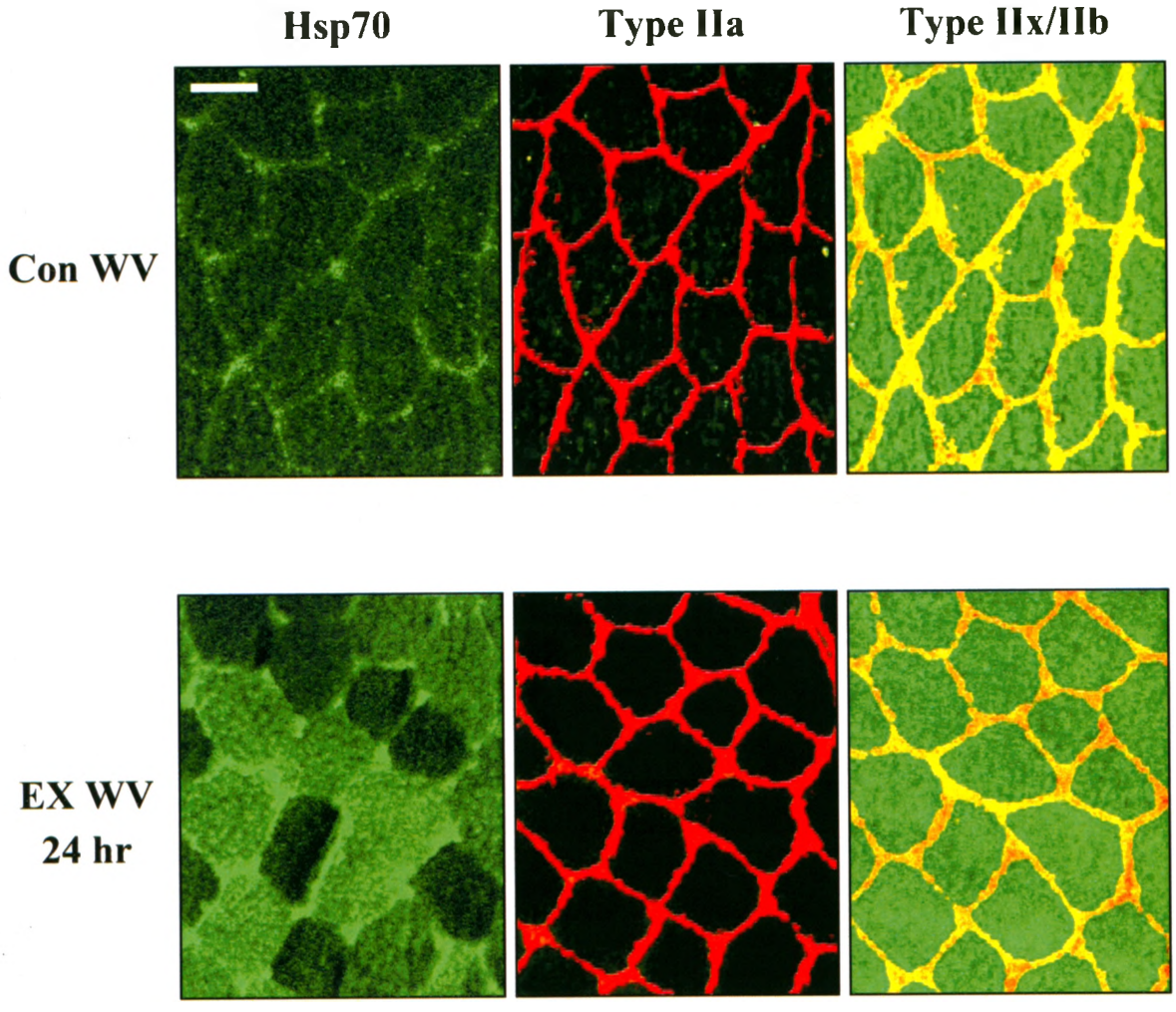
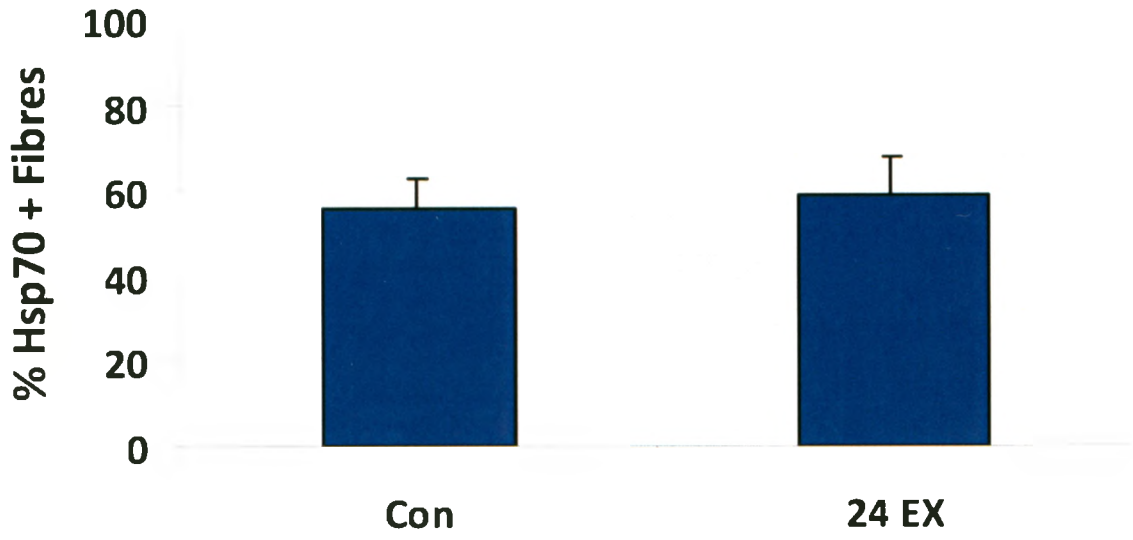

Hsp70
Type IIa
Type IIx/IIb
Con WV
EX WV
24 hr

Figure 3. A. Percentage of Hsp70-positive fibers evaluated on cross sections from plantaris (Plnt) muscles of control (Con) and 24 hr post-exercise (EX) rats. **B.** Percentage of Hsp70-positive fibers evaluated on cross sections from white portion of the vastus lateralis (WV) muscles of control (Con) and 24 hr post-EX (EX) rats. *Significantly greater than control, $P<0.05$.

A. Plnt


% Hsp70 + Fibres
100
80
60
40
20
0
Con
24 EX

B. WV

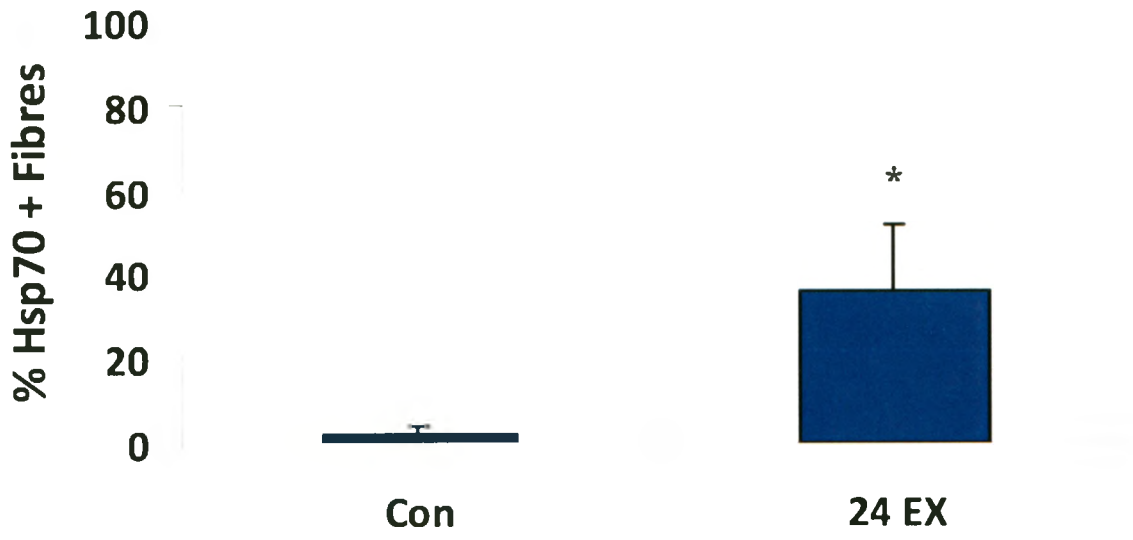
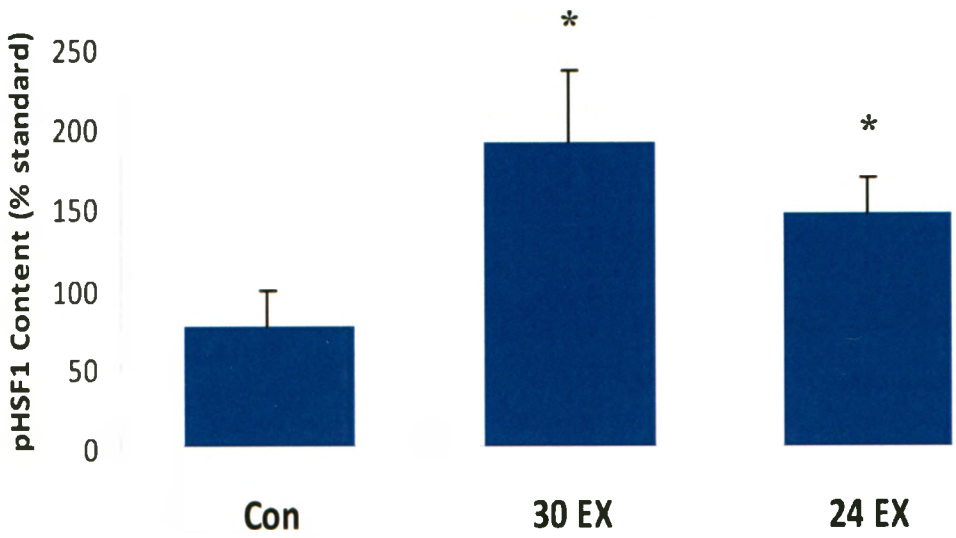
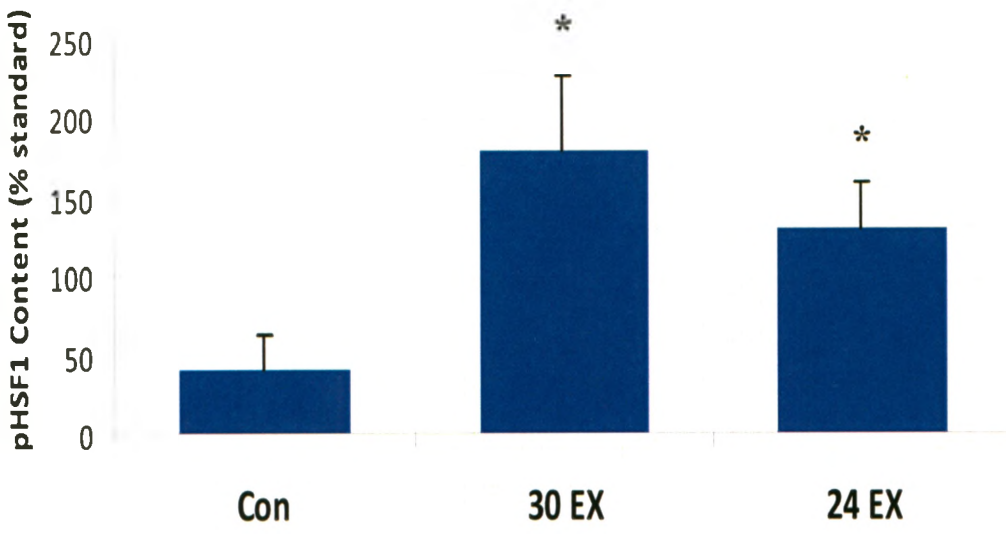

% Hsp70 + Fibres
100
80
60
40
20
0
*
Con
24 EX

Figure 4. pHSF1 levels in hindlimb muscles of control (Con) and exercised (EX) rats. **A.** pHSF1 expression in the plantaris (Plnt) as measured by Western blotting. Values are mean ± SE. *Significantly greater than Con, $P<0.05$. **B.** pHSF1 expression in the white portion of the vastus laterlis (WV) as measured by Western blotting. Values are mean ± SE. *Significantly greater than Con, $P<0.05$. **C.** Representative blots for pHSF1 from the Plnt and WV.

A. **Plnt**


pHSF1 Content (% standard)
250
200
150
100
50
0
*
*
Con
30 EX
24 EX

B. **WV**


pHSF1 Content (% standard)
250
200
150
100
50
0
*
*
Con
30 EX
24 EX

C.

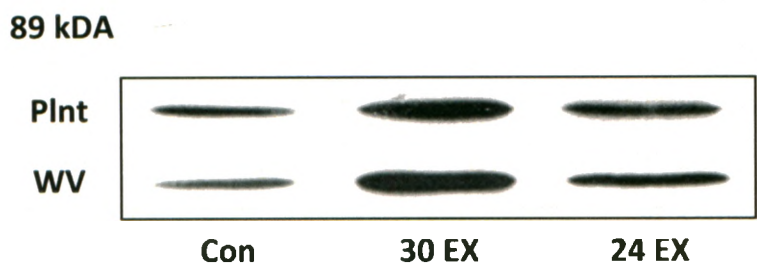

89 kDA
Plnt
WV
Con
30 EX
24 EX

Plate 6. Cross sections from the plantaris (Plnt) captured by confocal microscopy double-labeled with antibodies for Hsp70 (green) and pHSF1 (red), and the nuclear marker 4', 6-diamidino-2-phenylindole (DAPI) of control (Con) and exercised (EX) rats. pHSF1-positive nuclei (purple) were observed in all conditions, but greatest staining occurred 30 min post-EX. Inserts, indicated by arrows, are high magnification images of nuclear pHSF1 in Hsp70-negative fibers. Bar=100μm (all images, except inserts). Width of inserts=20μm.

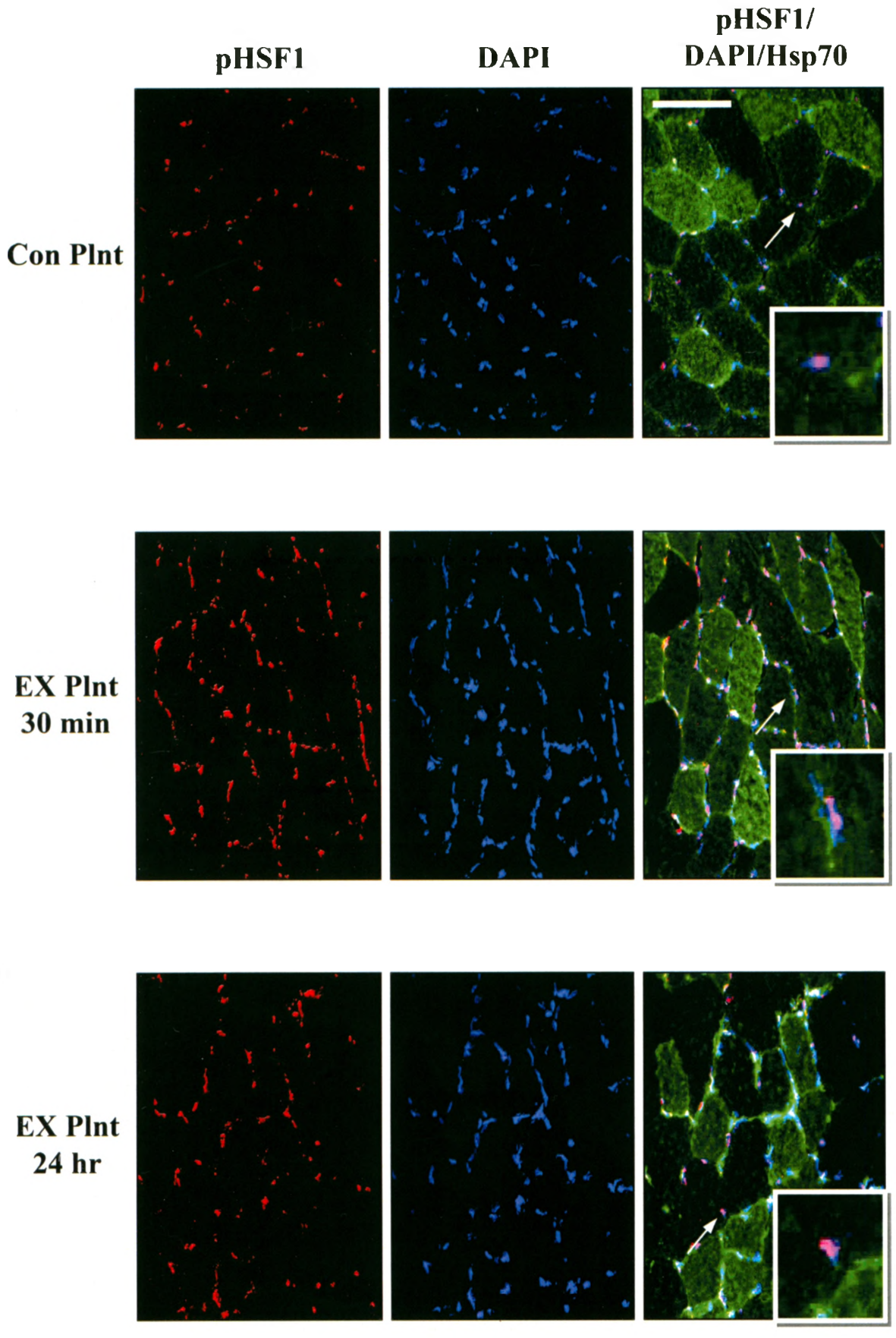

pHSF1
DAPI
pHSF1/
DAPI/Hsp70
Con Plnt
EX Plnt
30 min
EX Plnt
24 hr

Plate 7. Cross sections from the white portion of the vastus (WV) captured by confocal microscopy double-labeled with antibodies for Hsp70 (green) and pHSF1 (red), and the nuclear marker 4', 6-diamidino-2-phenylindole (DAPI) of control (Con) and exercised (EX) rats. pHSF1-positive nuclei (purple) were observed in all conditions, but greatest staining occurred 30 min post-EX. Inserts, indicated by arrows, are high magnification images of nuclear pHSF1 in Hsp70-negative fibers. Bar=100μm (all images, except inserts). Width of inserts=25μm.

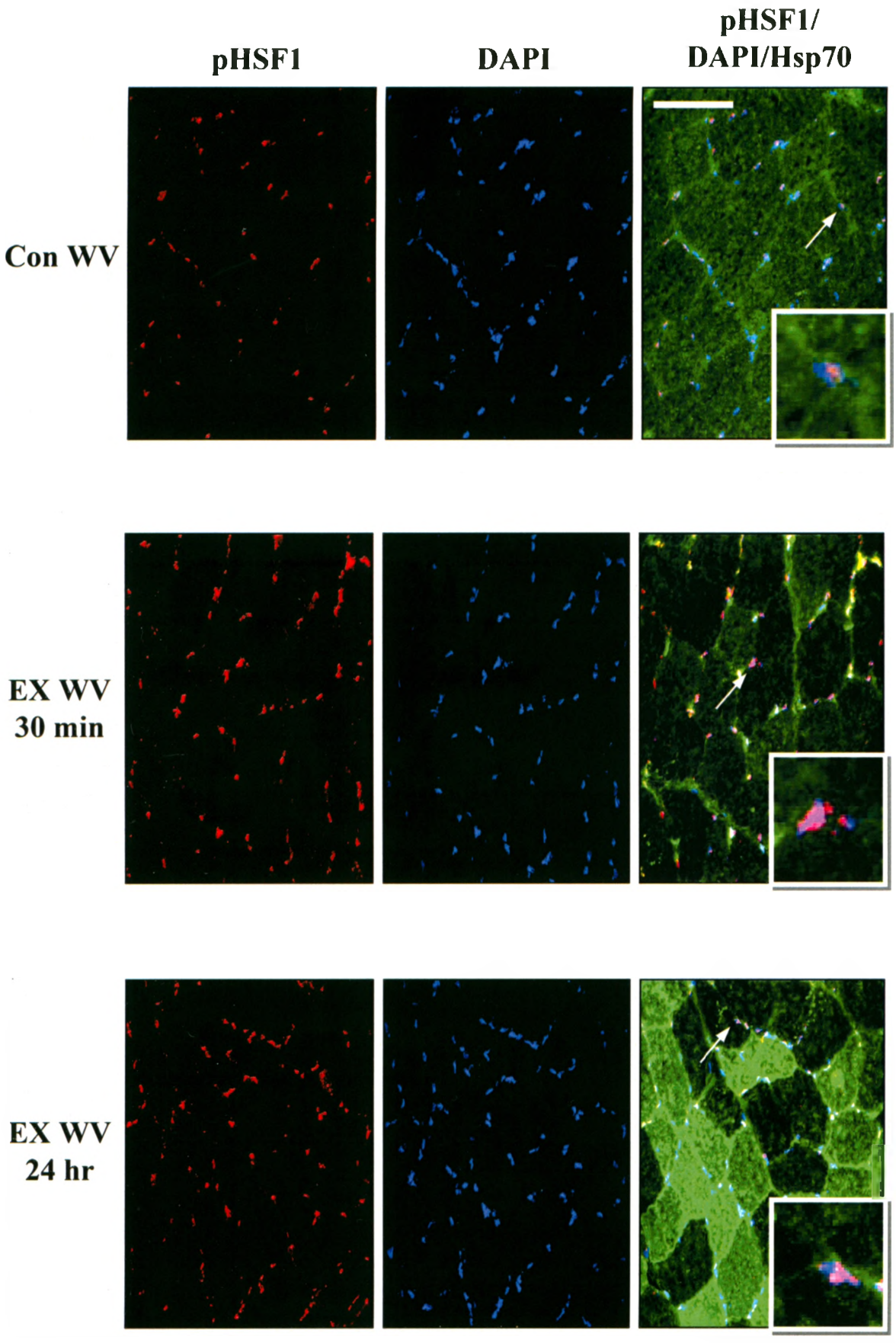
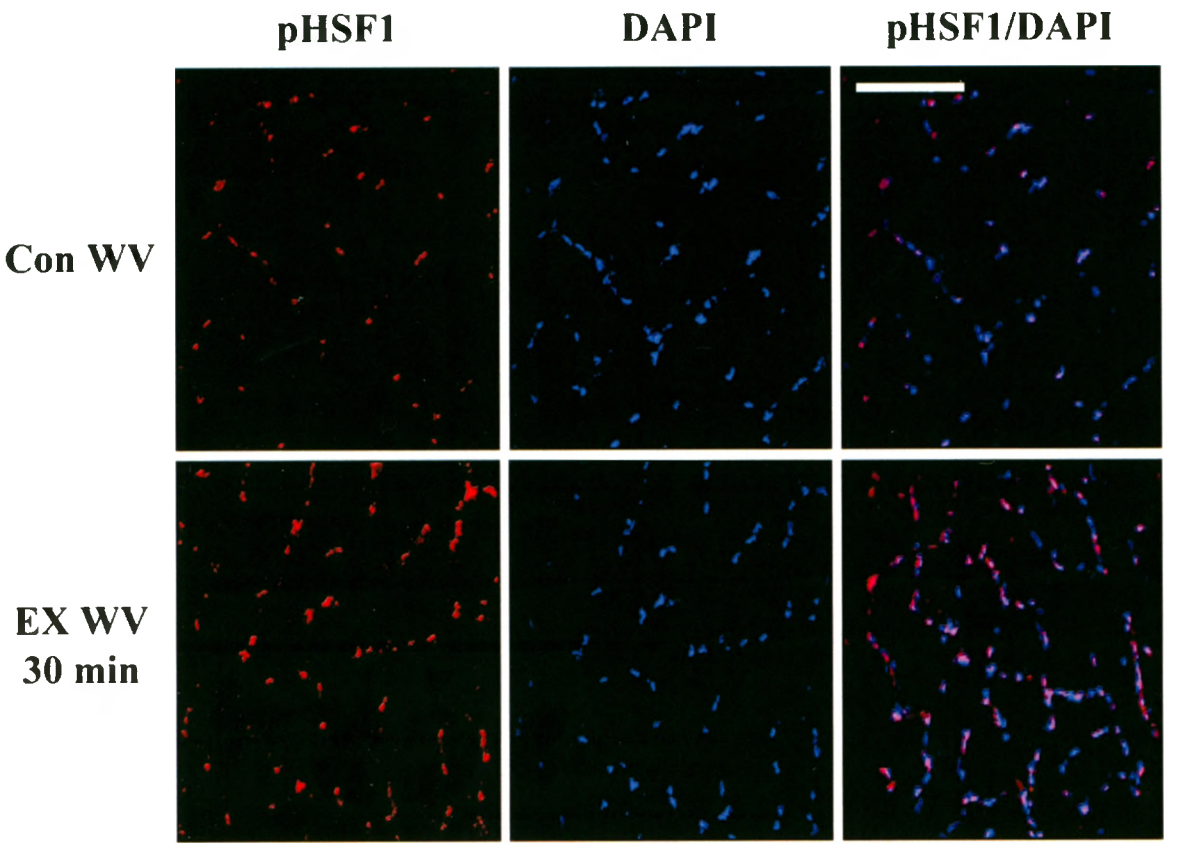
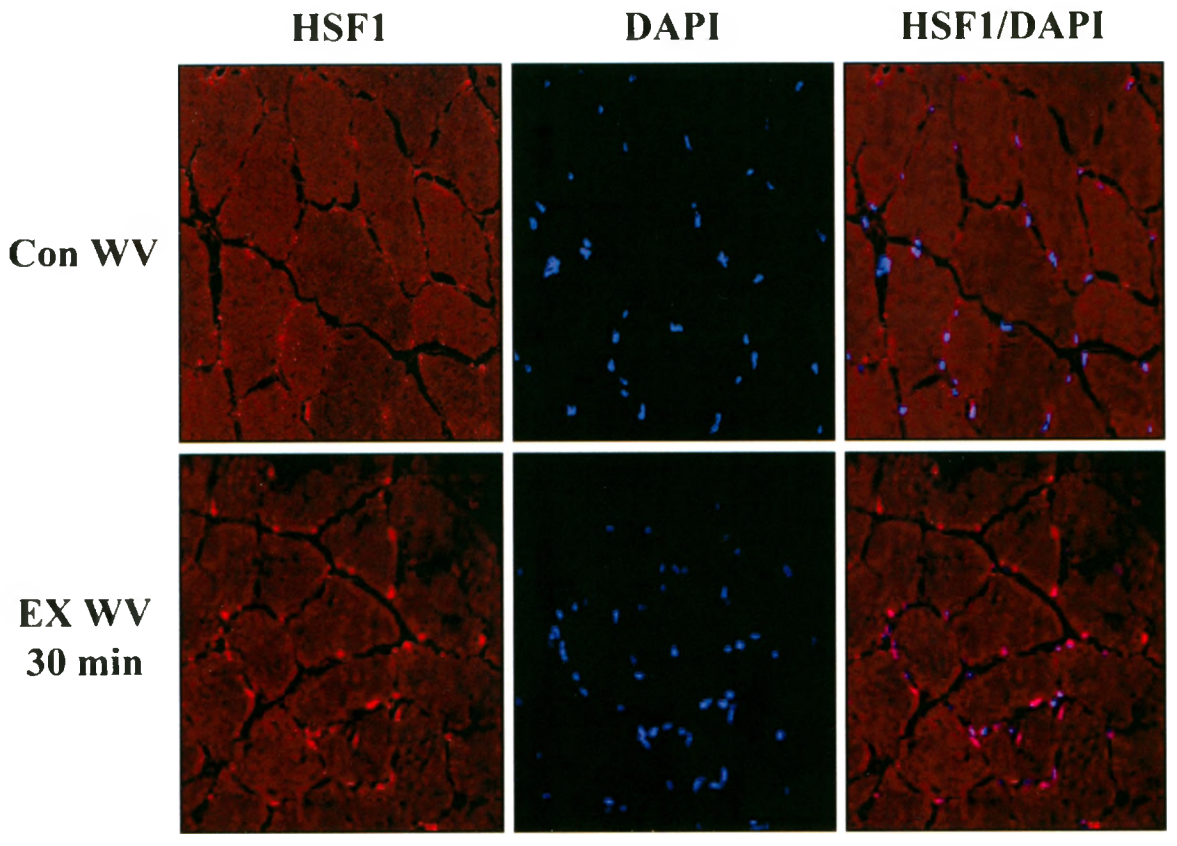

pHSF1
DAPI
pHSF1/
DAPI/Hsp70
Con WV
EX WV
30 min
EX WV
24 hr

Plate 8. Cross sections from the white portion of the vastus (WV) captured by confocal microscopy labeled with antibodies for pHSF1 and HSF1 (red), and the nuclear marker 4', 6-diamidino-2-phenylindole (DAPI) of control (Con) and exercised (EX) rats. Comparisons of pHSF1 and HSF1 in Con and 30 min post-EX (the time point in which pHSF1 expression was greatest after EX) rats revealed differing localization patterns. pHSF1 is localized exclusively in the nuclei, whereas HSF1 is also observed abundantly in the cytoplasm in addition to the nuclei. Bar=100μm (all images)


pHSF1
DAPI
pHSF1/DAPI
Con WV
EX WV
30 min


HSF1
DAPI
HSF1/DAPI
Con WV
EX WV
30 min

2.4 Discussion

This study investigated Hsp70 expression and localization in skeletal muscles of rats exposed to two stressful conditions, a 60 min bout of strenuous exercise and a 15 min bout of whole-body heat shock. Although both conditions significantly increased the relative Hsp70 protein level in hindlimb muscles, changes were more dramatic following heat shock. In exercised muscles, Hsp70 was increased in vascular cells when compared to controls and Hsp70 expression in the myofibers was observed in a fiber specific manner. In heat shocked muscles, Hsp70 increased to a greater extent throughout the vasculature compared to muscles from the exercised groups. Hsp70 expression in the myofibers was higher than under control conditions, however, no fiber specific expression was observed. The results support the original hypotheses that heat shock would induce Hsp70 in the vascular tissue of skeletal muscle as it does in the myocardium (16) and that exercise induces a fiber specific expression of Hsp70. Interestingly, like heat shock, exercise induced vascular expression of Hsp70 in both endothelium and smooth muscle. In addition, accumulation of the phosphorylated form of the heat shock transcription factor, pHSF1, was not restricted to only those fibers which ultimately expressed Hsp70, but rather to all muscle fibers post-exercise.

Heat shock induces Hsp70 in skeletal muscle in response to stress and this increase has been associated with an enhanced tolerance to ischemia-reperfusion injury (17) and reduced muscle atrophy during hindlimb unweighting (29). However, there is controversy as to whether increased Hsp70 is protective or not (19). Therefore, the role of Hsp70 in skeletal muscle is less understood than in the myocardium where increases of Hsp70 following heat shock have been clearly associated with numerous types of

protection including; enhanced tolerance to increased ischemia-reperfusion injury (26), inflammation (6), maintenance of the endothelial barrier (5), and survival of smooth muscle cells (14). As studies in the myocardium have demonstrated that Hsp70 accumulates almost exclusively in the blood vessels of the atria and ventricles following heat shock (1; 16), the aforementioned cardio protection may be partially a consequence of the vascular expression of Hsp70. In the present study, it is likely that the increases of Hsp70 in the endothelium and smooth muscle compartments of the skeletal muscle protect the vasculature and therefore the muscle itself in a similar fashion. Furthermore, the myofibers in the skeletal muscle appear to exhibit an increase in Hsp70 as opposed to the cardiomyocytes in the heart (16). This further suggests an important role following heat shock while demonstrating differences among tissues and/or organs.

In addition to the Hsp70 accumulation following heat shock, the present investigation also demonstrated exercise-induced Hsp70 in the skeletal muscle vasculature. As exercise gives rise to hyperthermia, it is not surprising that exercise-induced Hsp70 increases in the vasculature were observed. Given that our exercise protocol (30m/min, 60min, 2% grade) significantly increased the rat rectal temperatures (Table 2), it is likely that heat was a main factor for the Hsp70 increases in the vasculature (16). However, while increases in body temperature may accompany high-intensity exercise, exercise-induced Hsp70 has been shown to occur independently from changes in body temperature (30). Stary et al. (36) showed that exercise of isolated single muscle fibers maintained in a constant temperature bath increased Hsp70 mRNA. Furthermore, muscle heating to levels comparable to those occurring during exercise failed to induce a significant increase in Hsp70 levels in humans (28). As such, we

following hypoxia may have been responsible for the selective increase in Hsp70 displayed by the venous walls. It appears that, regardless of heat, shear or stretch stress, oxidative stress is capable of inducing Hsp70 in vascular cells and may aid in the vasculature expression of Hsp70 observed during exercise, further serving to protect the vessels and surrounding muscle as previously described.

In the present study, the exercise response was further characterized by noticeable fiber specific differences compared to the heat shock response. The exercise response appears to vary between different muscles, in that only an ~2 fold increase was observed in the Plnt (Figure 2A) compared to an ~5.5 fold increase observed in the WV (Figure 2B). Moreover, Hsp70 expression in the Plnt was not observed in IIx/IIb fibers (Plate 4) as it was in the WV (Plate 5), nor did the Plnt increase in percentage of Hsp70-positive fibers as did the WV. It is likely during the exercise protocol that the Plnt was not utilized to the same extent as the WV, and therefore may not have depleted its energy stores to the same extent (3). In addition, the Plnt expresses high basal levels of Hsp70 in muscle fibers that are most likely to be recruited during exercise. That is, unlike the WV which contains only IIx/IIb fibers, the Plnt has many type I and IIa fibers (9% SO-I, 50% FOG-IIa, 41% FG-IIx/IIb) (2) which express high resting levels of Hsp70. It has been previously demonstrated with muscle composed primarily of type I fibers (21) and in muscles from trained individuals (10) that if elevated levels of Hsp70 exist, increases in Hsp70 expression will not be robust following stress. Hence, it is possible that the Plnt did not show increases in Hsp70 accumulation to the same degree as the WV because constitutive levels of Hsp70 may have pre-protected the Plnt from exercise-induced stress. Undefined muscle specific differences may also exist, as prior work using a

longer term model of muscle loading has observed that increases in Hsp70 are also restricted to the Type I and IIa fibers in the Plnt (31).

Febbraio et al. (8) reported that following a 4-5 hr bout of cycling, Hsp70 accumulation occurred only in muscles that had been previously depleted of glycogen. Surprisingly, production of Hsp70 was not induced in the control/non-depleted leg despite the fact that post-exercise glycogen levels in this leg were approximately equal to pre-exercise levels in the depleted leg. It was suggested that since glycogen levels were lower in the depleted leg following the exercise bout, a threshold exists whereby glycogen must reach a critically low level before the stress response occurs. Therefore, in the current study, it is plausible that the fiber specific staining pattern was because only those fibers that were recruited during exercise, and thus depleted of carbohydrates, synthesized Hsp70. It should be noted that as glycogen repletion following exercise occurs much sooner than the production of Hsp70 (12), this study was unable to determine whether glycogen depleted fibers were the same ones that showed increased Hsp70 accumulation 24 post-exercise.

As a potential means of demonstrating fiber recruitment without examining glycogen depletion, the localization of pHSF1 and its possible fiber specific co-localization to Hsp70-positive fibers was investigated. It well known that HSF1 is hyperphosphorylated in stressed cells (27; 35), however, the regulatory importance of this phosphorylation is not yet fully understood. Cotto et al. (7) demonstrated that HSF1 trimerization and DNA binding occurred in the absence of serine phosphorylation, but phosphorylation at the serine residue was needed to acquire transcriptional activity. Furthermore, Holmberg et al. (13) demonstrated that phosphorylation of site ser230 on

HSF1 is essential for activation and largely contributes to the stress-induced transcriptional activity of HSF1, and hence the induction of Hsp70. The current study, which utilized this phospho-specific HSF1 antibody, found that even in Con muscles most nuclei of both the Plnt and WV contain pHSF1. This might be expected as there must be some basal level of Hsp70 expression occurring in all cells. Surprisingly, however, increased localization of pHSF1 following exercise was not confined solely to fibers exhibiting increased Hsp70 expression.

While phosphorylation of HSF1 may indicate transcriptional activation of the *hsp70* gene, post-transcriptional control by downstream regulators may inhibit the response in certain fibers. Adenovirus-infected cells have shown post-transcriptional regulation of Hsp70 expression through degradation of Hsp70 mRNA despite the continued transcription of the gene (38). Alternatively, the phosphorylation of HSF1 may be not be sufficient for stress-induced activation and other additional modifications may be required in order for pHSF1 to become transcriptionally competent. Nevertheless, it appears that the phosphorylation of HSF1 is a dynamic process that allows for fine-tuning of the transcription factor, rather than being a mechanism to only turn on and off its activity. While the manner by which pHSF1 regulates the expression of Hsp70 is unresolved, the fact remains that the presence of nuclear pHSF1 does not explain why Hsp70 appears in a fiber specific manner following exercise. It is apparent that nuclear localization of pHSF1 is associated with, but may not be sufficient for Hsp70 expression, suggesting additional regulatory steps.

2.5 Conclusion

In summary, the present data supports the hypothesis that upon exposure to either acute exercise or acute heat shock, distribution of Hsp70 in rat fast muscles will vary. In the myofibers, Hsp70 revealed stark differences between the stress conditions, whereas in the vasculature, both stressors increased Hsp70 with the greatest increases observed following heat shock. Interestingly, both stressors induced Hsp70 in the endothelium and smooth muscle compartments. Similar to the myocardium, increases in Hsp70 were observed in the vasculature, which may provide insights into the protection afforded to the skeletal muscle by these interventions. In addition, the Hsp70 response appears to vary between different muscles. The Plnt increased its relative Hsp70 levels ~2 fold and ~3.5 fold compared to the WV which increased its relative Hsp70 levels ~5.5 fold and ~9 fold, following exercise and heat shock, respectively. In the Plnt, exercise-induced Hsp70 was localized to type I and type IIa fibers and not type IIx/IIb fibers. In contrast, the WV, which lacks type I and type IIa fibers, accumulated Hsp70 in type IIx/IIb fibers. Unlike observations in heat-shocked muscles, this non-uniform staining pattern suggests that the induction of Hsp70 is a result of muscle fiber recruitment. The transcription factor for Hsp70, pHSF1, was examined to see if activation of the exercise response (i.e., phosphorylation of HSF1 at ser230 exhibited fiber specific differences similar to Hsp70 accumulation. Although Western blots and immunofluorescence revealed relative increases in pHSF1 and its nuclear localization, we observed no fiber specific differences following exercise. Further studies should explore Hsp70 mRNA via fluorescence in *situ* hybridization following exercise and heat shock. As this study has noted cellular differences between Hsp70 and pHSF1, it may be worthy in localizing Hsp70 mRNA to

determine if there is a global response as with pHSF1, or a non-uniform response, like observed with Hsp70.

2.6 References

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APPENDIX B
Double-labeling Immunofluorescence

DOUBLE-LABELING IMMUNOFLUORESCENCE PROTOCOL

Preparation

1. Collect 15 μm tissue sections on frosted, pre-cleaned slides. May be use coated slides, i.e., poly-L-lysine coated. Label well using a fine-tipped pencil.
2. Store at -20°C until use, in tightly sealed slide case.
3. Ensure that an adequate supply of the following are available:
    - Frosted, selected, precleaned microslides (25 x 75mm, VWR)
    - Poly-L-Lysine-coated microscope slides with frosted end (EMS product no. 63410-02 from Cedarlane)
    - coverslips (18 X 18mm,VWR)
    - humidity chambers (1 per 12 slides). We use 30 x 17x 9 cm plastic containers with lids with 16.5x 17 cm plexiglass inserts raised up on inverted plastic screws)
    - Vertical staining jars
    - ImmunEdge hydrophobic barrier pen (Vector)
    - 10X PBS
        - NaCl 112 g
        - KCl 2.8 g
        - Na_2HPO_4 20.18 g
        - KH_2PO_4 2.8 g
        - Dissolve in approximately 1300 mL of MilliQ and adjust pH to 7.4. Bring to a total volume of 1400 mL with MilliQ and split to glass bottles.
    - 1X PBS which has been re-adjusted to pH 7.4
    - Invitrogen ProGold anti-fade mounting medium (containing DAPI)
    - Triton-X
    - Normal goat serum for blocking solution (Vector)
    - Primary antibodies
        - SPA-810 (StressGen Hsp70, 1:100)
        - BA-D5 (MHC I, 1:400)
        - SC-71 (MHC IIa, 1:100)
        - 212F (MHC IIx/IIb, 1:200)
        - sc30443-R (Santa Cruz pHSF1, 1:200)
        - SPA-901 (StressGen, HSF1, 1:100)
        - L9393 (Dako laminin, 1:100)
        - A0082 (Sigma vWF, 1:200)
        - ab5694-100 (Abcam SM, 1:200)
    - Secondary antibodies (Invitrogen)
        - Alexa Fluor 488 Goat Anti-Mouse IgG (1:400)
        - Alexa Fluor 594 Goat Anti-Rabbit IgG (1:400)
4. Determine slide number/stain designation.

Day 1

5. Remove sections from freezer (keep in box to allow gradual warm-up).
6. Cover bottom of humidity chambers with d H_2O. Place slides on racks such that they overhang slightly as this facilitates their movement later. Allow to air dry at RT for ~ ½ hr.
7. Fixation: immerse sections in 80% Acetone/20% Methanol (4°C) for 15' a 4°C.
   Other Fixations that can be used if necessary:
   - 3.7% formaldehyde/PBS for 10' at RT
   - 100% Acetone for 10' at -20°C
8. Aspirate and wash 2 × 5' in PBS. Vertical staining jars work best.
9. Permeabilize: immerse sections in 0.05% triton-X/PBS for 5' at RT.
10. Aspirate and wash in PBS 3 × 5'.
11. Double circle sections with Image Pen.
12. Prepare blocking solution: 10% goat serum in PBS. Approximately 40 ul are needed for each section. Incubate at RT for 1 hr. Thaw primary antibodies at 4°C
13. Wash in PBS 3 x 5'.
14. Add primary antibodies. Suitably dilute primary antibodies (dilution shown above) and make up in PBS plus 0.1% goat serum. Apply both rabbit and mouse primary antibodies at same time for double label immunocytochemistry. Cover humidity chamber and incubate overnight 4°C

Day 2

15. Aspirate primary antibodies and wash in PBS 3 × 5'.
16. Apply secondary antibodies (apply in a darkened room). Dilute secondary antibodies in a 1:400 ratio in PBS. Allow incubation for 60 min at RT in the dark.
17. Aspirate secondary antibodies and wash in PBS 3 × 5'.
18. Mount with a fluorescent mounting medium. ProLong antifade with DAPI allows you to stain nuclei with the DNA intercalating fluorescent dye DAPI, which you can see on a fluorescence microscope fitted with appropriate blue filters.
19. Allow medium to harden. Place sections in an oven set for 37 °C to increase the speed of drying.
20. Store sections in dark folders (to minimize photo bleaching), at 4°C until use.
21. View on fluorescence microscope. We use confocal Zeiss LSM 410 mircroscopy.

Confocal microscopy

22. Before capturing images, place one slide on the stage to set initial brightness and contrast levels. Initial levels are set to the greatest intensity of fluorescent lighting occurring (most favourably the middle of the tissue). Brightness and contrast levels need to be created for each antibody used.
23. Next, place a slide on the stage with secondary antibody only. Adjust initial brightness and contrast levels until there is no evidence of secondary fluorescent lighting occurring. Nuclear fluorescent lighting from DAPI can be used locate middle of the tissue in regards to thickness.
24. Use the adjusted brightness and contrast levels when capturing images, this will ensure consistency among experimental groups. When changing magnifications new brightness and contrast settings need to be constructed.