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The effects of acute high- and low-intensity exercise on Hsp70 and Hsp90 accumulation in rat skeletal myofibres and vasculature

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Graduate Program in Kinesiology

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

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The effects of acute high- and low-intensity exercise on Hsp70 and Hsp90 accumulation in rat skeletal myofibres and vasculature

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By

Hana Kowalchuk

Graduate Program in Kinesiology

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

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THE UNIVERSITY OF WESTERN ONTARIO
ABSTRACT

Exercise induces cytoprotective stress proteins in blood vessels (BV) and skeletal myofibres. The localization and extent of induction with different exercise intensities is unclear. Rats (n=10 per group) were run at high (HIEX; 30m/min) versus low-intensity exercise (LOEX; 15m/min) for 1hr, sacrificed 24hr later. Sections of the white portion of the vastus were stained immunofluorescently for Hsp70 and Hsp90. It was hypothesized that: 1) a greater abundance of Hsp70 and Hsp90 would be observed in BV following HIEX, 2) due to earlier recruitment, larger BV would have a more robust response than small BV, 3) more myofibres surrounding BV expressing Hsp70 would express Hsp70. Ratio of BV with Hsp70/total vessels and Hsp70 density was greater in HIEX versus LOEX (p<0.05). Large vessels expressed Hsp90 similarly for LOEX and HIEX; more small vessels expressed Hsp90 at HIEX (p<0.05). The ratio of Hsp70 active myofibres/total myofibres was greater surrounding small BV (p<0.05). The Hsp70 response in BVs and Hsp70 localization to myofibres surrounding them suggests an intensity-dependent effect linking blood flow and myofibre recruitment to HSP activation.

**Keywords:** Heat Shock Protein, Hsp70, Hsp90, intensity-dependent, immunofluorescence
CO-AUTHORSHIP

Dr. Earl Noble was involved in the design of the experiment.
AKNOWLEDGMENTS

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I would also like to thank the rest of the Noble lab, as well as Dr. Jamie Melling, for their help.

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<tr>
<td>Ach</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>αSM</td>
<td>alpha smooth muscle actin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>calcium ion</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CON</td>
<td>control Sedentary</td>
</tr>
<tr>
<td>DAPI</td>
<td>1% 4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>GS</td>
<td>goat serum</td>
</tr>
<tr>
<td>HIEX</td>
<td>high-intensity exercise</td>
</tr>
<tr>
<td>HSE</td>
<td>heat shock element</td>
</tr>
<tr>
<td>HSF</td>
<td>heat shock factor</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein family</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat shock protein individual isoform within the family</td>
</tr>
<tr>
<td>hsp</td>
<td>gene and mRNA product</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol 3-phosphate</td>
</tr>
<tr>
<td>K(^{+})</td>
<td>potassium ion</td>
</tr>
<tr>
<td>kD</td>
<td>kiloDaltin</td>
</tr>
<tr>
<td>LOEX</td>
<td>low-intensity exercise</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cutting temperature</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered solution</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Triton-X phosphate buffered solution</td>
</tr>
<tr>
<td>PiP2</td>
<td>phosphatidyl inositol biphosphate</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SM</td>
<td>smooth muscle</td>
</tr>
<tr>
<td>SP</td>
<td>stress protein</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand Factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WV</td>
<td>white portion of the vastus lateralis</td>
</tr>
</tbody>
</table>
CHAPTER 1

1.1 Introduction

Heat shock proteins are a family of molecular chaperones, which protect against cellular homeostatic disruption. Although referred to as ‘heat shock’ proteins, heat stress is not the only stress that activates this response. Other cellular stressors indicated in the activation of heat proteins are acidosis, hypoxia, metabolite depletion, protein degradation, increased intracellular calcium concentration and many other homeostatic changes accompanying exercise (reviewed in (15, 46, 56, 60)). There are a number of heat shock protein subfamilies (HSP), categorized by molecular weight, with each family containing individual protein isoforms (Hsps) (60). The most commonly recognized and characterized HSPs are Hsp27, Hsp60, Hsp70 and Hsp90; of these, Hsp70 remains the best characterized (24, 60), and evidence is emerging of the importance of Hsp90 in vasculature cells (3, 7, 23, 27, 73). This literary review will focus on two heat shock protein families (HSP70 and HSP90) and their subsequent relationships and reactions to exercise within skeletal myofibres and the vasculature.

1.2 HSP Overview

1.2.1 HSP70 Family

The cytoprotective 70-kilodalton (kD) family is highly inducible in response to a wide array of stressors. HSP70 is found in all organisms and is necessary for cell function and survival (44). Of the specific isoforms of the HSP70 family, Hsp70 (72-kD) exhibits the greatest response to stress (24, 46, 56, 60). Although constitutively expressed in some
cells (44), Hsp70 has the capability of further activation in response to a disruption in homeostatic balance (19).

While involved in many cellular functions, Hsp70 is specifically involved in protein translation, translocation and refolding of denatured proteins (11, 30, 36, 74). As a molecular chaperone, Hsp70 acts to stabilize protein substrates and functions as a protector to mitochondrial and sarcoplasmic reticular function (4, 17, 29, 55, 83). Stress can interfere with proper formation and maturation of newly synthesized proteins and Hsp70 acts to eliminate the further production of non-native proteins by interacting with other molecular chaperones and adenosine triphosphate (ATP) to either degrade the protein or refold it to its original state (4, 8, 36, 54, 74). Early in vitro studies showed that when Hsp70 was produced following stress, cells could survive another bout of stress (heat shock) with little damage (as reviewed in (40)). The protective aspects of Hsp70 have been extensively explored in the heart; enhanced myocardial function and reduced infarct size post-induction of stress were seen when levels of Hsp70 were augmented (33, 62, 63, 69). In skeletal muscle it has been suggested that the induction of Hsp70 attenuates the inflammatory response, thereby potentially reducing muscle damage (10, 59).

1.2.2 Hsp90

Heat shock protein 90 (90kD) is an abundant cytosolic protein, which also acts in part as a molecular chaperone (3, 24, 67). As well as having highly specific binding partners, Hsp90 acts to coordinate trafficking and regulation of signaling proteins (23) and enables proper folding of its associated substrates (3). Hsp90 is constitutively
expressed within the vasculature and when suppressed, inhibits angiogenesis (3, 49). Another important interaction of Hsp90 is its association with the enzyme endothelial nitric oxide synthase (eNOS) which stimulates the production of nitric oxide (NO), a potent vasodilator (3, 20, 23, 90).

Hsp90 is responsible for aiding in the activation of eNOS by assisting with the coupling of eNOS monomers and their phosphorylation (20, 23, 61, 65, 79, 90). Hsp90 binds directly to eNOS when stimulated by factors such as fluid shear stress, histamine release, and increases in vascular endothelial growth factor (VEGF) concentration (3, 9, 20, 21, 23). The stimulatory effects of Hsp90 and eNOS will be explored below in 1.3.2 in relation to exercise.

1.2.3 Hsp70 and Hsp90 Response Regulation

The stress protein (SP) response is activated after an incident of stress of sufficient intensity (46) and Hsp70 can be detected 15min post-exposure to elevated temperatures (40). The response is regulated by a heat shock transcription factor (HSF) that binds to a protein sequence known as the heat shock element (HSE) (6, 13, 51, 54, 56, 64). Upon an incident of stress, pre-existing inactive HSF is converted to its active form to activate the synthesis of more Hsps (57). Under normal conditions Hsp70 and some Hsp90 are bound to HSF-, specifically HSF-1 (13, 45, 51, 54, 57, 91), but they dissociate once a sufficient stress occurs (51, 54) thereby converting the HSF into its active form (57). HSF-1 is then free to translocate to the nucleus, undergo phosphorylation and bind to the HSE (13, 36, 51, 54, 70). This leads to transcription of the Hsp70 and Hsp90 genes which results in
increased Hsp70 and Hsp90 protein leading to a feedback loop whereby the stress proteins rebind HSF-1, thus deactivating further transcription of HSPs (1, 54).

1.3 Exercise as an HSP Activator

1.3.1 Skeletal Muscle and HSPs

Physical activity is associated with the increase of HSPs in rodents (43). Exercise is responsible for many changes in cellular conditions including but not limited to: ATP depletion (5, 76), glycogen depletion (17, 23, 27, 52, 67, 70), fluctuations of the pH level (76, 86) often from the production of lactic acid and H\(^+\) ions, an influx in calcium ion release (76, 87), increased temperature, and production of free oxygen radicals (76, 77). These changes in cellular homeostasis are enough to cause an induction in HSPs.

Skeletal muscle is comprised of many different elements that work together to become a functional unit. Each muscle contains fibres that contract or lengthen as needed. There are different types of muscle fibres each with their own characteristics that have been titled as type I, IIa, IIx and IIb (Table 1) (28). Recruitment of muscle fibres during exercise depends on what type and intensity of exercise is being performed. Type I fibres are reliant on O\(_2\) as a main substrate and are slower contracting with a high resistance to fatigue (28, 59). In some type I fibres, HSPs have been shown to have higher constitutive levels in the unstressed basal state (42, 44, 59, 82). Type IIa fibres are capable of readily using energy from both oxidative and glycolytic pathways to provide energy for contraction. They contract at a high contractile speed but, like the type I fibres, are able to work for an extended period of time (28, 60). Unlike type I fibres, type IIa fibres are not chronically activated but used mainly for moderate activities such as
walking or running (28, 59). The fastest twitch muscles consist of primarily type IIb fibres, which have a large capacity for the glycolytic metabolic pathway but minimal resistance to fatigue so they tire quickly (28, 59). The more recently discovered IIx fibre type (37, 71) is metabolically between the IIa and IIb fibre types. Although all cells have a constitutive level of HSP expression, type II fibres have the lowest constitutive levels of HSPs and due to this, may show the greatest relative increase in HSP expression after utilization (26, 50).

<table>
<thead>
<tr>
<th>Muscle Type</th>
<th>Metabolic Pathway</th>
<th>Muscle Contractile Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>Oxidative</td>
<td>Slow</td>
</tr>
<tr>
<td>Type IIa</td>
<td>Oxidative/Glycolytic</td>
<td>Fast</td>
</tr>
<tr>
<td>Type IIx</td>
<td>Oxidative/Glycolytic</td>
<td>Fast</td>
</tr>
<tr>
<td>Type IIb</td>
<td>Glycolytic</td>
<td>Fast</td>
</tr>
</tbody>
</table>

During exercise, body temperature increases due to heat being released as a byproduct of skeletal muscle contraction. Temperature is a known inducer of HSPs in all cells, and the increase in temperature accompanying exercise causes the transcriptional process to begin. There has been debate as to whether the HSP response of exercise is due simply to the resulting increase in temperature or if other factors associated with exercise play a role. Skidmore et al. (76) exercised rodents in a cold environment and found that without an increase in core temperature Hsp70 levels in skeletal muscle still increased significantly (76). A separate study performed by Neufer et al. (58) used electrical stimulation of the motor nerve in the rabbit hindlimb to produce contraction
without a subsequent increase in muscle temperature (58). As in the previous study, Hsp70 levels increased in skeletal muscle without a temperature increase (58), (although their may have been minor local temperature increases associated with muscle contraction). These results were further supported by Silver et al. (75), who looked at hsp70mRNA levels from groups of exercised, non-exercised but heat shocked to 40°C (a temperature comparable to exercise temperatures) and non-exercised but heat shocked to 42°C rats (75). The results showed a more robust hsp70mRNA response in exercised rats compared to non-exercised but heat shocked to 40°C rats, indicating that the HSP response to exercise is not affected solely by exercised induced increases in temperature (75).

The fibre types of skeletal muscle enable different types and intensities of exercise to be performed. When using rats as the experimental subject, treadmill running is most commonly used. A higher running speed causes a more pronounced response of exercise-related cellular changes, as well as causing a greater need for muscle recruitment and O₂ uptake to sustain the higher intensity of exercise. Glycogen depletion as well as the reliance on the phospho-creatine system occur with exhaustive unsustained exercise and both induce HSPs (18, 24, 28). Type II fibres are typically recruited for faster, more exhaustive exercise and use the formation of lactic acid to provide energy when glycolytic metabolism is limited and free glucose is no longer readily available (28). Lactic acid however, decreases the cellular pH and stimulates HSP induction (28, 76, 86). As treadmill speeds increase there is a shift of recruitment from type I fibres to type IIa fibres, to type IIx fibres and finally to type IIb fibres (50). Milne and Noble (49) demonstrated that the lateral portion of the vastus lateralis (VW) (a known fast twitch
muscle) exhibited limited increases in Hsp70 levels until a speed of 24m/min. At speeds faster than 24m/min the WV exhibited an exponential increase in Hsp70 content (50). This is in accordance with high-intensity exercise leading to a greater recruitment of type IIb fibres as well as the observation that cells with initial low levels of Hsp70 show a greater relative increase in Hsp70 expression when exercise stressed (25, 50). Low-intensity exercise, although not as strenuous as high-intensity activity, still elicits a physical response from a rat, including increased heart rate, blood flow and changes in metabolites (38, 50). Laughlin and Armstrong (82), looked at different treadmill running speeds and the effect the changes in intensity had on specific muscle blood flow, heart rate and muscle recruitment (38). Their findings demonstrated that at 15m/min the rats were performing a fast walk, just before beginning to run, heart rate had increased significantly and blood flow to oxidative type I and type IIa muscles had increased but type IIx and IIb saw a decrease in blood flow (38). This absence of vasodilation suggests that the WV is not recruited to a significant extent or performing much work at exercise of low-intensity (38). To date, no studies have investigated the localization of HSPs at different intensities of exercise.

1.3.2 The Vasculature and HSPs

Hsp70 induction in the myocardium and its vasculature is cardioprotective (2, 14, 31, 52). Currie et al. (14) found with whole body heat shock, cardiac recovery improves following post-ischemic perfusion (14). Hsp70 increases in the myocardium are shown to aid in decreasing the impact of an infarct (31).
The increase in blood flow that accompanies the onset of exercise is controlled by resistance arterioles that determine the location of the influx of blood to the necessary organs (68). This increase in flow, although critical for the maintenance of exercise, also causes stress on the vessels. Vascular cell walls are subjected to stretch, circumferential and shear stresses (47, 84), which can cause an increase in the stress protein response. Heat shock is known to have positive effects on the vasculature (2, 39) and can potentially help protect the vasculature by preventing damage to smooth muscle and smooth muscle dysfunction (32).

The layer of endothelial cells that comprises the inside of a blood vessel exhibits the first response to the increase in shear stress. As cardiac output increases, the resultant regional increase in blood passage elicits shear stress on local endothelial cells (12, 16, 53, 84). In response to shear stress, membrane proteins are deformed and ion channels are opened and activated, releasing vasodilatory factors (66). As the endothelial membrane is subjected to blood flows, calcium (Ca\(^{2+}\)) activated potassium (K\(^+\)) channels, hyperpolarize the membrane allowing the entrance of Ca\(^{2+}\) into the cell and resultant stimulation of eNOS (12, 16, 53). eNOS synthesizes the conversion of L-arginine into NO, which diffuses into adjacent smooth muscle (SM) cells to stimulate vasodilation by decreasing SM intracellular Ca\(^{2+}\) through a series of pathways (88).

Hsp90 is known to act upon eNOS to upregulate the production of NO leading to further vasodilation; this relationship is enhanced by shear stress (20, 22, 23, 61, 65). Takahashi and Mendelsohn (80) investigated the interaction of Hsp90 and calmodulin (CaM- a messenger protein part of the calcineuron complex that transduces Ca\(^{2+}\) signals) on eNOS at different cellular levels of Ca\(^{2+}\). An increase in endothelial cellular Ca\(^{2+}\)
signals Hsp90 to activate CaM independently; thereby, maximizing the conversion rate of L-arginine to NO, further stimulating vasodilation (23, 27, 80, 81). Both Hsp90 and Hsp70 actively bind to CaM, although Hsp90 can upregulate calcineurin activity independently of CaM whereas Hsp70 uses a CaM-dependent path to activate calcineurin (78). As noted above Hsp90 also directly interacts with eNOS monomers helping them phosphorylate and dimerize into active conformation (3). The complete process of how Hsp90 binds to and influences the eNOS pathway is still under investigation.

Hsp70 can be activated through exercise-induced laminar shear stress as well and is thought to regulate vascular contractility via thick filament regulation (35). Hsp70 is thought to provide protection to the vasculature in a similar fashion as it does skeletal muscle (59); specifically through stimulation of anti-inflammatory cytokines (89), like the NfKB pathway (85), and inhibition of inflammatory cytokine release (34). Hsp70 also appears to be an important factor in angiogenesis (48).

Skeletal muscle arteriolar vasodilation is imperative to continue exercise. It is known that HSPs are induced in skeletal muscle vasculature in response to exercise (82) but until recently the magnitude of the response was not clear. In a recent study by Silver et al. (75) it was found that high-intensity exercise lead to rapid transcription of hsp70 mRNA in rodent skeletal muscle vasculature. Peak hsp70 mRNA signal was found 1hr post-exercise in the vessel walls indicating an immediate protective or regulatory response (75).

In the study by Silver et al. (75) different groups of rats were exercised or heat shocked to a temperature comparable to that reached during exercise. The interesting
finding from this procedure was that in relation to both skeletal myofibres and vasculature the HSP response was greater in the exercise group than in those animals heated to typical exercise temperatures. In a study by Milne et al. (52) it was found that post-exercise Hsp70 was localized mainly to the SM of the vasculature of the myocardium, whereas with HS Hsp70 was found both in the endothelium and the smooth muscle (52). This indicates that other factors are involved in the activation of Hsp70 besides a rise in core temperature (75).

1.4 Rationale, Purpose and Hypotheses

Although it has been demonstrated that altering exercise intensity can cause a differing HSP response (higher intensity elicits a greater HSP response), previous studies have generally employed a whole muscle analysis to measure protein (41, 50) rather than determining a distribution and localization of the HSP response. Many studies have documented a localization of HSPs to the vasculature in the myocardium (2, 31, 39, 52) but with few exceptions (75) this has not been looked at in skeletal muscle vasculature. Hence, the present study used differing exercise intensities to manipulate temperature and blood flow during exercise as well as the metabolic stress placed on the muscle fibres in order to identify whether there is a differential HSP response in the vasculature.

The purpose of this study was to investigate the distribution and localization of Hsp70 and Hsp90 in rodent skeletal muscle and intermyofibrillar vasculature in response to various exercise intensities. It was hypothesized that: I) Following high- and low-intensity aerobic exercise, all blood vessels would demonstrate increased Hsp70 and Hsp90 in an intensity-dependent manner, with high-intensity exercise increasing the
abundance of these proteins over low-intensity exercise, II) due to increased shear stress during exercise, larger blood vessels would have a more robust Hsp70 response within each exercise intensity, III) more myofibres expressing Hsp70 would more likely be found around small vessels already expressing Hsps than those around larger vessels and, IV) Hsp90 levels would not differ across intensity groups in skeletal myofibres.
1.5 Reference List:


CHAPTER 2

2.1 Introduction:

Exercise is known to improve overall health and provide protection to the vasculature (18, 49, 51, 67). The underlying mechanisms responsible for this protection are likely numerous and are still being investigated actively. Of potential protective agents, heat shock proteins (HSPs) have been found to be cardioprotective against ischemia-reperfusion injury (11, 53) and the protection may be associated primarily with the vasculature (34, 44). Heat shock proteins are a family of molecular chaperones that protect against disruptions to cellular homeostasis. The cytoprotective 72 kD heat shock protein (Hsp70) is the most commonly studied inducible HSP (36). Although expressed constitutively in certain cells (36), Hsp70 can be expressed further in response to stress and provides cellular protection through stabilization of protein substrates as well as being involved in protein translation, translocation and refolding of denatured proteins (6, 9, 12, 13, 20, 22, 30, 45, 58, 65). Another abundant cytosolic protein is Hsp90 (90kD). In addition to acting as a molecular chaperone, Hsp90 aids in the coupling and activation of eNOS in the vasculature, thereby increasing production of NO and enhancing vasodilation (15, 16, 50, 54, 56).

Exercise is known to increase skeletal muscle blood flow, leading to shear stress (38, 61), and exercise also elevates core temperature. Both of these stimuli could stress the vasculature of the skeletal muscle and induce HSP expression in rodent blood vessels (8, 14, 15, 35, 59). Although it is well known that an increase in temperature does activate an HSP response, the temperature increase accompanying exercise is not the sole reason for the exercise-associated increase in HSPs. Previous work by Silver et al. (59)
showed the *hsp70* mRNA response to be significantly greater in skeletal myofibers of exercised rats than the same muscle of rats heat shocked to an exercise relative temperature. However, the *hsp70* mRNA response appeared to be similar in the skeletal muscle vasculature between the two groups (59). Unlike in quiescent skeletal muscle fibres, the vasculature of heat shocked animals may be exposed to additional shear stress due to the increased cardiac output (69) or may experience increased adrenergic stimulation (39, 46) and this may account for the observed differences. It is likely that these are potent activators of the heat shock response in the vasculature (15, 54, 70).

Differing exercise intensities alter muscle recruitment and blood flow patterns to muscles (32). As intensity increases there is a progressive shift of recruitment from type I fibres to type IIa and type IIb fibres (42). Indeed, at lower running speeds there is an initial decrease in blood flow to muscles that are predominately type IIb fibres, such as the white portion of the vastus lateralis (WV) (32, 42), presumably as blood is shunted to the more active oxidative muscles. Blood flow to the WV starts to increase at speeds of 30m/min and then begins to increase drastically above critical power levels (generally above 50m/min) (10). The increased muscle fibre and blood flow recruitment in this muscle is reflected in the increase in Hsp70 levels once running speeds reach 27m/min and above (42). These data were collected in whole muscle however, and changes in the Hsp content in the vasculature in response to differing exercise intensities is unknown. One might assume however, that the increased blood flow would be directed to those fibres that are metabolically active and hence there might be an association between the increase in HSPs in the vasculature and the adjacent muscle fibres. Recently it was found that high-intensity exercise causes a rapid transcription of *hsp70* mRNA in blood vessels
in rat skeletal muscle with a peak signal occurring 1 hr post exercise which suggests an immediate stress, possibly protective, response (59). Hsp90 is found at constitutively greater levels in vessels than Hsp70 seemingly regardless of stress and temperature levels (5, 7, 35). Additionally there are no data available regarding the effect of exercise intensity on Hsp90 levels.

Given the important roles of Hsp90 in regulating angiogenesis (41) and eNOS activity (14, 71) and Hsp70 in vascular protection (52, 61), the purpose of the present study was to investigate the distribution and localization of Hsp70 and Hsp90 in rat skeletal myofibres and vasculature after high- and low-intensity exercise. It was hypothesized that: I) Following high- and low-intensity aerobic exercise, all blood vessels would demonstrate increased Hsp70 and Hsp90 in an intensity-dependent manner, with high-intensity exercise increasing the abundance of these proteins over low-intensity exercise, II) due to increased shear stress during exercise, larger blood vessels would have a more robust Hsp70 response within each exercise intensity, III) more myofibres expressing Hsp70 would more likely be found around small vessels already expressing Hsps than those around larger vessels and, IV) Hsp90 levels would not differ across intensity groups in skeletal myofibres.

2.2 Materials & Methods:

2.2.1 Animals:

This study was approved by The University of Western Ontario Council on Animal Care and was conducted in accordance with the Guidelines of the Canadian Council on Animal Care. Thirty, eight-week-old male Sprague-Dawley rats (~220g) were
obtained from Charles River, (St. Constant, Quebec) and housed 2 animals per cage in a climate-controlled facility with a 12h light/dark cycle. Rats were fed and watered *ad libitum* for a two-week period prior to the experiment.

**2.2.2 Experimental Treatment:**

Rats were divided randomly into 3 groups (n=10 each): sedentary control (CON), low- (LOEX) and high-intensity (HIEX) exercise. All animals underwent a period of familiarization to treadmill running, (2min at 15m/min, 4min at 24m/min, 2min at 30m/min and 2min at 15m/min), 4 and 2 days prior to the beginning of the experimental protocol. On the day of the experiment, the LOEX group was subjected to 1hr of treadmill running (2% incline) at 15m/min whereas the HIEX group ran for an hour at 30m/min (2% incline). Rectal temperatures (with the probe inserted 5cm) were measured pre- and post-exercise as well as at 15min intervals throughout the exercise period; rats were removed from the treadmill for approximately 1 minute to have temperatures recorded. The CON group was handled similarly to both LOEX and HIEX without performing exercise and rectal temperatures were only measured just before sacrifice. LOEX and HIEX animals were killed 24hr post-exercise with CON animals being taken in the same week as the other two groups.

**2.2.3 Sample Collection:**

After rats were sacrificed under anesthetic (sodium pentobarbital-65mg/kg), the white portion of the vastus lateralis (WV) was obtained. As a primarily fast twitch muscle (type IIx and IIx/b fibres only), the WV has low constitutive levels of Hsp70 and was chosen therefore as a sensitive marker for Hsp70 changes. Segments of the harvested muscles were mounted on cork with the fibres arranged perpendicularly in OCT (optimal
cutting temperature) compound. The mounted segments were then submerged in isopentane cooled by liquid N$_2$ and stored at -70$^\circ$C. Subsequently, muscle cross-sections, 8µm thick, were cut on a cryostat (CM350) and transferred to poly-L-lysine coated, positively charged slides and left to air-dry for 30min. Dry cross-sections were stored at -30$^\circ$C until use for immunohistochemistry. Although all groups began with an n equaling 10 animals, due to manual error including poor tissue freezing and cutting technique, the sample size was decreased in certain groups.

2.2.4 Immuno histochemistry Protocol:

After slides were removed from the freezer and allowed to come to room temperature (~30min) they were separated into coplin jars and washed and hydrated in a 0.1% Triton-X phosphate buffered solution (PBS-T) for 5min, followed by three 1XPBS washes (5min each). A 10% Goat Serum (GS) solution was used to block the tissues (1h at room temperature) followed by three more 1XPBS washes (5min each). Primary antibodies, rabbit polyclonal anti-Hsp70 (SPA-812, StressGen Hsp70; 1:100) and mouse monoclonal anti-Hsp90 (BD610418 BD Transduction Laboratories ™; 1:100) were added to 1% GS to reach the 1:100 dilution. After being added to the slides (50µL/section), primary solutions were incubated overnight at 4$^\circ$C. The following day slides were again washed three times with 1XPBS, 5min each, and secondary antibody was applied in the dark. Secondary anti-mouse (Alexa Fluor® 488 Goat Anti-Mouse IgG, Invitrogen, 1:400) and anti-rabbit (Alexa Fluor® 594 Goat Anti-Rabbit IgG, Invitrogen, 1:400) are combined together in a 1% GS solution to create the appropriate dilution and added to the tissues (50µL/section) before being incubated for an hour at room temperature. Following incubation, three more 1XPBS washes (5min each) were done
and the slides were then mounted with a fluorescent mounting medium (Prolong® Gold Antifade Reagent containing DAPI (1% 4’, 6-diamidino-2-phenyindole), Invitrogen), to counterstain for nuclei. Slides were stored at 4°C in a slide folder until use.

2.2.5 Vessel Fluorescent Imaging and Analysis:

In a dark room, fluorescent images were viewed using fluorescent and brightfield microscopy (Zeiss Axiovert S100) and analyzed using Northern Eclipse 6.0 and ImageJ software. All images were taken with the same magnification (40X) and microscope settings.

Each muscle section was thoroughly scanned and all blood vessels with visible smooth muscle were counted. Diameters of the whole vessel were each measured along both the long and short axes and averaged in order to estimate vessel diameter. Based upon their diameters, vessels were divided into 2 groups, large (22µm to 109µm) and small (3.5µm to 22µm) vessels. An image of each blood vessel was recorded.

In order to obtain a better estimate of the effect of exercise intensity on the expression of vascular HSPs, the area and pixel densities of representative vessel walls within sections from each subject were determined. To correct for background fluorescence, the selected vessel wall area was overlaid over a background area of the section and integrated density was again measured. The density from the area of non-specific binding was subtracted from that of the blood vessel to give the corrected fluorescence in the vessel. This value was then divided by the area of the vessel wall in order to standardize the intensity measurements. This process was repeated for each vessel with signal. Negative control slides were also imaged (muscle sections absent of primary antibody but still stained with a secondary) in an attempt to view the scope of
auto-fluorescence to be certain the stain was an accurate depiction of protein present (See Appendix B).

2.2.6 Myofibre Fluorescent Imaging and Analysis:

Imaging of skeletal myofibres was done in conjunction with vessel analysis and the same protocol was followed. Using ImageJ, fibres expressing Hsp70 were individually outlined; the area recorded and integrated pixel density measured. To correct for background fluorescence, the outlined myofibre area was shifted over a non-reactive area of the section and integrated density was again measured. The density from the area of non-specific binding was subtracted from that of the myofibre to give the corrected fluorescence in the myofibre.

In order to determine if there was any relationship between the appearance of HSPs in the vasculature and that of Hsp70 in the surrounding muscle fibers, muscle fibres surrounding each vessel with a detectable signal in a radius of 2 fibres deep were assessed for Hsp70 expression. Fibres expressing Hsp70 were divided by the total number of fibres surrounding each vessel to indicate the relative proportion of fibres per vessel (both large and small) that expressed Hsp70.

At first glance, Hsp90 was not easily identifiable in individual myofibres. Therefore, instead of imaging individual fibres a standardized area (the size of the captured image) was measured and used consistently for each image throughout Hsp90 analysis in ImageJ.

2.2.7 Statistical Analysis:

Group mean and standard deviation (SD) were calculated for body mass and temperature recordings. A two way repeated measures analysis of variance (ANOVA)
with a one factor repetition of time was used to determine significant difference between temperatures of the HIEX and LOEX groups. An independent sample t-test was done between temperatures at 15min for exercise groups and CON temperature. Two way ANOVAs were used to determine significant differences for all comparisons between vessel size and exercise condition. A Tukey test was used as a post-hoc test for all ANOVAs.

2.3 Results:

2.3.1 Animal Masses & Temperatures:

Resting body masses and temperatures were measured and recorded for all treatment groups prior to exercise (Tables 1&2) (n=10 per group); no statistical difference was found between groups (p>0.05). A significant interaction of temperature between exercise condition and time was found (p<0.05). By 15 minutes of exercise, temperatures of HIEX (37.4° ± 0.2) and LOEX (36.8° ± 0.9) groups were significantly greater than CON (35.0° ± 0.5) and HIEX was significantly greater than LOEX (p<0.05). Both exercise conditions were higher than CON and HIEX remained significantly elevated (p<0.05) in comparison to LOEX, for the duration of exercise (Refer to Table 2).

2.3.2 Quantification of Hsp70 and Hsp90:

Plate 1 shows representative images of CON Hsp70 and Hsp90. Plates 2 and 3 are representative of Hsp70 and Hsp90 respectively with both LOEX and HIEX images in each plate. More Hsp70 is visible in the HIEX condition, specifically in the large blood vessels compared to LOEX. Hsp90 appears relatively uniform across both LOEX and HIEX conditions.
Figure 2.1 shows the ratio between blood vessels expressing Hsp70 and total blood vessels visible within the WV cross-section. A significant interaction between exercise and vessel size was found (p<0.05). In both large and small vessels, HIEX has a significantly greater proportion of vessels expressing Hsp70 than LOEX and CON (p<0.05). Significantly more large vessels in LOEX exhibited Hsp70 protein expression than in CON (p<0.05) and large vessels within both LOEX and CON express significantly more Hsp70 than small vessels in the same condition (p<0.05). These differences between large and small vessels disappeared in the HIEX condition. Integrated densities per unit area, in blood vessels containing Hsp70 are shown in Figure 2.2. A main effect was found in the exercise condition as well as the vessel size (p<0.05). HIEX, in both large and small vessels, had significantly greater protein content per unit area than in CON (p<0.05).

Figure 2.3 shows the ratio between blood vessels expressing Hsp90 and total blood vessels visible in the WV. A significant interaction between exercise and vessel condition was found (p<0.05). A significantly higher number of large vessels in both HIEX and LOEX express detectable Hsp90 than large vessels in CON (p<0.05). A significantly higher number of small vessels in HIEX had detectable Hsp90 than small vessels in LOEX and CON (p<0.05). Both HIEX and LOEX have significant differences between large and small vessels, with more large vessels expressing detectable Hsp90 than small vessels (p<0.05). Integrated density of immunofluorescent Hsp90 protein levels within vessels are shown in Figure 2.4. Unlike with Hsp70 (Fig 2.2), vessels expressing Hsp90 contained similar levels of Hsp90 and there were no differences across vessel size or exercise condition (Fig. 2.4).
Hsp70 expression in muscle fibres surrounding large or small blood vessels is demonstrated in Figure 2.5. Around 20% of muscle fibres within a 2 fibre diameter area around small blood vessels had Hsp70 present in the HIEX condition. There was a significantly interaction between exercise condition and vessel size (p<0.05). Significantly more muscle fibres around small blood vessels than large blood vessels expressed Hsp70 in the HIEX condition (p<0.05). More muscle fibres around small vessels expressed Hsp70 in HIEX than in either the LOEX or CON groups (p<0.05). The amount of Hsp70 present in those fibres surrounding blood vessels in which Hsp70 was detectable was significantly greater in HIEX than in LOEX or CON (Refer to Fig. 2.6) (p<0.05).

Hsp90 abundance in muscle fibres is shown in Fig.2.7 and there were no differences between exercise conditions (p>0.05).
### Table 2.1. Animal Masses (g) (Pre-exercise)

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>LOEX</th>
<th>HIEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Mass</td>
<td>305.45 ± 21.81</td>
<td>314.43 ±16.52</td>
<td>317.18 ±15.89</td>
</tr>
</tbody>
</table>

All values expressed as mean standard deviations (SD).

### Table 2.2. Rectal temperatures (°C)

<table>
<thead>
<tr>
<th></th>
<th>PRE</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>POST</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>35.04 ± 0.47</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOEX</td>
<td>35.20 ± 0.32</td>
<td>36.81 ± 0.93*</td>
<td>37.13 ± 0.48*</td>
<td>37.18 ± 0.53*</td>
<td>37.14 ± 0.66 *</td>
</tr>
<tr>
<td>HIEX</td>
<td>35.31 ± 0.18</td>
<td>37.43 ± 0.15†</td>
<td>37.93 ± 0.59†</td>
<td>38.04 ± 0.77†</td>
<td>38.12 ± 0.74 †</td>
</tr>
</tbody>
</table>

All values expressed as mean standard deviations (SD).* Significantly greater (p<0.05) than CON temperatures. † Significantly greater (p<0.05) than LOEX and CON temperatures.
Plate 1. Representative image of CON animals showing Hsp70 and Hsp90, respectively. Line is equal to 50 μm.
**Plate 2.** Hsp70 stains at LOEX and HIEX showing small and large blood vessels. Arrows show vessels and * labels reactive myofibres. Line is equal to 50 μm.
Plate 3. Hsp90 stains showing LOEX and HIEX large and small vessels. Arrows show vessels. Line is equal to 50 μm.
Figure 2.1. Ratio of blood vessels with detectable Hsp70 to total blood vessels in cross-section. #Significantly greater than CON and LOEX in vessels of the same size (p<0.05). *Significantly greater than CON in vessels of the same size (p<0.05). †Content of Hsp70 in vessels of different sizes are significantly different from each other (p<0.05). Mean values ± SD. n=8 rats for HIEX and LOEX. n=7 rats for CON.
Figure 2.2. Integrated density measures of Hsp70 protein in blood vessels. *Significantly greater than CON (p<0.05). Mean values ± SD. n=7 rats for HIEX. n=6 rats for LOEX and CON.
**Figure 2.3.** Ratio of blood vessels with detectable Hsp90 to total blood vessels in cross-section. #Significantly greater than CON and LOEX in vessels of the same size (p<0.05). *Significantly greater than CON in vessels of the same size (p<0.05). † Content of Hsp90 in vessels of different sizes are significantly different from each other (p<0.05). Mean values ± SD. n=8 rats for HIEX and LOEX and CON.
Figure 2.4. Integrated density measures of Hsp90 protein in blood vessels. No differences were found between any exercise conditions or vessel sizes (P>0.05). Mean values ± SD. n=5 rats for HIEX and CON. n=7 rats for LOEX.
Figure 2.5. Ratio of myofibres surrounding Hsp70 positive blood vessels to total myofibres (2 layers diameter) surrounding blood vessels. #Sig. greater than CON and LOEX for vessels of the same size (p<0.05). †Number of Hsp70 positive fibres around different size blood vessels within the same exercise condition are sig. different from each other (p<0.05). Mean values ± SD. n=7 rats for each group.
Figure 2.6. Integrated density measures of Hsp70 expressing myofibres surrounding blood vessels with Hsp70 present. The measures have been normalized to fibre area. #Sig. greater than CON and LOEX (p<0.05). Mean values ± SD. n=7 rats for each group.
Figure 2.7. Integrated density measures of Hsp90 across a cross-sectional view of a standardized area of muscle. No differences were found between any groups (p>0.05). Mean values ± SD. n=7 rats for each groups.
2.4 Discussion:

This study investigated the effects of differing exercise intensities on Hsp70 and Hsp90 expression and localization in skeletal muscle vasculature and myofibres. Hsp70 was induced by both HIEX and LOEX but HIEX caused the greatest response in both skeletal muscle fibres and vasculature. Within the WV sample, the HIEX condition induced a detectable Hsp70 signal in all large blood vessels and 98% of the small vessels as well, significantly greater than the response elicited during LOEX. More large vessels than small vessels expressed Hsp70 in LOEX but when the vessels exhibiting Hsp70 were quantitatively analyzed as Hsp70 per unit area, there was no difference between large or small vessels. There was a trend for small LOEX vessels to express less protein than the large ones though (p=0.10). This is true as well for HIEX, although unlike LOEX no differences were observed between the percentage of vessels of different sizes, expressing protein. These results are in agreement with the first hypothesis in terms of Hsp70 and partially in agreement for Hsp90. The proportion of vessels expressing detectable Hsp90 increased regardless of the exercise condition, however, while LOEX produced the maximal response in the large vessels, a greater proportion of small vessels in the HIEX condition expressed Hsp90 after exercise than in the LOEX group. In those vessels that did express detectable Hsp90 no differences per unit area were observed. Hypothesis II was not supported as HIEX had no difference in vessel activation between large and small vessels and even in LOEX (which saw a greater number of large vessels express Hsp70) when expressed as pixel density per unit area, there was no difference between larger and smaller vessels. In support of hypothesis III, in 20% of myofibres surrounding small vessels, Hsp70 was present post-HIEX and the amount of measured
Hsp70 present was significantly greater than in LOEX and CON. In accordance with hypothesis IV, no myofibrillar Hsp90 changes were detected supporting part IV of the hypothesis.

Whole body heat shock has been found to improve cardiac recovery after an infarct or cardiac event (11, 24). Within the cardiac vasculature, Hsp70 has been shown to accumulate within the smooth muscle and endothelium post-exercise or heat stress and may provide protection by preventing smooth muscle damage and dysfunction (1, 25, 26, 34, 44). Skeletal muscle vasculature may be an important target of the HSP response as well but there are limited data in this regard and none concerning differences in response to various exercise intensities. It has been shown that post-exercise, at a speed of 30m/min, Hsp70 concentration (61) and hsp70 mRNA (59) increased in the vasculature of skeletal muscle. The trigger for this HSP elevation likely results not only from an increase in core temperature (35) but also from an increase in blood flow leading to increased shear stress (55, 57). Two exercise intensities, which would elicit differing cardiovascular and metabolic challenges, were chosen in an effort to address the hypotheses raised in this experiment. Milne et al. (42) found 33m/min treadmill running to be above the lactate threshold for rats but a speed of 30m/min to be just below. The heart rate of a group of treadmill-exercised rats was found to be above 90% of the maximal heart rate at a speed of 28m/min (42). The lactate values and maximal heart rate values determined by Milne et al. (42) supports 30m/min treadmill running as a high-intensity speed (approximately 80-90% of VO$_2$ max). A speed of 15m/min for rats has been likened to a fast walk slightly below the speed where trotting begins (approximately 50-60% VO$_2$ max) (32).
The two exercise conditions elicited different loads on the muscle as animals in the HIEX groups exhibited a 3°C raise in rectal temperature over the course of the exercise session and at 15min of exercise and thereafter their core temperature significantly surpassed the LOEX group and remained elevated above CON. Rat temperatures appeared to be slightly hypothermic to begin with and were only raised to normothermic levels by the end of exercise, much different than temperatures typically seen with heat shock (35, 37, 40, 42). However, the change in the exercise-induced increase in temperature and accompanying cardiovascular and metabolic load was stressful enough to cause both Hsp70 and Hsp90 induction in LOEX and HIEX when compared to CON, and it appears as though temperature is not the sole reason for HSP increases during exercise (43, 59). A previous study by Silver et al. (59) found that exercise and not just the temperature change seen with exercise, would in fact cause a greater HSP response, and it has been previously shown that a temperature increase with exercise is not needed to induce Hsps (59).

The induction of exercise results in an increase in blood flow to the working muscles (2, 27, 32) which would result in an increased shear stress (38) with potential activation of the heat stress response (15, 38, 61). It has also been demonstrated that muscle activation results in the release of vasoactive substances, which would also increase blood flow to vessels in the vicinity of the active muscle fibres (19, 32, 40). Consequently, in the present study it was hypothesized that in the white portion of the vastus lateralis muscle (WV) induction of HSPs would be more evident in the HIEX versus the LOEX condition as a result of greater muscle recruitment. Moreover, it was anticipated that the HIEX condition would recruit smaller vessels as nearby muscle fibres
activated vasodilatory mechanisms. Previous studies have examined bulk blood flow and presumably muscle recruitment in the WV of rats running on a treadmill (10, 33) but the results are somewhat equivocal. Laughlin and Armstrong (32) found a significant decrease in blood flow to the WV at 15m/min from pre-exercise and no significant increase in blood flow until speeds reached at least 45 to 60m/min (32). In contrast, Copp et al. (10) found an approximately 3.5 fold increase in blood flow for rats that were running at 20m/min at a 10% grade. The reason(s) for these differences are unclear, however Laughlin and Armstrong (33) observed significantly higher resting blood flows than did Copp and colleagues (10) and this may account for the lack of increase in blood flow until very high running speeds. In other studies either using western blots to examine total tissue Hsp70 (42), or proxy markers of muscle activity such as cytochrome c (3), these markers begin to increase between 20 and 30m/min running speed suggesting recruitment of the WV. In the present study, muscle blood flow was not measured, however, if one assumes that some increase in blood flow occurred with recruitment, it clearly would have been greater in the animals in the HIEX group.

Interestingly, although there was no difference in the proportion of large versus small vessels expressing Hsp70 in the HIEX group, fewer small vessels in the LOEX group expressed protein. Although small arterioles receive increased blood flow prior to increased conduit flow, the conduit’s still have greater levels of shear stress, which might account for the differences seen between vessel sizes in the LOEX group (4, 23, 62). With increased exercise intensity, sympathetic tone may also be increased, leading to additional α-adrenergic stimulation resulting in the greater Hsp70 response seen with HIEX over LOEX. α-adrenergic stimulation has been shown to induce the hsp70 gene
promoter in immune cells of oysters as well as activating an increase in HSF activity in rats (31, 48, 70). Although the α-adrenergic vasoconstriction would be overcome by local vasodilators in the working muscle, the combination of adrenergic stimulation and shear stress would act as a powerful activator of the stress response and subsequent increases in Hsp70.

One of the questions that the present study addressed was whether the apparent “checkered” (meaning a non-uniform response) pattern of the Hsp70 induction in specific muscle fibres during exercise (63, 64) might be associated with enhanced blood flow to the area. It was observed that significantly more skeletal myofibres were found to contain Hsp70 surrounding small vessels than large vessels during HIEX and across intensity groups as well; myofibrillar Hsp70 was not uniformly induced across the muscle section. It has been suggested that differential Hsp70 activation in myofibres is based on myofibre recruitment and possibly glycogen depletion during exercise (13, 32, 42, 47). This is supported by the current findings of a greater number of Hsp70 stained fibres found in HIEX compared to LOEX and CON in that HIEX causes a greater stress on the muscle and is also at an intensity level where the WV is beginning to be more heavily recruited. Like the vasculature, Hsp70 density was significantly greater in HIEX than LOEX and CON implying HIEX to be of greater stress to the muscle than LOEX. The smaller diameter vessels in this study would include primarily 2nd to 4th order arterioles and potentially capillaries, whereas the larger vessels would represent 1st order arterioles as well as veins (19, 21, 23). Interestingly, a greater proportion of muscle fibres around smaller sized vessels expressed Hsp70 than around larger sized vessels. These observations would support the speculation above that while the larger vessels act more
as conduits for bulk flow through the muscle and are controlled to a larger extent by blood borne factors; the smaller vessels may be responding to muscle derived vasodilatory signals as adjacent muscle fibres are activated.

Regardless of the stimuli for the selective increase in Hsp70 in the vasculature of blood vessels, the role of this protein is still unclear. Generally, Hsp70 has been thought to protect cells in which it is located. In the case of the vasculature, Hsp70 may enhance vascular contractility and responsiveness (29), activate anti-inflammatory cytokines (68) suppress the release of inflammatory cytokines (28) and suppress oxidative stress and inflammatory NfKB signaling (66). Further studies will need to be conducted to determine the exact function of this exercise-induced increase.

The vascular content of Hsp90 appears to be responsive to exercise but unlike Hsp70, moderate exercise appears to produce a maximal response (at least for the intensities examined) in the larger vessels. The smaller vessels demonstrated a more graded response to exercise with a larger increase seen in the HIEX condition than LOEX. Under neither condition did the proportion of smaller vessels expressing Hsp90 reach that of the larger vessels. Approximately 70% of large vessels induced Hsp90 post-HIEX compared to the 100% Hsp70 induction after HIEX. Since Hsp90 has been associated with the activation of eNOS, and the subsequent release of NO, a potent vasodilator (14, 15, 17), it was anticipated that Hsp90 might increase in the vasculature with exercise. The pattern of vascular induction of Hsp90 observed in the present investigation could be a consequence of smaller vessels being less dependent upon NO release for vasodilation than those of larger diameters (21). Regardless, exercise appears to act as a potent activator of Hsp90 induction in the vasculature and could have a
significant impact on vasculature function. Importantly the proportion of the vasculature that is influenced by exercise appears to be dependent upon exercise intensity. Hsp90, like Hsp70 is susceptible to shear stress, although it appears to have a different sensitivity for protein synthesis.

No apparent *de novo* synthesis of Hsp90 was seen in the skeletal myofibres between CON, LOEX and HIEX. Unfortunately, little is known of the functional role of Hsp90 in skeletal myofibres.

### 2.5 Conclusion:

In summary, blood vessels, both large and small, showed an increase in Hsp70 in an intensity dependent manner. Once the stress response was achieved the amount of Hsp70 per vessel area was similar between vessel sizes but still increased in an intensity-dependent manner (HIEX was greater than LOEX). The Hsp70 induction in vessels can likely be attributed to the increased blood flow that happens with exercise and the resultant increase in fibre recruitment as exercise intensity increases. Hsp90 is found in large blood vessels in equal amounts across LOEX and HIEX and it is theorized that its presence is in attempt to upregulate eNOS (14, 15, 17) to further increase vasodilation in an attempt to increase muscular perfusion to account for the increased fibre recruitment and stress seen with exercise.
2.6 Reference List:


63. **Travis J.** Cellular localization and expression of HSP70 and HSF1 in rat skeletal muscles following exercise and heat shock. The University of Western Ontario. (Masters Dissertation), 2008.


CHAPTER 3

3.1 Limitations and Future Prospects:

This study found interesting and novel results. In accordance with the original hypothesis, large and small blood vessels showed an increase in Hsp70 in an intensity dependent manner. Interestingly, once the stress response was activated the generated quantity of Hsp70 was similar between vessel sizes, although still following an intensity-dependent pattern (HIEX was greater than LOEX). Hsp90 is found in similar quantities in large blood vessels both in LOEX and HIEX. A potential explanation for this finding is the presence of Hsp90 is needed in attempt to upregulate eNOS (1–3) to aid in muscle perfusion and increase muscle blood flow.

Experimental limitations as well as potential further examinations can be found within the methodology of this study in such that without the use of Western blots the measurement of protein quantification is not as accurate a measure as it could be. Western blots should be performed in conjunction with immunofluorescent protein density measures for a better idea of the level of the heat shock response. The use of a variety of muscles rather than just the fast twitch WV would have been an interesting addition as well to see the heat shock response in a more frequently recruited muscle, like the soleus for example. Greater increases in blood flow is found to increase in oxidative slow twitch muscles at the running speeds used in this experiment (4), as well as generally showing greater constitutive levels of Hsp70 already (5), and it would be interesting to see if the response of Hsp70 in the vasculature is similar.

Colocalization of Hsp70 and Hsp90 to the endothelium and smooth muscle portions of the vasculature was attempted but issues with the signal saturation with
certain antibodies did not provide for a clean enough image to accurately use this procedure. Further attempts should be made to determine the specific localization of both stress proteins.

Further studies should investigate the response of both Hsp70 and Hsp90 at higher intensities when blood flow becomes significantly increased in the WV. An interesting addition would be the use of eNOS markers to attempt to measure Hsp90 interaction as well as a potential α-adrenergic blocker to mitigate different blood flow and Hsp70 responses. Different modalities of exercise including resistance training or a resistance/cardiovascular training combination may provide different benefits and potentially a different pattern of recruitment.
3.2 Reference List:


APPENDIX A
Ethics Approval

AUP Number: 2008-095
PI Name: Noble, Earl
AUP Title: Innovation to Reduce Cardiovascular Complications of Diabetes at the Intersection of Discovery, Prevention and Knowledge Exchange

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2008-095 has been approved.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than those through this system must be cleared through the ACVBS office.
   Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Thompson, Shara H
on behalf of the Animal Use Subcommittee

The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, London, Ontario, CANADA – N6A 5C1
PH: 519-661-2111 ext. 86768 • FL 519-661-2028
Email: aucum@uwo.ca • http://www.uwo.ca/animal/website/
APPENDIX B
Negative Controls

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<thead>
<tr>
<th>Hsp70 Negative Control</th>
<th>Hsp90 Negative Control</th>
</tr>
</thead>
</table>

**Negative Controls:** Stained with secondary antibodies only, no primary. Line denotes 50 μm.
APPENDIX C
Immunofluorescence Protocol

Preparation:

1. Collect tissue samples (8 µm thick) on poly-L-lysine coated slides
   - Only collect consecutively cut slices of muscle
   - Label slides clearly with pencil
2. Store at -30°C until ready for use in a sealed slide-case
3. Have all solutions and instruments ready beforehand:
   - Coverslips (VWR)
   - Humidity chambers (1 per 12 slides)
   - Coplin jars
   - ImmunEdge hydrophobic barrier pen
   - 10X PBS solution:
     - NaCl 112 g
     - KCl 2.8 g
     - Na₂HPO₄ 20.18 g
     - KH₂PO₄ 2.8 g
     - Dissolve in approximately 1300 mL of ddH₂O and adjust pH to 7.4
     - Bring to a total volume of 1400 mL with ddH₂O and split to glass bottles
   - 1X PBS (adjusted to pH 7.4)
   - 10% Goat Serum
   - 1% Goat Serum
   - Triton-X
   - Primary Antibodies:
     - Hsp70: SPA-812, StressGen Hsp70
     - Hsp90: BD610418 BD Transduction Laboratories™
   - Secondary Antibodies (Invitrogen):
     - Alexa Fluor® 488 Goat Anti-Mouse IgG
     - Alexa Fluor® 594 Goat Anti-Rabbit IgG

Protocol:

4. Remove necessary slides from freezer and thaw, keeping slide box sealed until room temperature is reached
5. Rinse humidity chambers and place moistened paper towel in bottom
   - Use ddH₂O of 1XPBS (referred to from now as just PBS)
6. Place slides in coplin jars with 0.05% Triton-X (with PBS)
   - Do 1 wash, 5 min each
7. Wash in PBS
   - 3 washes, 5 min each
8. Dry every slide (front and back) and make grease circles around each cut
   - KIM wipes are best for drying
- If sections are being stained the same way make large circle around how many you are staining but make sure to make a separate circle for the control section of the slide

9. Prepare 10% goat serum in PBS
   - ~50 µL/section

10. Pipette goat serum on each cut and leave slides in sealed humidity chambers at room temperature for 1hr

11. Prepare primary antibody solution in 1% goat serum in PBS
    - ~50 µL/section
    - Dilution for primary antibody should be 1:100
    - All slides and containers should be clearly marked for each specific antibody

12. After 1hr, tap goat serum off slide and dry slides

13. Add primary antibody solution to each cut

14. For control slide use 1% goat serum

15. Refrigerate (4°C) overnight in sealed humidity chambers

16. Tap off primary antibody solution from every slide and dry

17. Wash in PBS
    - 3 washes, 5 min

18. Turn off the lights for the rest of the procedure

19. Prepare secondary antibody solutions in PBS (no goat serum)
    - ~50 µL/section
    - 1:400 for secondary solutions

20. After washes are complete dry
    - Reapply grease circles if necessary

21. Add secondary antibody solution to slide (including control section)

22. Incubate in sealed humidity chambers at room temperature for 1hr

23. After 30min, remove DAPI from freezer

24. After 1hr, tap off secondary antibody solution and dry

25. Wash in PBS
    - 3 washes, 5 min each

26. Apply DAPI (mounting medium) to coverslips
    - Enough DAPI to cover all the tissue
    - TIP: Use a pipette tip to smear DAPI over cover slip to allow for less waste

27. After washes are complete dry slides and apply coverslips

28. Let sit in humidity chambers (moist paper towel removed) for 30min to allow for drying time

29. If not immediately using slides for the microscope, remove from humidity chamber and into a slide folder
    - Store at (4°C) until use
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