Abstract
Effects of Aerobic Capacity Phenotype on Adaptive Responses to Ischemic Stress
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Ischemic disease leads to increased tissue stress by decreasing supply of nutrients adequate to meet energy demands. To maintain functionality, compensatory mechanisms for diminished vascular supply are induced by numerous tissues factors associated with ischemia. Compensatory responses include both remodeling of the vascular network to sustain substrate delivery and adaptive responses within affected tissue to sustain function despite diminished substrate availability. Active aerobic exercise has been shown to improve both vascular and metabolic remodeling within and around ischemic tissues. However, active exercise programs, while consistently beneficial, still generate a very heterogeneous response, suggesting the potential for an important contribution from the genetic composition determining intrinsic aerobic exercise capacity. A novel rat strain, developed using forced artificial selection for intrinsic endurance running capacity, provides a unique tool that enables assessment of intrinsic aerobic capacity and the effects of these phenotypes on adaptive responses to ischemic stress. Untrained low endurance running capacity (LCR) rats were found to have a higher incidence of risk factors for ischemic disease and may have differing vascular and metabolic responses to peripheral artery occlusion. The overall goal of this dissertation was to determine the influence of intrinsic exercise capacity on the vascular and metabolic adaptive responses to ischemic stress. This study tested the hypothesis that the LCR would show altered vascular and metabolic adaptive responses in response to

peripheral arterial occlusion, compared to HCR counterparts. Muscle samples from both the ischemic and the non-ischemic limb in both strains were compared metabolically for their relative capacity to oxidize fatty acid, histologically for the anatomical vascular capillarity supporting perfusion, and functionally using both perfusion tracers to track blood flow, and direct muscle stimulation to test fatigue characteristics. Biomarkers obtained using PCR were obtained to suggest potential pathways accounting for differences in response between the phenotypes. Results indicate that intrinsic aerobic phenotype does alter both the resting tolerance for demand induced ischemia, as well as the adaptive compensatory responses to chronic anatomic arterial obstruction. The LCRs showed both a reduced capacity for demand induced workloads, as well as responses to chronic obstruction that were both delayed in onset, and delayed in induction compared to HCR counterparts. These data may have implications for better structuring active exercise programs to achieve intended outcomes in limiting consequences associated with cardiovascular disease risk factors.

Effects of Aerobic Capacity Phenotype on Adaptive Responses to Ischemic Stress

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DEDICATION

I dedicate this dissertation to my family, my husband, and the Air Force family. I would like to thank my parents and siblings for always lending an ear when I needed to vent and for giving me the confidence to believe in myself. To my husband Jay, you have my heart! You have been my biggest fan and provided me the calming effect when I needed it most. To my AF family, thank you for giving me the courage to do this crazy adventure.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

The underlying basis for ischemic disease, one of the most deadly and costly diseases of the Western world, is a mismatch between the oxygen supplied by the bloodstream and the oxygen demands of the tissue. The two predominant forms of ischemic disease that affect millions of Americans annually are coronary artery disease and peripheral arterial occlusive disease (PAOD)(67; 73; 96; 103). The American Heart Association has found that there are differences between ethnic groups and their predisposition for cardiovascular diseases to include susceptibility for ischemic disease (85). This data suggest that there are phenotypic influences on the development for ischemic disease and more research may be necessary to better understand how phenotype may influence development and progression of disease. Both cardiac and peripheral tissues employ compensatory mechanisms in response to ischemia. These mechanisms primarily include a vascular remodeling process that increases perfusion capacity to offset any deficits in supply (61; 62; 80) and remodeling of metabolic pathways, including changes in the activity of oxidative enzymes (17; 84) enabling tissue to better tolerate chronic deficits in vascular supply.

The heart is one of the most versatile tissues in the body, capable of utilizing multiple substrates to meet energy demands. Under normal resting conditions, cardiac muscle derives approximately 70% of its energy from fatty acid oxidation (34). However, fatty acid oxidation depends on a ready supply of oxygen, such that under ischemic conditions, as in progressive coronary artery occlusive disease, cardiac metabolism shifts towards using more anaerobic glycolytic mechanisms to meet energy demands (34). Unfortunately, the energy yield of glycolysis is insufficient to meet the

energy requirements of cardiac demand, and glycolysis produces lactic acid, a potential cause of cardiac pain. It has been suggested that within as little as 30 minutes from the onset of acute coronary ischemia, alterations in adenine concentrations and other metabolic oxidative stressors can lead to cardiac cell death (88). Studies of post ischemic myocardium have reported that carnitine treatment (19), adenine nucleotide reuptake inhibitors (57), malate/aspartate solutions (utilizing transamination pathways) (46), glucose/insulin/potassium solutions (89), mitochondrial ATPase targeted drugs (37), adenosine (74), and delta opiod agonists (37) all have shown to enhance recovery from cardiac ischemic challenges, all at least in part due to effects on metabolic pathways. Further complicating the analysis is that, while diabetes is a major risk factor for cardiovascular disease, insulin insensitivity is a much less essential component of substrate utilization in cardiac muscle (13; 63), leaving open the question regarding whether the primary effect of diabetes is directly related to metabolic alterations, or secondary to atherosclerotic processes and hypertension, with which diabetes is also associated, and to which the heart is highly vulnerable. The complexity of cardiac metabolism suggests that shifting substrate preference is a major component of the dynamic response to ischemia (13; 63), perhaps more than oxidative capacity of any particular substrate. When one considers that heart tissue also is much less accessible to recurring assessment during chronic conditions, and tissue availability often occurs in the setting of an acute critical ischemic event superimposed on the chronic conditions, it becomes clear that studying metabolic responses in the heart to challenges such as exercise or disease is complex.

In peripheral arterial occlusive diseases, which are just as prevalent as coronary artery disease, the basic factors that stimulate vascular and metabolic remodeling are the same, but the tissue is much more accessible to study, and the metabolic characteristics of skeletal muscle fibers are more precisely defined, and significantly less variable than in cardiac muscle. Skeletal muscle responds to arterial insufficiency by generating a vascularization stimulus that utilizes the same critical factors to stimulate remodeling as cardiac muscle. However, skeletal muscle also has the ability to alter enzyme activity and to change muscle fiber type towards a more anaerobic phenotype (18; 75).

Regular aerobic active exercise is one of the few interventions that have been shown to consistently slow the progression of occlusive arterial diseases, and to enhance the recovery from them. Exercise induces many effects, including lowering serum lipids and raising plasma HDL levels (33), both of which are thought to decrease the risk of atherosclerosis in most arterial systems (25; 33). Regular aerobic exercise lowers resting blood pressure (48), which limits the resting energetic demands of the heart, and limits the progression of atherosclerosis in both cardiac and peripheral locations. Regular aerobic exercise increases tissue responsiveness to insulin, and stimulates increases in key enzymes regulating metabolic pathways, promoting substrate uptake and utilization in peripheral tissues (7; 35), and presumably in cardiac tissue as well. Aerobic exercise also increases vascular capillarity (81) and increases vascular responsiveness to vasoactive substances such as nitric oxide (23), which serves both to increase anatomic flow capacity, as well as dynamic flow responsiveness. For all that has been reported with regard to the benefits of active aerobic exercise, little is known about how the intrinsic capacity to exercise, independent from any active exercise

component, influences the vascular or metabolic remodeling responses to occlusive arterial disease.

Given the complexities involved in analyzing the metabolic demands of cardiac muscle, peripheral ischemia is a more attractive model for investigating how tissues respond to ischemic conditions, and has been used extensively for that purpose. However, most studies to date have concentrated on studying either the vascular component of ischemic disease, or the metabolic component, while few have examined the integrated relationship between skeletal muscle capillarity and metabolic adaptations to chronic occlusion within the same muscle tissue, and none have done so in the context of the intrinsic exercise capacity phenotype.

Therefore, the aim of this dissertation was to study the influence of intrinsic aerobic exercise capacity on vascular and metabolic adaptive responses to ischemic stress, and **tests the overall hypothesis that the low aerobic endurance running capacity (LCR) phenotype will show altered vascular and metabolic adaptive responses to peripheral arterial occlusion when compared to the high aerobic endurance running capacity (HCR) phenotype.**

Peripheral Arterial Occlusive Disease (PAOD)

Peripheral artery occlusive disease (PAOD) affects 8-12 million Americans and is difficult to treat (67). It has been suggested that ethnicity plays a role in the development of this disease with Blacks and Hispanics having higher risk for development compared to Asians and Caucasians(6). This data suggest that phenotype influences the development and progression of ischemic disease. PAOD occurs mainly in

the lower extremities, and its incidence and prevalence are nearly equal to that of coronary artery disease (103). Approximately 15% of adults over age 55 have detectable hemodynamic impairments attributable to PAOD (103). There are two major clinical presentations of PAOD: intermittent claudication and critical limb ischemia. Intermittent claudication presents as pain during exercising conditions when blood flow is insufficient to meet tissue demands, but which resolves with time after return to resting conditions. Critical limb ischemia is characterized by blood flow that is inadequate to meet demands at resting conditions, leading to leg pain at rest, marked disability, and impaired quality of life (103).

Treatments for PAOD include pharmacological agents that reduce atherosclerotic risk-factors, and delay the development of occlusive plaques. Surgical treatments for PAOD include mechanical revascularization, either by percutaneous methods, or by direct bypass (103). Revascularization is an option for advanced disease but is not realistic in patients with multiple obstructions (94). In addition, revascularization studies have demonstrated that gender and race are synergistic determinants in vein graft failure suggesting that phenotype is an important factor behind PAOD and clinical outcomes(69). Surgical therapy carries a risk of perioperative morbidity and mortality, and a large number of patients are unable to undergo surgical therapy. In addition, surgical therapy is not always successful (100). Occlusions may recur and patients can again progress towards pain with walking and other clinical symptoms of PAOD. New and more efficacious treatments options for PAOD clearly are needed.

Considering the limits of surgical revascularization as a treatment option, other non-invasive treatments that increase perfusion to downstream ischemic tissue are being investigated. Understanding the processes and pathways of capillary and artery development in adults is paramount for development of these new therapies, and most new experimental treatments emphasize re-establishing perfusion. Several years ago, treatment strategies targeting gene mediated revascularization in PAOD showed initial promise in limited clinical trials, but have since not proven consistently efficacious, and the basis for the variability in responsiveness was not clear (5; 98). The working focus of experimental and clinical studies in POAD has been on reestablishing perfusion, as that is the anatomic deficit. However, there are no therapies that directly target improved function of distal tissues as an alternative, or an adjunct to re-establishing vascular supply. Evidence from aerobic exercise training studies suggested clinical PAOD patients increase capillary density and oxidative capacity with time (101; 107), but the underlying mechanisms that cause the changes in oxidative capacity in conjunction with changes in capillary density is not understood well at this time. Research on the adaptive and remodeling characteristics of ischemic tissue may provide new cell based treatments for PAOD.

Hind limb femoral artery ligation is a well-established popular model for the study of chronic ischemia and PAOD (70; 100; 103), and provides a relevant conceptual framework to assess the pathways of ischemia-induced changes in the vasculature and surrounding tissue. Hind limb femoral artery ligation is an accepted model of intermittent claudication, and has been used to study potential mechanism underlying the beneficial effects of aerobic exercise training (70; 103). Recent studies by Lloyd *et al.*

(61; 62), utilized the femoral artery ligation model to assess angiogenic growth factor expression in rats in response to a chronic ischemic injury during an active aerobic exercise training protocol lasting three weeks (61; 62), reporting that pro-angiogenic gene signals were increased significantly within days after occlusion, and that aerobic exercise increased the overall capillary remodeling response following occlusion. Roberts *et al.* (84) utilized this injury model coupled with aerobic exercise training and studied changes in oxidative capacity and capillarity in plantaris and soleus muscles, and demonstrated that arterial insufficiency caused vascular and metabolic remodeling in both red and white skeletal muscles fiber types, and that aerobic exercise training improved upon these adaptations (84). However, the influence of pre-existing intrinsic aerobic exercise capacity on these adaptations to ischemia was not established and remains poorly understood. **In summary, there is a well-established model for PAOD that has been used to study components of vascular and metabolic adaptations occurring in skeletal muscle in response to ischemia, and the effects of exercise, that would be appropriate for studies of intrinsic aerobic capacity influences.**

Ischemic Induced Adaptations and Remodeling of Skeletal Muscle

Skeletal muscle responsiveness to ischemia or depletion of oxygen is dependent on the muscle fiber type (64). Skeletal muscle has three distinct fiber types. Slow twitch (Type I) fibers have a high concentration of mitochondria and myoglobin (64). Fast twitch fibers (Type II) fibers are subdivided further into two distinct subtypes: Type IIA and Type IIB (64). Type IIA fibers are fast oxidative fibers that exhibit a faster contractile velocity than Type I but also contain a high myoglobin and mitochondrial content. Type IIB fibers are fast glycolytic fibers with less myoglobin and fewer

mitochondria. Type IIB fibers have a higher storage content of glycogen (64) and rely heavily on anaerobic metabolism to meet their energy demands. Because fiber types and their distributions within a muscle are genetically influenced, individuals differ in terms of baseline oxygen supply and utilization within a given muscle. These genetic differences in fiber type distribution provide a basis for intrinsic difference in intrinsic aerobic exercise capacity.

In the rat, different skeletal muscles have distinct fiber type populations. In addition, rat skeletal muscle also has a Type IID/X fiber which is similar to other Type II or fast twitch fibers in their myosin heavy chain composition but has distinct physiological and biochemical properties. Generally, the deepest extensor muscles have the highest percentage of Type I fibers and the lowest percentage of Type IIB fibers (4). However, muscle fibers also are capable of transforming from one fiber type extreme to another in response to altered functional demands, hormonal signals, or changes in neural input (75). Delp *et al.* found that both the soleus (84%) and adductor longus (87%) muscles contain a high proportion of Type I fibers (24). The soleus muscle was found to have no Type IIB fiber expression. Different portions of the gastrocnemius contain different fiber concentrations. The gastrocnemius muscle is typically analyzed as red, mixed and white sections. However, the delineation is not as clear as the naming might suggest, and indicates only the dominant fiber type. The red gastrocnemius muscle was 51% Type I, 35% type IIA, and 14% Type IIB which is still approximately 86% percent of this muscle section to be described as oxidative fibers, while the mixed gastrocnemius muscle was 1% Type I and 3% Type IIA fibers, and 28% Type IID/X and 68% Type IIB fibers. White gastrocnemius was found to have no Type I or Type IIA fibers (24).

Thus the soleus, and white portions of the gastrocnemius muscle appear to be relatively homogeneous muscles that correspond to the well defined characteristics of slow oxidative, and fast glycolytic fiber classification (24). This demonstrates that rat gastrocnemius muscle provides distinct fiber populations that can allow a deeper understanding of how different fibers respond to stressors.

Skeletal muscle is remarkably adaptable to ischemia and the stress of endurance exercise training (Figure 1.1) compensating for reduced oxygen availability by increasing mitochondrial volume density (volume of mitochondria per volume of muscle fiber) and citrate synthase activity (36; 64; 99). Ischemic conditions also cause skeletal muscle to shift substrate utilization away from fatty acid oxidation towards glucose oxidation, which improves the yield of ATP generated per mole of oxygen consumed (40; 64; 87). If ischemia persists, skeletal muscle can undergo fiber type switching towards a more glycolytic phenotype (Figure 1.1) (75). This phenotype switch is normally a slow twitch to fast twitch fiber switch leading to a decrease in Type I fibers and an increase in the Type II fibers (75). Muscle fibers, once considered a static entity or tissue, are now understood to be dynamic structures with extraordinary adaptive potential. **In summary skeletal muscle adaptations to ischemic conditions, many of which are at least partially transcription dependent phenomena, include changes in substrate utilization pathways, oxidative enzyme activities, mitochondrial volume density and, if warranted, fiber type phenotypic switching. However, the influence of intrinsic aerobic phenotype on these processes is unknown.**

Skeletal muscle also responds to ischemic challenge via vascular remodeling. In response to ischemia, skeletal muscle may increase the expression of vascular endothelial growth factor (VEGF) mRNA, which in turn can lead to increases in VEGF protein (18). Increased VEGF expression has been shown to ameliorate peripheral blood flow insufficiencies and promote angiogenesis (14). VEGF has also been shown to produce dose-dependent release of nitric oxide (NO). NO, in addition to other factors, including VEGF and angiopoietin (Ang) 2, stimulates both angiogenesis and arteriogenesis, reflecting the development of new blood vessels, primarily capillaries, and the remodeling of existing blood vessels to develop/expand collateral perfusion, respectively. Angiogenesis and arteriogenesis are presumed to work in concert, promoting opportunities to maintain or sustain bulk flow at an arteriolar level, in concert with changes in capillarity that modify residual flow distribution to individual fibers, together restoring skeletal muscle perfusion towards baseline conditions and alleviating ischemic conditions (90).

Exercise

Understanding the complexities of exercise requires more than discrimination between active aerobic exercise training and/or resistance training. There are two distinct entities that contribute to exercise capacity, broadly characterized as genetic and environment influences. The environmental component represents the imposed conditions that cause the skeletal muscle to react and adapt, and can be represented by such things as diet and exercise training programs. The genetic component relates to the genes that regulate intrinsic aerobic exercise capacity and genes that respond to active aerobic or resistance training stimuli. It is thought that 70% of the variation in intrinsic

aerobic capacity is determined genetically (9). A variety of genes may participate in the determination of intrinsic aerobic exercise capacity, and these genes are capable of influencing every physiological system within the human body.

Intrinsic Aerobic Exercise Capacity

Low aerobic capacity, or low cardiorespiratory fitness, is a strong predictor of early mortality (97). Aerobic capacity is determine by a variety of factors that encompass both environmental and genetic influences. Twin and family studies support heritability of intrinsic exercise capacity, VO_{2max}, and the inducible aerobic capacity, ability to increase VO_{2max} with aerobic exercise training (9).

It has been difficult to determine the role intrinsic aerobic capacity plays in the progression of human disease states because it is nearly impossible to control for environmental influences. Twin studies have provided valuable insight into the way genes influence intrinsic exercise capacity (9). The HERITAGE family study, which evaluated VO_2max during sedentary and training phases, suggested that there are several specific chromosomal regions linked to individual VO_{2max} (8). These twin and family studies have pointed towards 17 mitochondrial genes and 165 autosomal genes that appear to influence fitness and performance phenotypes (8). Within metabolic disease, these studies have provided insight into the pathogenesis by demonstrating a down regulation in expression of genes involved in aerobic metabolism. Conversely, these markers of aerobic metabolism appear to be upregulated in individuals with increased physical capacity. This literature supports the need for a better understanding of how genes influence both intrinsic exercise capacity and the ability to respond to active exercise training.

It is widely accepted that individuals differ drastically in aerobic capacities in the absence of a training stimulus and in their inherent capacity to adapt to exercise (8-10). Research clearly shows that individuals with diminished aerobic capacity have increased morbidity and decreased quality of life (30; 48; 65). Genes that enhance intrinsic aerobic exercise capacity are believed to provide protection against disease, decrease complications associated with disease, and allow the individual to respond favorably to different types of stress. **In summary, while clearly present, the genetic influence on aerobic capacity largely has been overlooked primarily due to modeling difficulties.**

Aerobic Exercise Training

Dynamic exercise training has been shown to improve and treat various chronic diseases, including coronary heart disease, hypertension, osteoporosis, obesity, and metabolic disease (11; 47; 55; 58; 77). Physiologic adaptations that improve muscle function, delivery and use of fuel sources are just a few of the changes associated with improved exercise capacity (84). In ischemic diseases, aerobic exercise training has been shown to induce beneficial physiological adaptations both prior to symptoms of disease onset, and also in advanced disease states.

The physiological adaptations associated with aerobic exercise training include both metabolic and vascular adaptations within skeletal muscle. Aerobic exercise training has been shown to stimulate increases in angiogenic and arteriogenic pathways that lead to tissue remodeling and better perfusion of the tissue (81; 82). This remodeling has been shown to alleviate symptoms of PAOD (100). Endurance exercise training improves the utilization and extraction of oxygen from erythrocytes, influences blood viscosity, and raises pain thresholds (81; 82). Aerobic exercise training also improved

endothelial function (11) increasing endothelial release of vaso-active factors that increase intrinsic control of resistance (11).

The precise mechanisms by which aerobic exercise training induces arteriogenesis and angiogenesis are unknown, but in animal studies, by day 12 of an exercise training protocol, angiogenic processes are histologically evident (61; 62), especially in the low oxidative, less vascularized white gastrocnemius tissue, which is primarily Type IIB fibers, which exhibited the greatest increase in capillarity (62). A single bout of acute aerobic exercise training has been shown to trigger immediate upregulation of VEGF mRNA (81; 82). There also appears to be a decrease in muscle VEGF protein expression and a corresponding increase in VEGF protein in the surrounding interstitium (81; 82). Furthermore, aerobically exercising muscles show increases in both VEGFR-1 and VEGFR-2 mRNA expression (32). Together, these studies suggest that VEGF and its receptors are important parts of the mechanism by which exercise promotes angiogenesis, but the exact upstream triggers and downstream targets of this pathway under these conditions remains unclear.

Other angiogenic factors that may influence intrinsic aerobic exercise capacities response to ischemic challenge are angiopoietin 1 and 2 (Ang1 and Ang2) and nitric oxide (NO) (Figure 1.2). These three factors are known to be key players in exercise induced and ischemia induced angiogenesis as well as the combination of both stressors (29; 32; 38; 62; 107). The angiopoietins are important cytokines that assist in vascular development and remodeling (29). Ang 1 promotes maturation and stabilization of vessels and is expressed widely throughout tissues, while Ang 2 displaces Ang 1 and

activates the Tie2 receptor. The Tie2 receptor is expressed at sites of vascular remodeling. Thus Ang1 dominance is associated with a stable vasculature, while Ang2 dominance is associated with active angiogenesis. In response to aerobic exercise training, Ang2 to Ang1 ratio was more affected in highly oxidative fibers compared to low oxidative fibers, suggesting that these Ang factors may have more pronounced effects on vascular remodeling in highly vascularized sections.

NO can be increased with activation of VEGF receptors, VEGFR2 or kinase dependent receptor (KDR) (29). The increase in NO production can be critical to VEGF signaling and the remodeling response of the skeletal muscle (29). Training elevates endothelial nitric oxide synthase (eNOS) expression, one enzyme that controls NO production, and eNOS knockout mice show a requirement for NO in skeletal muscle angiogensis. Studies suggest that angiogenesis in response to aerobic exercise training may rely heavily on NO (29). **In summary, while there are a variety of factors that influence angiogenesis in response to exercise alone, ischemia alone, and the combination of both, there are likely pathways for which the importance of intrinsic aerobic exercise capacity on the angiogenic response to ischemia could be related, but as yet has not been established.**

Aerobic exercise training, in addition to its vascular and angiogenic alterations within ischemic tissue, has been found to increase long chain fatty acid (LCFA) availability and transport. Aerobic exercise training also appears to increase the enzymes of the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (1; 97). With these alterations, subjects with enhanced aerobic capacity can use more fat for energy

production at both the same absolute and at the same relative exercise intensities (41; 99). This is advantageous for any disease condition because these tissues maintain energy output under stressful conditions. Aerobic exercise training also triggers a change in muscle fiber type from fast to slow. In particular, fiber type changes characterizing aerobic endurance exercise training primarily shifts type IIB toward type IIA (75). Increased exercise intensities that has induce a more pronounce hypoxic event have been shown to force transitions beyond the fast fiber type population (75). Fiber type phenotypic transitions occur due to a variety of influences, including changes in gene expression and altered transcription and translational activities. However, an exact mechanism responsible for phenotypic transitions is still unknown. Evidence suggests that Ca^{2+} and calcineurin may play an important role in the control of gene expression (75) (Figure 1.1) within skeletal muscle in response to exercise, but multiple pathways may exist.

Aerobic exercise training is one treatment option that improves quality of life and tissue perfusion characteristics, presumably by inducing controlled, reversible demand ischemia, leading to increased capillary density (101). Aerobic exercise training has been shown to increase capillary development within healthy skeletal muscle through the process of angiogenesis (61; 62; 81; 82; 107) that involves the vascular endothelial growth factor (VEGF) driven pathway (61; 62; 81; 82; 107) (Figure 1.2). The potential involvement of NO, Ang1 or Ang2 has not been determined at this time, but there is strong evidence to suggest that VEGF cannot act independently. The mechanisms by which aerobic exercise training improves intermittent claudication in PAOD patients remains unclear (92; 93). Clinical data of PAOD shows that patients limited by

intermittent claudication who engage in any amount of weekly physical activity beyond light intensity have a lower mortality rate than their sedentary counterparts who perform either no physical activity or only light-intensity activities (30). This study also found that the protective effect of physical activity persists even after adjusting for other predictors of mortality, including age and body mass index (BMI). Thus, aerobic exercise training appears to induce adaptive remodeling within ischemic skeletal muscle, but these pathways and mechanisms of improvement are not well characterized.

Aerobic exercise training appears to be a potent stimulus of angiogenesis through VEGF and other angiogenic growth factors that within skeletal muscle can alter capillary density and increase potential tissue perfusion. In addition to vascular adaptive changes, aerobic exercise training influences skeletal muscle oxidative capacity through alterations in mitochondrial volume and enzymatic activity. In addition to changes within the muscle fiber, aerobic exercise training also appears to have the capacity to induce muscle fiber phenotypic switches. These adaptations, both vascular and metabolic, are mechanism that can provide protection against development of arterial insufficiency and in the event of ischemic disease may improve patient quality of life.

Rat Model and Low and High Capacity Running Rats

A model to investigate the contributions of intrinsic aerobic exercise capacity versus adaptations induced by aerobic exercise training has been developed. Using a genetically heterogeneous outbred stock of N:NIH rats, two rat strains, low endurance running capacity (LCR) rats and high endurance running capacity (HCR) rats were developed by forced artificial selection, using a forced endurance treadmill running test

(51; 53). Genetic variance among the population has been maximized by not selecting among brother and sisters. At 10 weeks of age, rats undergo a protocol to estimate aerobic running capacity. The rats complete a forced run treadmill protocol which allows for the highest and lowest running rats to be selected from each sex and randomly paired for mating. Over several generations, two divergent rat strains have been produced such that by the 11th generation, a difference in running capacity of 347% was present (53; 105). The difference can be translated to the LCR rats on average, as a decreased running capacity of 16 meters per generation, while the HCRs increased by an average of 41 meters per generation. The divergence has further increased to a difference of 461% by the 21st generation (53; 105). This animal model is valuable in the study of the interaction between aerobic capacity and chronic diseases because the differences observed in aerobic capacity between these strains is evident under sedentary conditions, which removes the conflicting effects of training adaptation due to daily exercise training (45; 97; 106).

Previous work has demonstrated that these rat strains have different risk profiles for cardiovascular disease. Wisloff *et al.* utilized rats from the 10th and 11th generations to measure differences in cardiovascular disease risk factors between the two phenotypes (106). LCR rats were found to be more insulin resistant, with increased visceral adiposity to body weight ratio, higher serum free fatty acids, and increased blood pressure. Noland *et al.* (72) in our laboratory utilized generation 13 rats to assess specific differences in fatty acid disposal by skeletal muscle as a function of intrinsic oxidative capacity under normal and high fat diet conditions. The results confirmed basic characteristics of relative insulin resistance in the LCRs as reported previously by

Wisloff *et al.* (106), but in addition demonstrated differences between the phenotypes in adaptive responses to the stress of a high fat diet, suggesting possible differences between phenotypes in response to other environmental stresses other than high fat feeding, such as ischemia.

Previous work in our lab utilized this model to investigate whether differences in intrinsic aerobic exercise capacity offered protection against acute ischemic reperfusion injury (AIRI) (unpublished data). Findings from this study indicated that there were phenotypic differences in infarct size to AIRI when ischemic challenge is 15 minutes but these phenotypic differences disappear when ischemic challenge increases to 30 minutes. This data suggests the potential for intrinsic exercise capacity to influence the response of tissue to ischemic challenge, but if the challenge is too severe, intrinsic protective pathways in the HCR disappear. Because intermittent claudication is not as a severe ischemic injury, it provides a model for investigating if the protective intrinsic ischemic pathways associated with the HCR phenotype are preserved.

Goals of Current Study

The purpose of this project is to gain a better understanding of the effect of intrinsic exercise capacity on the adaptive responses to ischemic disease. Previous studies have found that the LCR phenotype has decreased capillary density when compared to the HCR counterparts (43; 105) and increased risk to disease (72; 97), this suggests that the LCR phenotype will have a decreased tolerance to a peripheral ischemic injury and demonstrate maladaptive responses from vascular and metabolic pathways. **We will test the hypothesis that the low aerobic endurance running capacity (LCR)**

phenotype will show altered vascular and metabolic adaptive responses to peripheral arterial occlusion than the high aerobic endurance running capacity (HCR) phenotype.

Figure 1.1. Proposed ischemia and aerobic exercise training induced changes in skeletal muscle

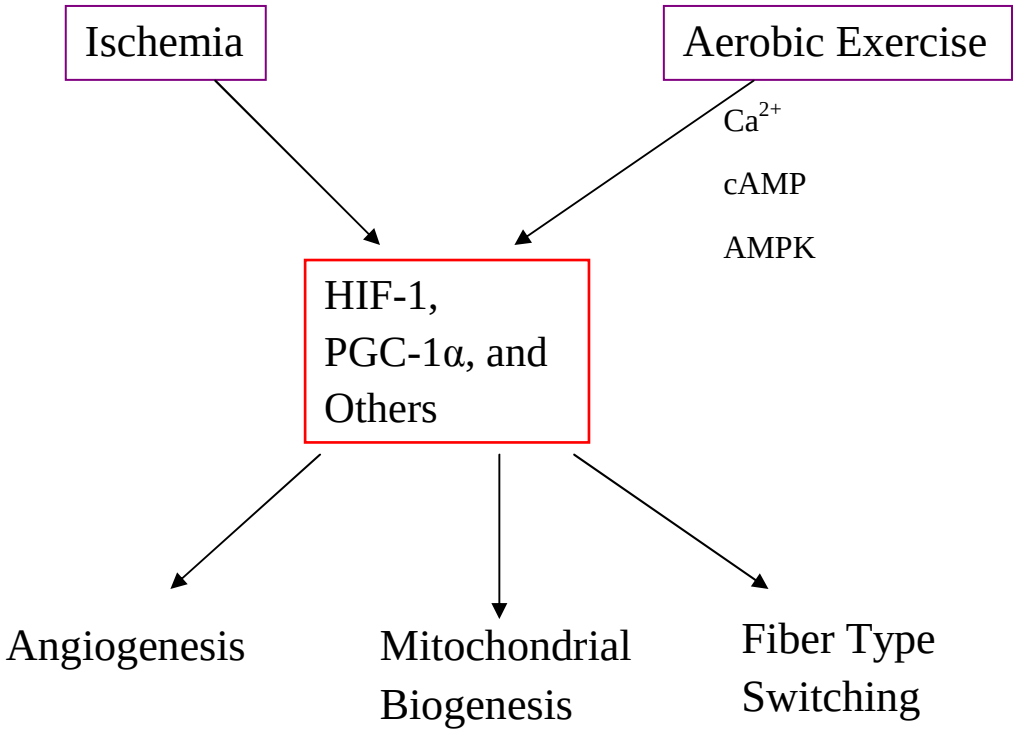
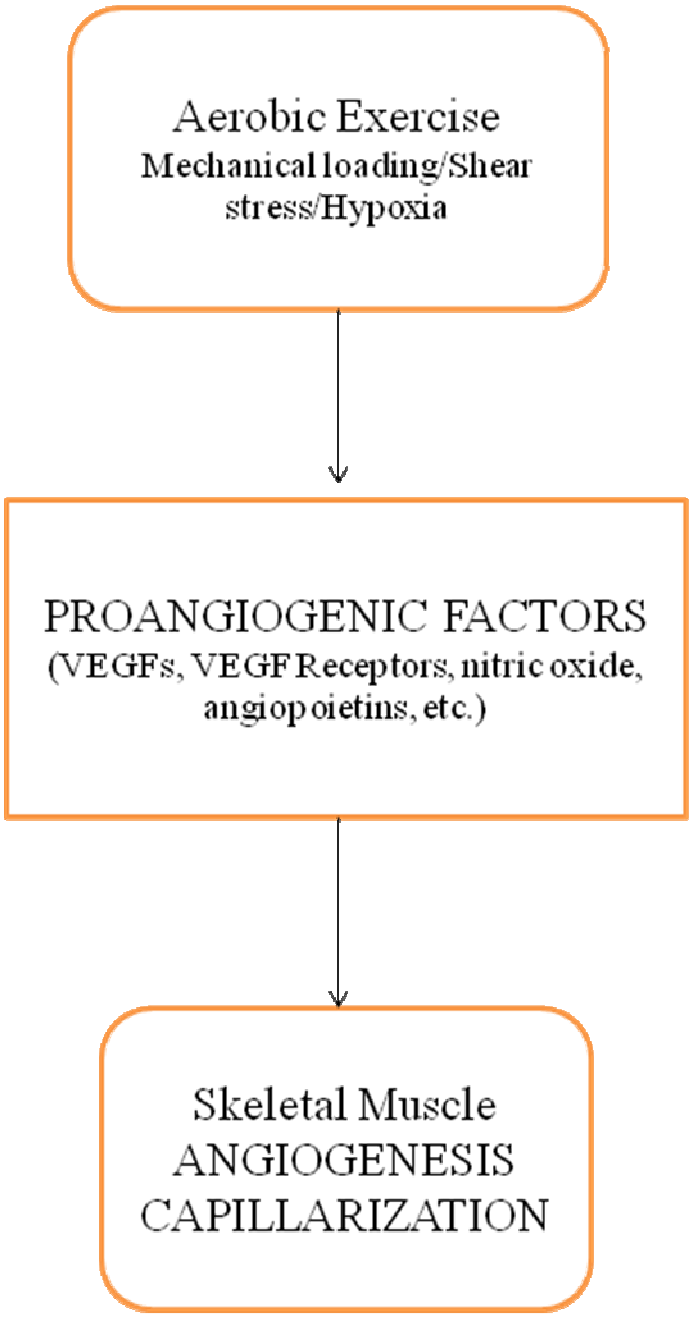

Ischemia
Aerobic Exercise
Ca^{2+}
cAMP
AMPK
HIF-1, PGC-1α, and Others
Angiogenesis
Mitochondrial Biogenesis
Fiber Type Switching

Figure 1.2. Proposed aerobic exercise training induced pathway to increase capillarization


Aerobic Exercise
Mechanical loading/Shear stress/Hypoxia
PROANGIOGENIC FACTORS
(VEGFs, VEGF Receptors, nitric oxide, angiopoietins, etc.)
Skeletal Muscle
ANGIOGENESIS
CAPILLARIZATION

CHAPTER 2: METHODS

Animal Strains. 40 LCR and 40 HCR generation 22 rats were obtained from Drs. Lauren Koch and Steven Britton at the University of Michigan. Those authors have previously described the selection process of artificial selection(12; 52; 54). Briefly, two-way forced artificial selective breeding was used to create low capacity runner (LCR) and high capacity runner (HCR) strains that were divergent for treadmill running capacity (run time to exhaustion on a graded treadmill exercise test). The 13 lowest and 13 highest running capacity rats of each sex were selected from the founder population (N:NIH stock) and randomly paired for mating. At all other times, animals had no exercise other than spontaneous cage activity. The only exposure to exercise for these animals was during the five day window at 10 weeks of age when they underwent the treadmill protocol to establish phenotype. Once phenotyping was verified, animals were prepared for shipping at 14 weeks of age, or as soon after as weather conditions (airport tarmac temperatures < 85°F) permitted. Once received by the Department of Comparative Medicine at ECU, the animals were maintained under mandatory quarantine for 10 weeks before they were released for study. Therefore, all animals were at least 24 weeks of age before they were available to be enrolled in a protocol. Rats were provided standard rat chow and water *ad libitum*, and were kept on a 12 h light/ 12 h dark time schedule until sacrifice. Animal procedures were conducted in accordance with American Physiological Society guidelines for the humane and safe use of animals, and all protocols involving animals used for these experiments were approved by the East Carolina University Animal Care and Use Committee.

Generation 22 animals were used in the current study. The phenotypic characteristics (best run time, distance and body weight) for individual rats used in this study can be found in Tables 2.1 (LCR group) and 2.2 (HCR group). Generation 22 LCRs averaged 23.3 ± 0.155 minutes for best run time while the HCRs averaged 67.01 ± 0.285 minutes (Figure 2.1A). The LCRs achieved an average best distance on this treadmill test of 357.33 ± 3.29 meters while the HCRs performed an average best distance of 1759.76 ± 12.32 meters (Figure 2.1B). Similar to previous studies, animals in the cohort for these experiments differed significantly in their body weight at 10 weeks of age, with the LCRs weighing 332.23 ± 4.25 grams while the HCRs were averaging 242.84 ± 4.19 grams.

Table 2.1. LCR generation 22 rat phenotypic characteristics.

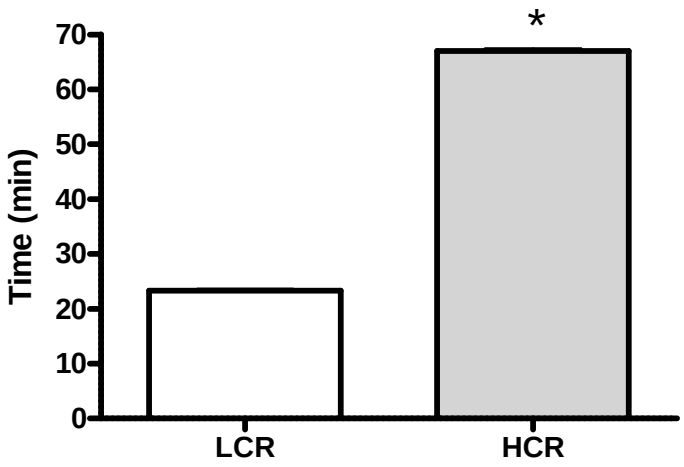
Rat #	Body Weight	Run Time (min)	Run Distance (m)	Rat #	Body Weight	Run Time (min)	Run Distance (m)
22889	343.33	21.93	328.60	22952	338.67	23.20	354.40
22850	314.33	21.93	328.60	22900	306.33	23.32	356.97
22871	344.00	21.93	328.60	23107	319.33	23.32	356.97
22912	334.00	21.97	329.30	22905	364.33	23.60	363.20
22887	353.67	22.15	333.15	22807	313.33	23.68	365.03
22839	283.67	22.20	334.20	22845	341.00	23.70	365.40
23100	381.00	22.25	335.25	22954	345.33	23.85	368.70
23106	313.00	22.25	335.25	22911	307.67	23.90	369.80
22820	349.33	22.40	338.40	22922	273.00	23.97	371.27
22864	335.67	22.43	339.10	22935	333.33	24.05	373.10
22956	349.33	22.43	339.10	22934	303.67	24.32	378.97
22938	327.00	22.53	341.20	22945	359.33	24.32	378.97
22925	298.67	22.62	342.95	22937	328.00	24.38	380.43
23108	328.33	22.68	344.35	22898	279.67	24.40	380.80
22999-23195	403.67	22.73	345.40	22913	376.33	24.42	381.17
22921	327.67	22.73	345.40	22906	362.00	24.58	384.83
22978	336.67	22.90	348.90	22852-22980	346.33	24.72	387.77
22851	356.67	23.05	351.10	22914	343.33	24.87	391.07
22944	323.33	23.15	353.30	22806	277.33	25.03	393.77
22946	347.00	23.15	353.30	22863	320.33	25.10	395.30

Table 2.2. HCR generation 22 rat phenotypic characteristics.

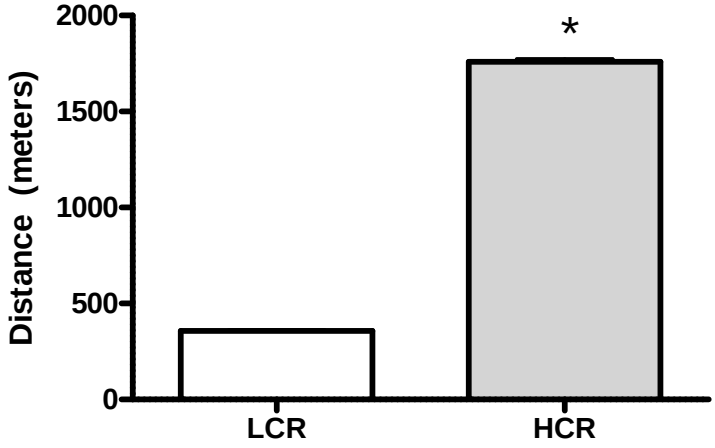
Rat #	Body Weight	Run Time (min)	Run Distance (m)	Rat #	Body Weight	Run Time (min)	Run Distance (m)
13208	242.67	70.33	1905.00	13167	238.67	66.48	1736.78
13108-13300	245.33	70.27	1902.00	13226	188.67	66.47	1736.07
13117	253.67	69.72	1877.25	12978	287.67	66.30	1728.90
12927	244.67	69.70	1876.50	12908	220.33	66.23	1726.03
12951	284.33	69.52	1868.25	12994	257.33	66.18	1723.88
13213	255.33	69.33	1860.00	13164	224.33	66.15	1722.45
13148	217.33	69.10	1849.50	12974	232.33	65.92	1712.42
13188	228.33	68.95	1843.80	13154	302.33	65.87	1710.27
13165	229.33	68.60	1828.40	12966	160.33	65.75	1705.25
12993	256.00	68.43	1821.07	12991	250.67	65.58	1698.08
13239	268.67	68.35	1817.40	13189	221.00	65.45	1692.35
13234	230.67	68.32	1815.93	13209	230.67	65.20	1681.60
12998-13215	227.67	67.98	1801.27	13233	239.33	65.12	1678.02
13129	248.00	67.93	1799.07	12928	266.33	65.08	1676.58
13214	235.33	67.78	1792.47	12995	250.00	64.93	1671.20
12917	258.67	67.73	1790.27	12992	267.67	64.73	1662.80
13230	237.67	67.42	1776.33	12907	270.33	64.70	1661.40
13191	217.33	67.35	1773.40	13241	247.67	64.57	1655.80
13106	243.67	67.22	1767.53	13175	283.33	64.38	1648.10
13210	225.00	66.78	1749.68	13254	225.00	64.37	1647.40

Figure 2.1. Comparison of generation 22 best run time and distance. A. Comparison of LCR and HCR best run times (min). LCR achieved a significantly less best time for forced treadmill run test compared to HCR (*, $p<0.05$). B. Comparison of LCR and HCR best run distance (meters). LCR performed significantly less meters during the forced treadmill run test compared to HCR (*, $p<0.05$).

A.


*
70
60
50
40
30
20
10
0
Time (min)
LCR
HCR

B.


*
2000
1500
1000
500
0
Distance (meters)
LCR
HCR

Hind Limb Femoral Artery Occlusion. Femoral artery occlusion was produced as outlined by Lloyd *et al*. (61; 62). Briefly, rats were anesthetized with 90:10 ketamine/xylazine solution (dosage 0.08-0.1ml mixture per100g body weight i.p.). The fur was shaved from the inner thigh and the surgical sight was cleaned with Betadine and 70% alcohol. Utilizing a small incision, the right femoral artery was isolated and three ligatures of 3.0 surgical silk were placed to cause an ischemic condition in the downstream tissue. One ligature was placed 5-6mm distal to the inguinal ligament, a second was placed on a collateral artery arising from the inguinal fat pat, and the third was placed 5-6 mm distal to the first ligature. When all three ligatures were in place, the incision was closed in layers and the animal was given a Buprenex injection (dosage 0.1ml mixture per 100g body weight i.p.) and placed on a warming blanket under a heating lamp to recover. Once spontaneous movement and sternal recumbancy was observed, the animals were returned to their cages and returned to the animal facility. Animals were sacrificed at 7 (18 HCR and 18 LCR) or 14 days (10 HCR and 10 LCR) following placement of the ligatures.

Microsphere Injections. To determine relative bulk perfusion in ischemic and non ischemic muscles, colored tracer microspheres were utilized. The underlying rationale for this technique is that microspheres, when injected into the left ventricle, will distribute in relative proportion to blood flow to any given region. The size of the microsphere determines the anatomic vascular level at which the flow determination is made. Typically, microspheres of between 10 and 20 µm in diameter are used, leading to first pass clearance of all injected microspheres at the capillary level. It has been estimated that a typical microsphere injection occludes approximately 5% of a given

capillary bed, and that any capillary bed has 30-40% excess perfusion capacity. Therefore, as many as 6 different microsphere tracers may be injected before the procedure itself is expected to alter tissue perfusion patterns. In the present studies, a maximum of two injections was performed.

To perform the injections, a PE50 catheter was positioned in the left ventricle by retrograde advancement under continuous pressure guidance after insertion in the right carotid artery. Proper positioning of the catheter in the left ventricle was determined by observing the characteristic transition from arterial pressure to left ventricular pressure values, and was verified postmortem by visual inspection of catheter tip placement in the left ventricle. Yellow, and persimmon (15 um) Dye-Trak microspheres (Dye-Trak; Triton Technology, San Diego, CA) were dispersed by sonication and vortex mixing. The number of microspheres for any given color (mean diameter, 15um) injected into the left ventricle was about 900,000/0.3ml. The microspheres were loaded into the catheter and given as a bolus injection followed by a flush of 0.5ml of saline.

Microsphere injections were conducted on the day of euthanasia, 7 or 14 days following surgery to occlude the right femoral artery. Yellow microspheres were injected first as a control, which allowed comparison of flow between the non-ischemic left gastrocnemius muscle, and the right, post-occlusion gastrocnemius. Following the first injection, the left femoral artery was ligated acutely, at similar locations to those used previously for the right femoral artery. Immediately after ligation, the persimmon microspheres were injected. Comparison of tracer densities obtained using this microsphere were used to determine the difference between native collateral flow

Microsphere Tissue Digestion and Recovery. Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Inc., St. Louis, MO). All reagent solutions were generated in-house following manufacturer's directions for microsphere recovery and processing (Dye-Trak; Triton Technology, San Diego, CA). Following the last injection of microspheres, gastrocnemius, soleus and plantaris muscles were excised and fixed in formalin for 1 hour. Tissue was weighed (~1.0-2.0g) and then subjected to tissue digestion and processing as outlined by Dye-Trak. Once a pellet of blanched microspheres and any remaining debris was obtained, the supernate solution was used for dye analysis. Photometric absorption of each dye solution was determined by UV/Visible Spectrophotometer (wavelength 300-700nm with 1nm optical band width). The composite spectrum of each dye solution was resolved into the spectral of the single constituents by a matrix inversion technique, using formulas provided by the manufacturer for that purpose. The absorption spectrum of each dye was measured separately from a control sample of each colored microsphere, and was used as a reference for the matrix inversion, determining the contribution of each color to the measured composite spectra at 440, 495, 545, 672 nm. In the present study, the various vascular interventions precluded access to a reference withdrawal arterial blood sample that would have allowed final expression of the microsphere data in mls/min/gram tissue. Instead, the raw data, expressed as number of microspheres/gram tissue was used to report flow. As a control to verify uniform distribution of blood flow, the right and left kidneys also were obtained and processed for microspheres. Differences in microsphere distribution of greater than 7% between right and left kidney were used as a technical

criteria for ignoring all values obtained with that injection in that animal. No data were excluded on that basis.

High Frequency Stimulation. As a gross functional test of differences between HCR and LCR phenotypes, demand ischemia was induced by direct electrical stimulation of the gastrocnemius muscle. The pacing protocol was similar to that described by Keeton *et al.* (49). Briefly, high frequency electrical stimulation was accomplished using a pair of wire electrodes, approximately 5 mm apart, inserted directly into the body of the gastrocnemius muscle. Electrical impulses were delivered using a stimulator (Grass Instruments, Columbus OH) set to deliver repeated single pulses at a frequency of 5 Hz, 200-400 mV, and a pulse duration of 1-ms duration. Preliminary experiments were used to establish the threshold voltage for stimulation response (100-200 mV), and a stimulation frequency that would produce spontaneous exhaustion of muscle contraction. The electrical stimulation protocol lasted until the muscle failed to contract with enough force sufficient to cause a visible displacement of the foot. Eight HCR and 8 LCR rats underwent high frequency pacing prior to hind limb femoral artery occlusion to establish baseline differences between the phenotypes, and 13 HCR and 14 LCR rats received pacing at the time of euthanasia to establish functional differences in the effective remodeling following femoral artery ligation.

Rosenblatt Staining and Analysis. Gastrocnemius muscles for each animal not used for microsphere studies of blood flow were cut in half and quickly frozen. One half was placed in optimal cutting temperature (O.C.T.) compound while the other half was stored for protein isolation. The muscle in the OCT was cut into transverse sections (thickness

10um) and underwent capillary staining as originally defined by Rosenblatt *et al*. (86). Briefly, Rosenblatt staining allows capillary visualization for subsequent photographs under 20 x magnification. From the images, measurements were obtained in 5 fields per section, 50 myocytes/field, and in at least five separate sections, with care taken to avoid repeated sampling of the same field in sequential sections. The following indices were measured: 1) number of capillaries around a fiber (NCAF), 2) the capillary to fiber ratio on an individual fiber basis (Cap/Fi), 3) the number of fibers sharing each capillary (share factor (SF)), and 4) capillary density (CD). This stain also provides the ability to distinguish between different muscle fiber types. Slow twitch fibers were counted and are presented as a percentage of the total fiber number.

Fatty Acid Oxidation. Red and mixed gastrocnemius tissues were quickly excised from anesthetized rats and placed in ice-cold isolation buffer (in mM: 100 KCl, 40 Tris·HCl, 10 Tris base, 5 $MgCl_2 \cdot 6H_2O$, 1 EDTA, and 1 ATP; pH 7.4). Homogenates were prepared according to the methods of Kim *et al*. (50). Briefly, ~50–100 mg of tissue were thoroughly minced with scissors in 200 µl of buffer containing (in mM) 250 sucrose, 1 EDTA, 10 Tris·HCl, and 2 ATP (pH 7.4), and then the buffer volume was brought up to yield a 20-fold (wt/vol) diluted sample. This was transferred to a 3-ml Potter-Elvehjem glass homogenization vessel. Muscle suspensions were homogenized on ice with a Teflon pestle at 10 passes over the course of 30 s at 1,200 rpm. Homogenates were kept on ice until oxidation experiments were performed. The liberation of $^{14}CO_2$ from [1-^{14}C]palmitate was used to assess mitochondrial fatty acid oxidation as previously described (21; 71). Briefly, palmitate (200 µM) was bound to 0.5% BSA (final concentration) and brought up in reaction buffer to yield the following final

concentrations (in mM): 100 sucrose, 10 Tris·HCl, 10 KPO_4, 100 KCl, 1 $MgCl_2 \cdot 6H_2O$, 1 L-carnitine, 0.1 malate, 2 ATP, 0.05 coenzyme A, and 1 dithiothreitol (pH 7.4). Aliquots (40 µl) of the appropriately diluted tissue homogenates were plated in quadruplicate in a modified 48-well cell culture plate (Costar, Cambridge, MA). A small groove was engineered between adjacent wells so that CO_2 could freely diffuse between the incubation and trap wells. Reactions were started by the addition of 160 µl reaction buffer. Culture plates were sealed with parafilm and a siliconized rubber gasket and allowed to incubate in a shaking water bath at 37°C. Reactions were terminated after 30 min by addition of 100 µl of 70% perchloric acid to the incubation wells. The incubation plate was transferred to an orbital shaker, and $^{14}CO_2$ was trapped in 200 µl of 1 N NaOH for 1 h at room temperature. Radioactivity of CO_2 was determined by liquid scintillation counting using 4 ml Uniscint BD (National Diagnostics, Atlanta, GA).

Citrate Synthase Activity Assay. Citrate synthase activity was determined using the method previously described methods (28; 71). Briefly, 10ug homogenates were incubated in the presence of oxaloacetate, acetyl-coenzyme a, and 5,5'-dithiobis (2-nitrobenzoic acid (DTNB)). Spectrophotometric detection of reduced DTNB as a wavelength of 412nm served as an index of enzyme activity.

Western Blot. Whole gastrocnemius muscle was homogenized in RIPA lysis buffer supplemented with a cocktail of protease and phosphatase inhibitors (P2714, P2850 and P5726, Sigma, St-Louis, MO), and centrifuged at 15,000 Xg for 25 min at 4°C. The protein content in the supernatant was determined using BCA protein assay kit (# 23227, Pierce, Rockford, IL). Proteins were resolved by SDS-PAGE and transferred onto

nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked overnight at 4°C in a blocking buffer made with Tris-buffered saline containing 0.1 % Tween 20 (TBS-T) and 4 % Bovine Serum albumin. Subsequently, the membranes were incubated for 2h with the indicated antibody in the blocking buffer. After 3 washes of 5 min each with TBS-T, the membranes were incubated with an HRP conjugated secondary antibody for 1h, followed by 3 washes of 5 min each with TBS-T. The immunoreactive bands were visualized by enhance chemiluminescence (GE Healthcare, Piscataway, NJ) using the Typhoon Phosphorimager (Molecular Dynamics).

RNA isolation, reverse transcriptase-PCR, and real-time PCR. Total RNA was extracted from harvested gastrocnemius muscle using TriReagent (Sigma-Aldrich, USA) and cDNA was generated using the High Capacity cDNA kit from Applied Biosystems (Foster City, CA) following the manufacturer's protocol. Real-time PCR was performed using specific primers (Table 2.3) (Invitrogen, La Jolla, CA) and SYBR Green mix (Applied Biosystems, Foster City, CA) following the manufacturer's protocol and using GAPDH as the reference gene as CT values for this gene did not change with treatment. The Real-Time PCR detection system used was the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). LCR control samples were used for all data to be normalized with the $\Delta\Delta^{ct}$ method.

Table 2.3. Real Time PCR Primers sequences.

Primer	Forward Sequence	Reverse Sequence
VEGF	TTCAAGCCGTCCTGTGTGC	TCCAGGGCTTCATCATTGC
Flt-1	CCTCGCCAGAAGTCGTATGG	CCTCGCCAGAAGTCGTATGG
Ang1	AGATACAACAGAATGCGGTTCAAA	TGAGACAAGAGGCTGGTTCCTAT
Ang2	TGGCTGGGCAACGAGTTT	TGGATCTTCAGCACGTAGCG
eNOS	GTGCTGGCATACAGAACCCA	CCATGTGGAACAGACCCCA
GAPDH	GCTGAGTATGTCGTGGAGTC	GTCAGATCCACAACGGATAC

Statistics. Statistical analysis was performed using SPSS software (SPSS, Chicago, IL). Data are expressed as means ± SEM, and a p value <0.05 was considered statistically significant. Student unpaired t-test were used to compare intrinsic differences between LCR and HCR run time to exhaustion and run distance. For microsphere, real time PCR, and histology variables simple effects (LCR control vs LCR acute, LCR acute vs LCR 7D, LCR 7D vs LCR 14D, HCR control vs HCR acute, HCR acute vs HCR 7D, HCR 7D vs HCR 14D, LCR control vs HCR control, LCR acute vs. HCR acute, and LCR 7Dvs HCR 7D, LCR14D vs HCR14D) were the comparisons of interest. A mixed model analysis of variance (2x3 ANOVA, 2 groups (LCR and HCR) x 3 time points (control, acute, 7D for microsphere data and control, 7D, 14D for all other data sets)) was performed using SPSS on all variables between LCR and HCR control, seven day, and 14 day endpoints. Irrespective of a significant interaction, the simple effects defined were compared. High frequency stimulation data was collected in two separate experiments so the post ligation data was analyzed with a student t-test as was the pre ligation data. A generalized linear model of variances using SPSS was performed on these two data sets with the intent to assess if there was a main effect for ischemia. When there was a significant interaction, post hoc test were done with Bonferroni adjustment but irrespective of a significant interaction, the simple effects were compared without adjustments for multiple comparisons. However p-values were less than 0.01 by simple effect analysis, which provides a level of confidence at least as powerful any adjustment for four comparisons would provide using Bonferroni.

CHAPTER 3: EFFECTS OF HIND LIMB ISCHEMIC CHALLENGE ON VASCULAR ADAPTATIONS IN LOW AND HIGH CAPACITY RUNNING RATS

Summary

Untrained low endurance running capacity (LCR) rats have been found to have a higher incidence of risk factors for cardiovascular disease than their high endurance running capacity (HCR) counterparts. Previous work with this model suggests that the LCR phenotype may have an inability to tolerate and generate a vascular response to ischemic stress imposed by unilateral hind limb femoral artery occlusion. We examined the levels of VEGF, Flt-2, eNOS, Ang1 and Ang2, capillary density, NCAF, Cap/Fib ratio, and SF under control conditions and following 7 and 14 days of unilateral hind limb femoral artery occlusion. Capillary density of the LCR rats was less and appeared to be less primed for angiogenesis compared to the HCR counterparts. Following occlusion, HCR rats generated an angiogenic response at 7 days while the LCRs generated a later angiogenic response that does not translate to alterations in capillary density. Therefore it appears that a lower intrinsic exercise capacity appears to be less tolerant of an ischemic injury but maintains the ability to generate a delayed angiogenic response.

Introduction

Peripheral artery occlusive disease (PAOD) is a major health problem affecting 8-12 million Americans with limited treatment options (67). The pathophysiology of PAOD is characterized by an impaired perfusion to the lower extremities. Exercise training is one treatment option that improves quality of life and tissue perfusion characteristics through inducement of the ischemic tissue to increase capillary density (101) which is thought to increase surface area for exchange of oxygen and other

substances between capillary and muscle fibers (95). Exercise training has been shown to increase capillary development within healthy skeletal muscle through the process of angiogenesis that involves the vascular endothelial growth factor (VEGF) driven pathway (15; 81; 82; 107). The mechanisms by which exercise training improves intermittent claudication in PAOD patients remains unclear (92; 93). Clinical data of PAOD shows that patients limited by intermittent claudication who engage in any amount of weekly physical activity beyond light intensity have a lower mortality rate than their sedentary counterparts who perform either no physical activity or only light-intensity activities (30), even after adjusting for other predictors of mortality, which include age, and body mass index (BMI). Thus, exercise training appears to induce adaptive remodeling within ischemic skeletal muscle, but the pathways and mechanisms responsible for this remodeling not well characterized.

Exercise is a complex stimulus with multifactoral outcomes, determined by at least two broad cohorts of genetic control. One cohort establishes the innate aerobic exercise capacity or genetic component that each individual possesses irrespective of any subsequent training or activity, and the second genetic component establishes the pattern of responses to active exercise training. It is estimated that up to 70% of the variation in exercise capacity are due to the genetic predisposition of an individual (9). Many studies have focused on the active exercise training component, but even within these studies, there is a heterogeneous response in subjects to training protocols. It is this finding that suggests the importance of investigating intrinsic exercise capacity and its impact on the response to ischemic stress.

Active exercise training in healthy individuals utilizes the ischemic stress of increased energy output with insufficient oxygen availability from inadequate perfusion to generate a remodeling stimulus for angiogenesis. Vascular endothelial growth factor (VEGF) appears to be one of the signaling molecules that stimulates this angiogenic pathway (61; 81; 82). There are three known VEGF receptors, VEGFR-1 (Fms-like tyrosine kinase 1 (FLT-1)), VEGFR-2 (Kinase insert domain-containing receptor (KDR) also known as fetal liver kinase (FLK-1)), and neuropilin-1 (NP-1) (61; 81; 82). VEGFR-1 appears to play an important role during development, while VEGFR-2 appears to play an important role in mediating angiogenesis in the adult. VEGFR-2 appears to be the receptor that responds following exercise training and ischemic challenge independent of any active exercise (61; 62; 81). It is not known whether these VEGF receptors contribute to differences in capillary density between animals that differ in their intrinsic exercise capacity.

Other potential angiogenic factors that may influence intrinsic exercise capacity associated responses to ischemic challenge are angiopoietin 1 and 2 (Ang1 and Ang2) and nitric oxide (NO) (32; 38; 62; 107). Both factors are known to be involved in exercise training induced angiogenesis and ischemia induced angiogenesis, as well as the combination of both stressors. The angiopoietins are important cytokines that assist in vascular development and remodeling (29). Ang 1 promotes maturation and stabilization of vessels and is expressed widely throughout tissues, whereas Ang 2 competes with Ang 1 by displacing it from the activating Tie2 receptor. The Tie2 receptor is expressed at sites of vascular remodeling. Thus Ang1 dominance is associated with a stable vasculature while Ang2 dominance is associated with active

angiogenesis (29). NO can be increased with VEGFR2 or KDR activation (29). The increase in NO production can be critical to VEGF signaling and the remodeling response of the skeletal muscle. Thus, there are a variety of factors that influence angiogenesis in response to exercise alone, ischemia alone, and the combination of both. The influence of intrinsic exercise capacity on the ischemic expression and angiogenic response has not been studied and serves as the basis questioning the extent to which the gene profile that determine intrinsic aerobic exercise capacity phenotype also influence the basal and inducible expression of these important angiogenic factors.

Recently, Koch and Britton (51), have developed a model to investigate the role of intrinsic aerobic exercise capacity on responses and adaptations to chronic disease. Low aerobic capacity or low cardiovascular fitness, is a strong predictor of early mortality within PAOD patients (30; 92; 101) and recent studies with this model show that the low endurance running capacity phenotype (LCR) rats are more prone towards hepatic steatosis (97) and high fat diet induced insulin resistance (71). These LCR rats from the 11^{th} generation displayed a higher incidence of both cardiovascular and metabolic syndrome risk factors than the high endurance running capacity (HCR) rats(105). All these studies demonstrate that different phenotypic backgrounds are associated with different susceptibility to disease that can be linked to skeletal muscle and its ability to utilize oxygen and meet energy demands. These studies also show that this animal model is valuable in the study of the interaction between intrinsic exercise aerobic capacity and chronic disease. Previous studies have found that the LCR phenotype has decreased capillary density when compared to the HCR counterparts (44; 106), which suggest that the LCR may have a decreased tolerance to this injury that

mimics PAOD, and studies of skeletal muscle responses to the stress of high fat feeding indicate that while the LCR's are less tolerant, the inducible responses are largely intact (72).

The relationship between intrinsic aerobic capacity and the changes that occur in response to occlusion-induced ischemia in skeletal muscle is the focus of the present study. Specifically, the hypothesis is that the low endurance aerobic exercise capacity phenotype will have an altered angiogenic adaptive response to ischemic stress compared to the high endurance aerobic exercise capacity phenotype. To model ischemic stress similar to that seen in PAOD patients, we utilized an established unilateral hind limb femoral artery occlusion (61; 62).

Results

To provide a functional measurement for comparison between the phenotypes, high frequency electrical stimulation of the gastrocnemius muscle was performed prior and following ligation in two separate experiments. Independent t-test were done on the data sets to assess if there was a significant difference between the phenotypes. A generalized linear model of variances was done but did not show there was a significant interaction. The t-test on the data set following ligation did show there was a significant difference with the LCR gastrocnemius muscle time to exhaustion different from the HCR (#, $p < 0.05$, Figure 3.1). The t-test on the data prior to ligation did not demonstrate differences between the phenotypes with an unstressed circulation. The generalized linear model did not find when the data sets are assessed together a significant interaction. A significant main effect for the ischemic condition was found

in both strains, such that following placement of the ligature on the femoral artery, both strains decreased the time to muscle exhaustion with pacing (*, $p < 0.05$, Figure 3.1). These data demonstrate that the injury produced a similar response in both phenotypes, but also showed that the ischemic injury uncovered phenotypic differences that were not present under baseline conditions.

Microsphere injections and subsequent colored dye-extraction has been demonstrated to be an effective means to measure perfusion in skeletal muscle and other organs (108; 109). The initial microsphere injection allowed for resting perfusion to be measured and was normalized for tissue weight. This measurement provided information on resting, acute and seven day ischemic perfusion time-points to better characterize the underlying perfusion and effects ischemic stress placed on the phenotypes. There simple effect comparisons were the designed comparisons for this experiment. The mixed model linear analysis of variance did not reveal a significant interaction. Further analysis showed there was a significant main effect for phenotype and condition. Not surprisingly, the condition main effect demonstrated that ligation of the artery produced a decrease in blood flow in both the LCR and HCR strains (* $p < 0.05$, Figure 3.2). The phenotypic main effect was evident in that the LCR animals had significantly less perfusion of the muscle at acute and seven day ischemic time-points, compared to the HCR animals. Simple main effect comparisons between the LCR and HCR acute measurements demonstrated that the LCR had a significantly more severe loss of perfusion with occlusion (# $p < 0.05$, Figure 3.2). Following seven days of ischemic challenge, the LCR continue to have less perfusion compared to the HCRs (#, $p<0.05$, Figure 3.3). This data indicate that while both phenotypes altered tissue perfusion in

response to the hind limb ligature, the extent of perfusion deficit, and the pattern of recovery at seven days was significantly different as a function of the phenotype.

Figure 3.1. Comparison of high frequency electrical stimulation time in LCR and HCR gastrocnemius muscles prior and following femoral artery ligation. Both LCR and HCR animals significantly decreased time to exhaustion with ligature placement (*, $p<0.05$). LCR time to exhaustion following femoral artery ligation was significantly less compared to HCR time (#, $p<0.05$).

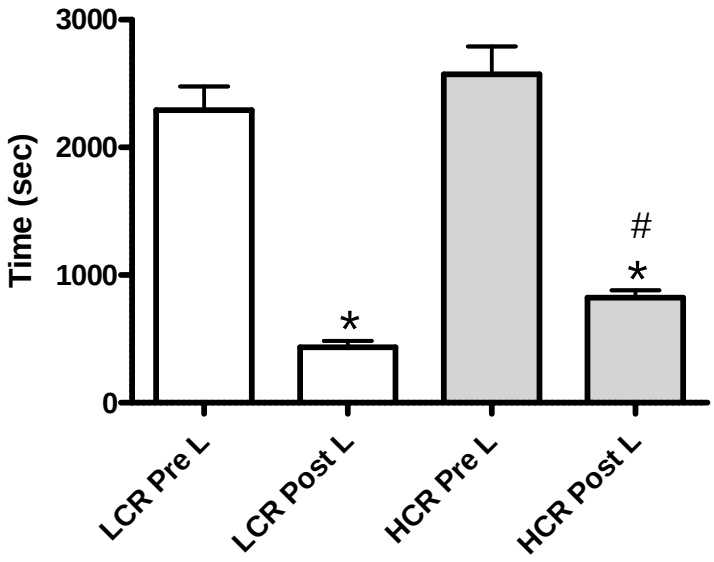

3000
2000
1000
0
Time (sec)
*
#
*
LCR Pre L
LCR Post L
HCR Pre L
HCR Post L

Figure 3.2. Perfusion of muscles prior to, and following ligations of the femoral artery. LCR and HCR animals both significantly decreased perfusion following ligation (*, $p<0.05$), and LCR perfusion was significantly less than HCR (#, $p<0.05$).

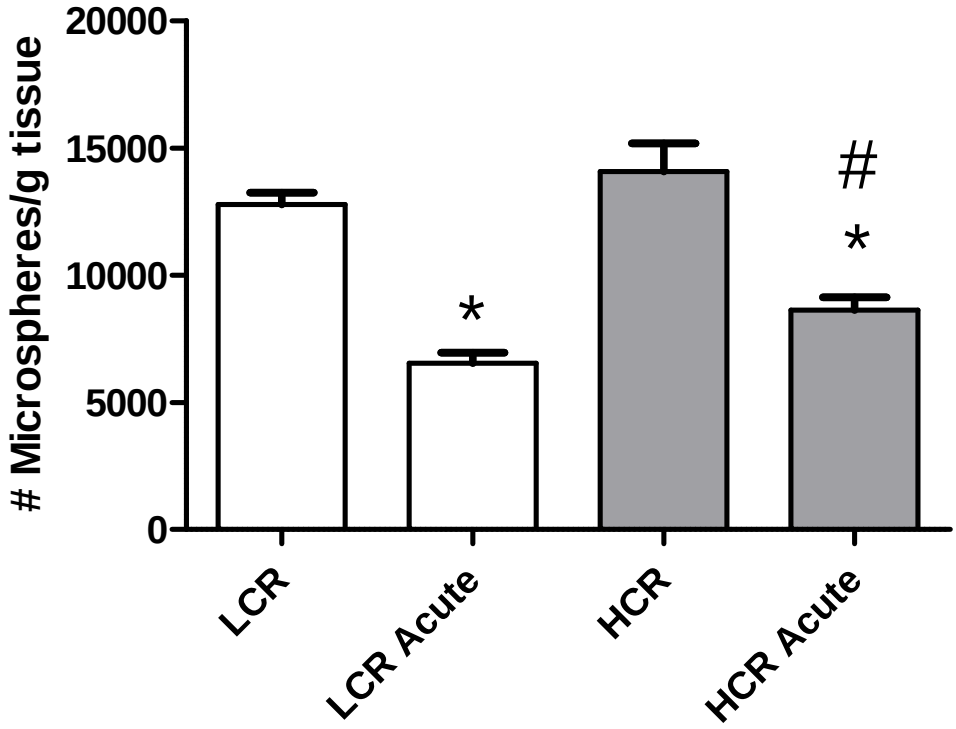

Microspheres/g tissue
20000
15000
10000
5000
0
*
#
*
LCR
LCR Acute
HCR
HCR Acute

Figure 3.3. Perfusion of muscles immediately following, and 7 days after femoral artery ligation. LCR animals had significantly lower perfusion both immediately and seven days after occlusion compared to HCR animals (#, $p<0.05$), suggesting phenotypic differences in either in pre-existing anatomic collateral blood vessels, or their control, and as well as phenotypic differences in the in the inducible response at seven days.

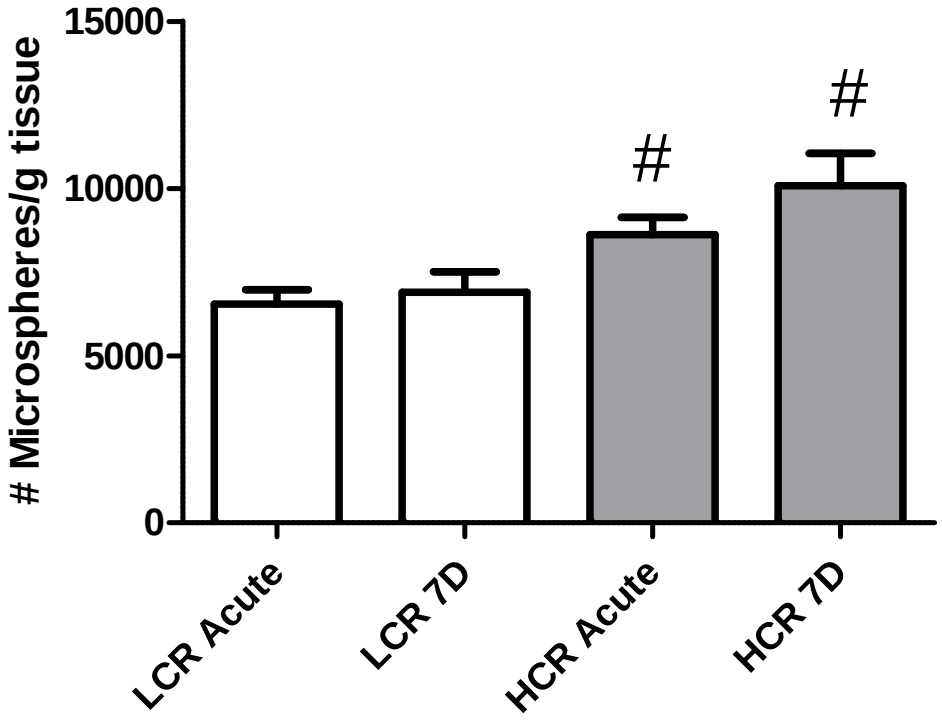
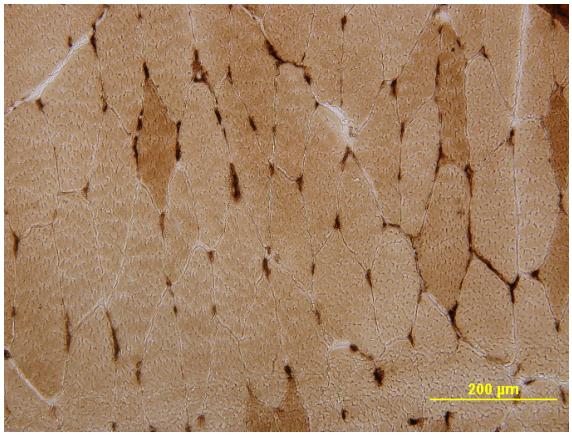

Microspheres/g tissue
15000
10000
5000
0
#
#
LCR Acute
LCR 7D
HCR Acute
HCR 7D

Figure 3.4. Representative Rosenblatt stained gastrocnemius muscles of LCR and HCR animals. A: LCR gastrocnemius muscle, B: HCR gastrocnemius muscles.

A.



B.

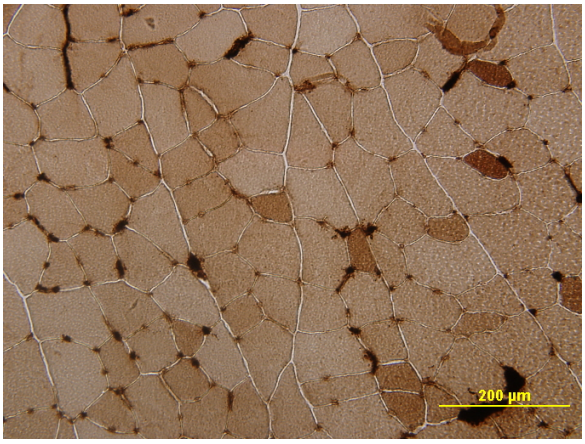
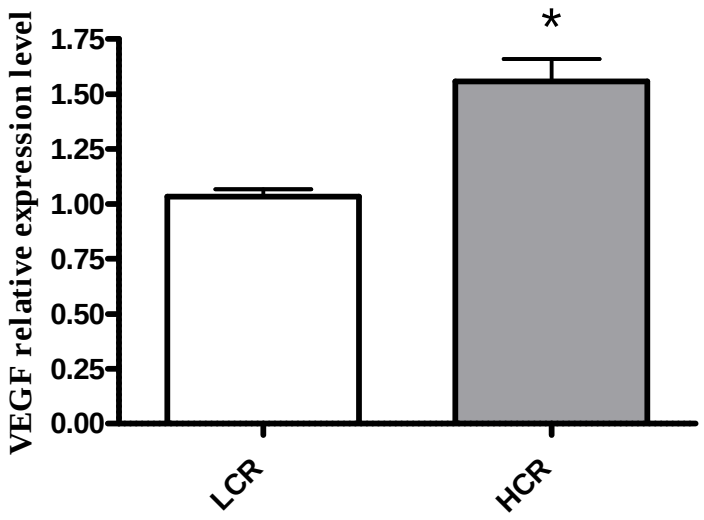


Figure 3.5. Comparison of Real Time PCR results for VEGF (A) and Flt-1 (B) mRNA expression in control gastrocnemius muscles from LCR and HCR animals. A: LCR rats had significantly less VEGF mRNA expression compared to HCRs (*, $p<0.05$). B: LCR rats did not significantly differ in mRNA expression of Flt-1 compared to HCRs.

A.


VEGF relative expression level
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
LCR
HCR

B

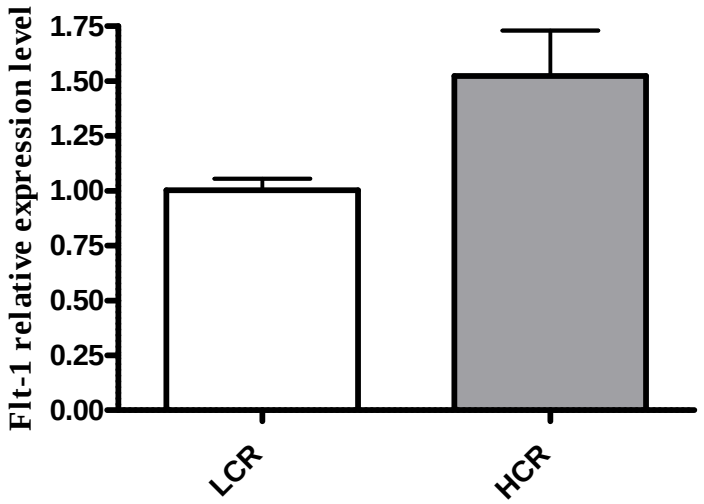
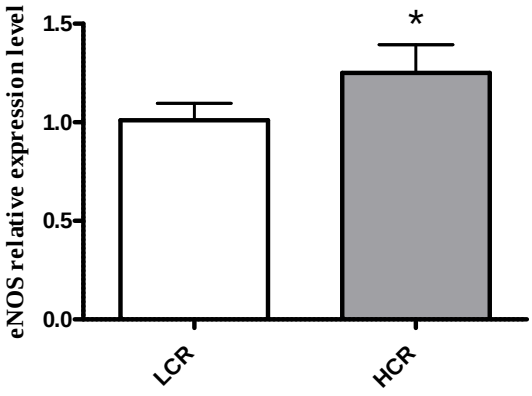
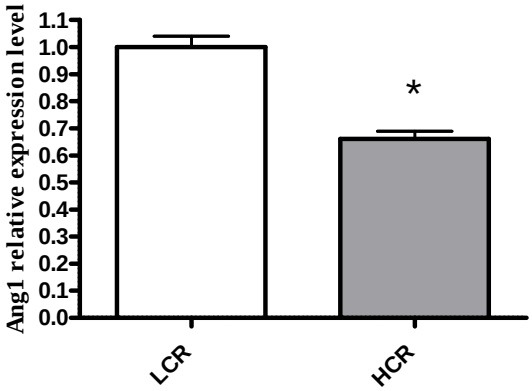

Flt-1 relative expression level
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
LCR
HCR

Figure 3.6. Comparison of Real Time PCR results for eNOS (A), Ang1 (B), and Ang2 (C) mRNA expression in control gastrocnemius muscles from LCR and HCR animals. A: LCR rats had significantly less eNOS mRNA expression in control gastrocnemius muscle compared to HCRs (*, $p<0.05$). B: LCR rats had significantly more Ang1 mRNA expression compared to HCRs (*, $p<0.05$). C: LCR rats had significantly less Ang2 mRNA expression compared to HCRs (*, $p<0.05$)

A.


eNOS relative expression level
1.5
1.0
0.5
0.0
*
LCR
HCR

B.


Ang1 relative expression level
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
LCR
HCR

C.

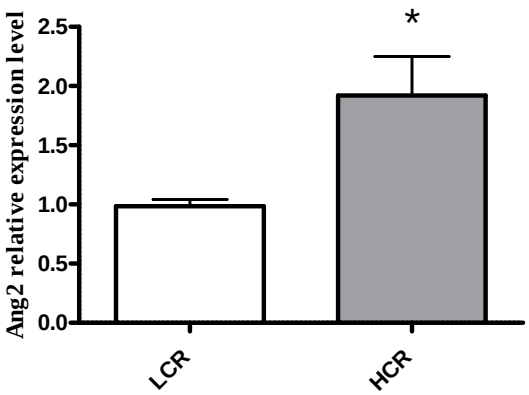
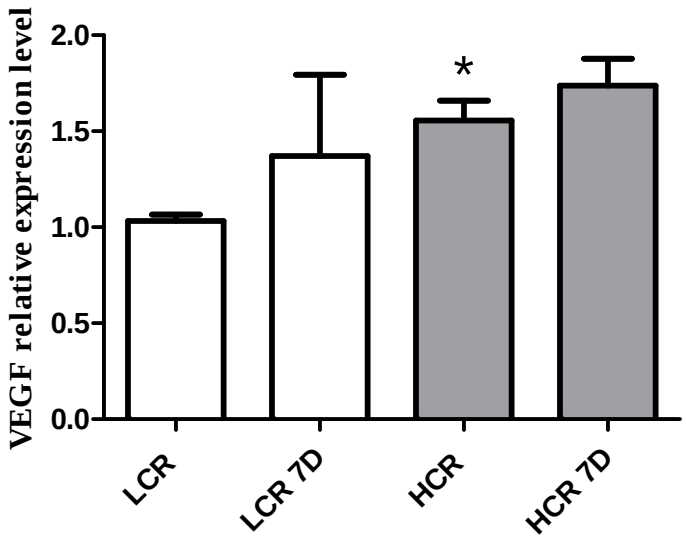

Ang2 relative expression level
2.5
2.0
1.5
1.0
0.5
0.0
*
LCR
HCR

Figure 3.7. Comparison of Real Time PCR results for VEGF (A) and Flt-1 (B) mRNA expression in control and 7 day (7D) post-ischemic gastrocnemius muscles from LCR and HCR animals. A: Neither the LCR nor the HCR animals demonstrated significantly altered VEGF mRNA expression following 7D of ischemic challenge and baseline differences disappeared between the phenotypes. B: Neither the LCR nor the HCR animals demonstrated significantly altered Flt-1 mRNA expression following 7D ischemic challenge.

A.


VEGF relative expression level
2.0
1.5
1.0
0.5
0.0
*
LCR
LCR 7D
HCR
HCR 7D

B

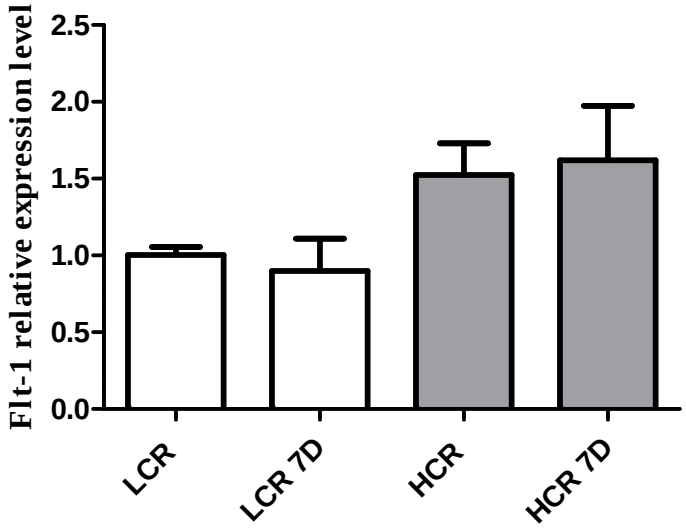
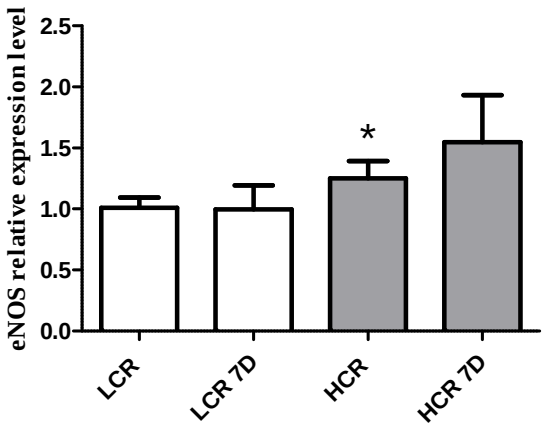
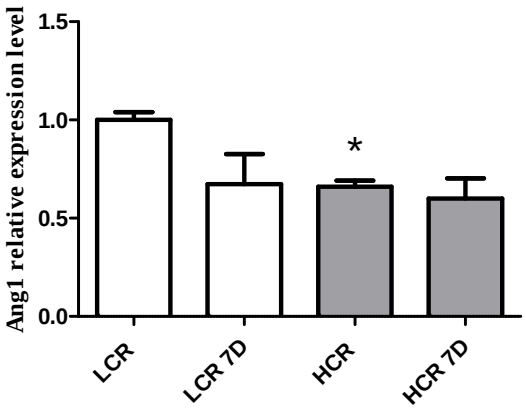

Flt-1 relative expression level
2.5
2.0
1.5
1.0
0.5
0.0
LCR
LCR 7D
HCR
HCR 7D

Figure 3.8. Comparison of Real Time PCR results for eNOS (A), Ang1 (B), and Ang2 (C) mRNA expression in control and seven day (7D) post-ischemic gastrocnemius muscles, from LCR and HCR animals. Significant differences between phenotypes in all three genes were present under control conditions (*, $p < 0.05$), but not seven days after ischemia, and none of the genes demonstrated an inducible response seven days after occlusion in either phenotype.

A.


eNOS relative expression level
2.5
2.0
1.5
1.0
0.5
0.0
*
LCR
LCR 7D
HCR
HCR 7D

B.


Ang1 relative expression level
1.5
1.0
0.5
0.0
*
LCR
LCR 7D
HCR
HCR 7D

C.

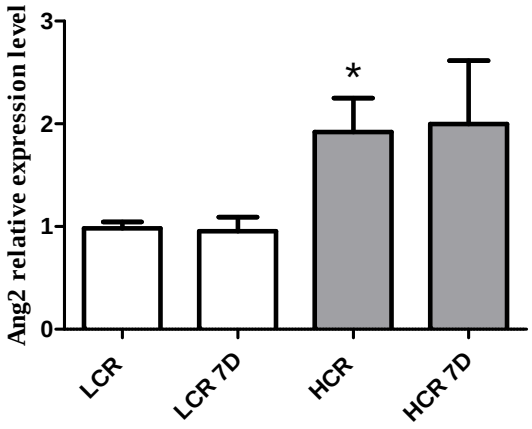
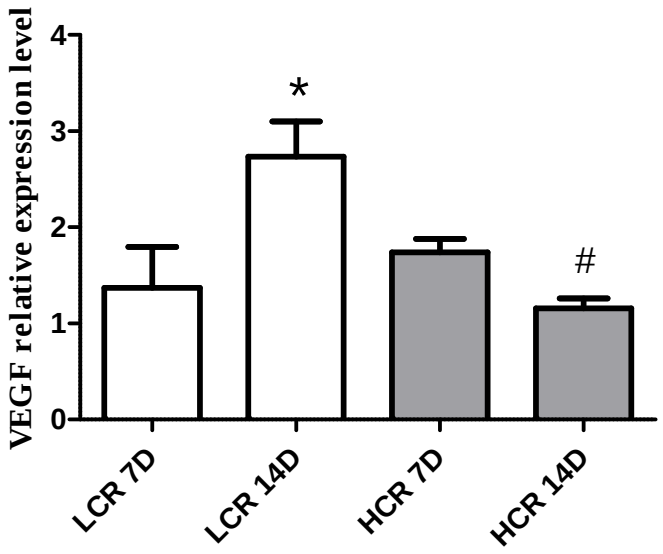

Ang2 relative expression level
3
2
1
0
*
LCR
LCR 7D
HCR
HCR 7D

Figure 3.9. Comparison of Real Time PCR results for VEGF (A) and Flt-1 (B) mRNA expression seven days (7D) and fourteen days (14D) after ischemia, in gastrocnemius muscle from LCR and HCR animals. A: LCR 14D ischemic gastrocnemius muscle significantly increased VEGF mRNA expression compared to 7D ischemic levels (*, $p<0.05$), and LCR 14D VEGF mRNA expression was significantly greater than HCR 14D ischemic gastrocnemius muscle (#, $p<0.05$). B: LCR 14D ischemic gastrocnemius muscle significantly increased Flt-1 mRNA expression compared to 7D (*, $p<0.05$), and LCR 14D ischemic gastrocnemius muscle Flt-1 mRNA expression was significantly more than HCR 14D levels (#, $p<0.05$).

A.


VEGF relative expression level
4
3
2
1
0
*
#
LCR 7D
LCR 14D
HCR 7D
HCR 14D

B.

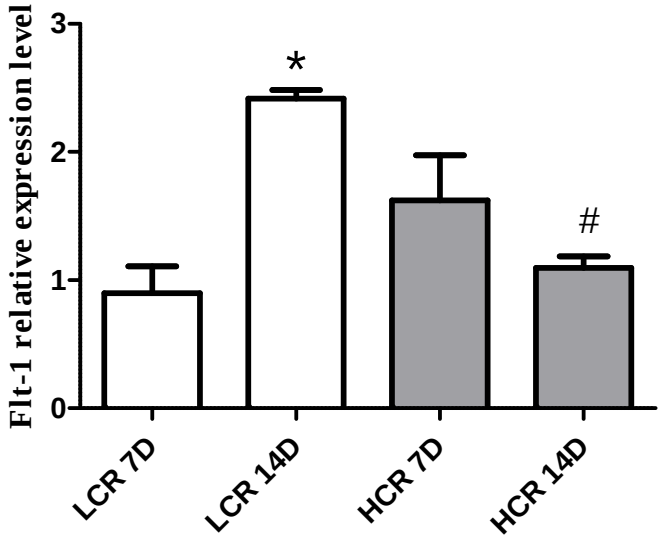
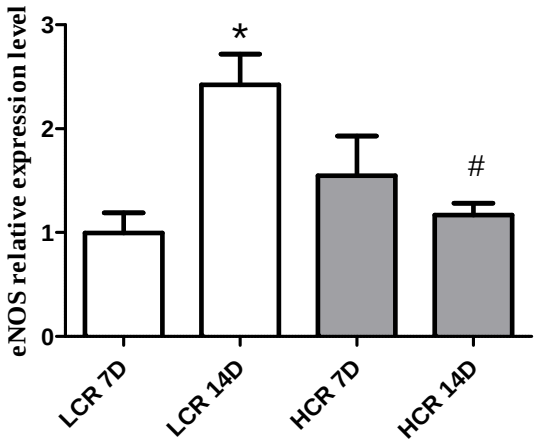
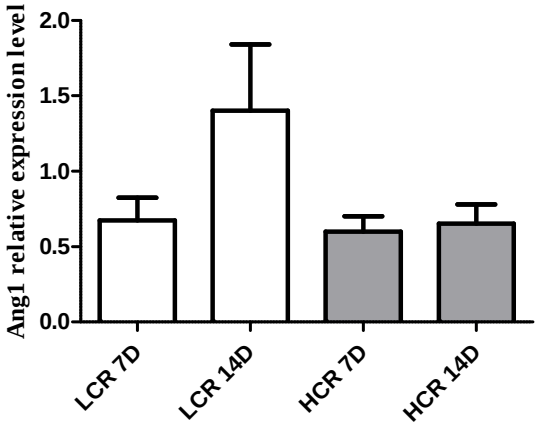

Flt-1 relative expression level
3
2
1
0
*
#
LCR 7D
LCR 14D
HCR 7D
HCR 14D

Figure 3.10. Comparison of Real Time PCR results for eNOS (A), Ang1 (B), and Ang2 (C) mRNA expression seven days (7D) and fourteen days (14D) after ischemic occlusion in gastrocnemius muscle from LCR and HCR animals. A: LCR 14D ischemic gastrocnemius muscle eNOS mRNA expression significantly increased compared to 7D levels (*, $p<0.05$), and LCR 14D eNOS mRNA expression was significantly higher than the HCR 14D ischemic gastrocnemius muscle (#, $p<0.05$). B: No statistically significant alterations in Ang1 mRNA expression level were observed between phenotypes or within the phenotypes at the different time points. C: LCR 14D Ang2 mRNA expression was significantly increased compared to 7D ischemic expression, but there were no statistically significant differences observed between the phenotypes at these time points.

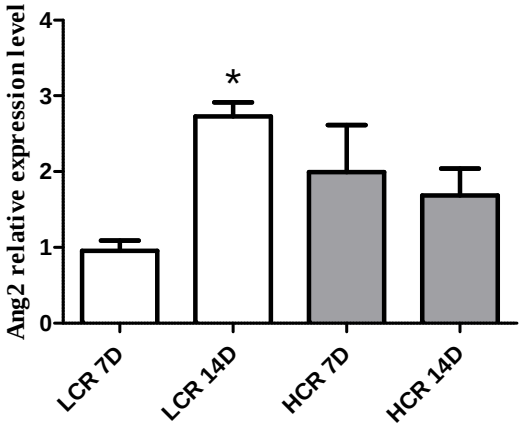
A.


eNOS relative expression level
3
2
1
0
*
#
LCR 7D
LCR 14D
HCR 7D
HCR 14D

B.


Ang1 relative expression level
2.0
1.5
1.0
0.5
0.0
LCR 7D
LCR 14D
HCR 7D
HCR 14D

C.


Ang2 relative expression level
4
3
2
1
0
*
LCR 7D
LCR 14D
HCR 7D
HCR 14D

Analysis of histology data in the present study generate results consistent with previously reports indicating that the LCR has reduced capillary density (CD) compared to the HCR in non-ischemic gastrocnemius muscle (11; 26) (*, $p<0.05$, Table 3.1) (*, $p<0.05$, Figure 3.11B). In addition, these data demonstrate that there are significant interactions between phenotype and occlusion for CD, NCAF, and Cap/Fib. There was no significant interaction detected for SF. Simple effects were analyzed for SF and no significant differences were found. Further analysis of the simple effects and conducting post hoc testing, using a Bonferroni adjustment, demonstrated that with respect to CD, the HCR control muscle is significantly lower than both the 7 day (7D) and 14 day (14D) measurements (#, $p<0.05$, Table 3.1)(#, $p<0.05$, Figure 3.14B), displaying an increase in capillary density seven and fourteen days after ischemia. Significant differences in capillary density (CD) between the phenotypes were present at all time points (*, $p<0.05$, Table 3.1) (LCR control vs HCR control, LCR 7D vs HCR 7D, and LCR 14D vs HCR 14D), and the LCR phenotype does not significantly increase the capillary density within the ischemic tissue.

With respect to NCAF, there were no differences between phenotypes under control conditions. Each phenotype increased NCAF significantly in response to ischemia, with the HCR greater at both the 7 day and 14 day time point measurements (#, $p<0.05$, Table 3.1)(#, $p<0.05$, Figure 3.15A), and the LCR significantly elevated only at the 14 day endpoint (#, $p<0.05$, Table 3.1) (#, $p<0.05$, Figure 3.15A). The final NCAF values 14 days after ischemia were significantly greater in the LCR compared to the HCR animals (*, $p<0.05$, Table 3.1)(*, $p<0.05$, Figure 3.15A).

The capillary to fiber ratio on the individual fiber basis (Cap/Fib) demonstrated a significant increase at 7 days compared to control in the HCR (#, p<0,05, Table 3.1)(#, p<0.05, Figure 3.15B), but a similar increase above control levels was not seen in the LCR animal until 14 days(#, p<0.05, Table 3.1) (#, p<0.05, Figure 3.15B), at which point the LCR values were higher than the HCR animals (*, p<0.05, Table 3.1)(*, p<0.05, Figure 3.15B).

The percentage of slow twitch muscle fibers in the mixed gastrocnemius muscles from the LCR and HCR animals was compared (Figure 3.13). The LCR rats had a significantly lower percentage of slow twitch fibers in the control gastrocnemius muscle compared to the HCRs (*, p<0.05, Figure 3.13). With ischemic challenge, the LCR rats did not significantly alter the percentage of slow twitch muscle fibers (Figure 3.16), but the HCRs significantly increased the percentage of slow twitch muscle fibers between the 7D and 14D ischemic time points ($, p<0,05, Figure 3.16).

Assessment of mRNA for angiogenic markers was conducted on whole gastrocnemius muscle. Under baseline conditions, relative expression of VEGF mRNA was significantly lower in the LCR rats compared to the HCRs (*, p<0.05, Figure 3.5), but VEGFR-1or Flt-1 mRNA expression were not different. The LCR rats also had significantly less mRNA expression of eNOS and Ang2, and significantly more Ang1 (*, p<0.05, Figure 3.6), consistent with a more stable vasculature, or less remodeling potential, than the HCR counterpart. With 7 days of ischemic challenge neither phenotype significantly altered mRNA expression (Figure 3.7, Figure 3.8), while at 14 days, only the LCR animals demonstrated significant alterations, increases VEGF, Flt-1,

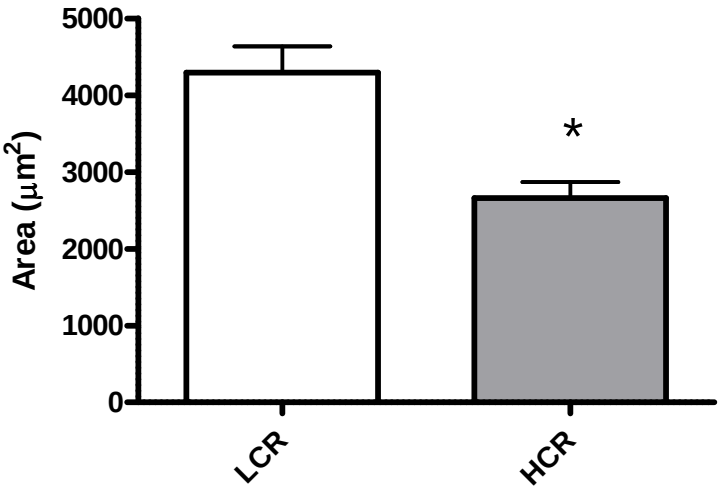
eNOS, Ang2 mRNA expression(*, $p<0.05$, Figure 3.8, Figure 3.9). These increases of the LCR for VEGF, Flt-1, and eNOS at 14 days differed significantly from the HCR suggesting the LCR did generate an angiogenic response to ischemia.

Table 3.1. Summary of skeletal muscle morphology and capillarization under control, and seven days (7D) and fourteen days (14D) after ischemia in gastrocnemius muscle from LCR and HCR animals. (*, $p<0.05$ compared to respective LCR value; #, $p<0.05$ 7D vs control values for the same variable within the phenotype; $, $p<0.05$ vs 14 day 7 day values for the same variable within the phenotype).

	LCR	HCR
Area μm^2		
Non-Ischemic Gastrocnemius	4349.8 ± 281.3	2783.6 ± 236.3*
7 Day Ischemic Gastrocnemius	3031.4 ± 372.7#	2433.3 ± 369.8
14 Day Ischemic Gastrocnemius	4938.5 ± 788.6$	2292.7 ± 622.2*
Perimeter μm		
Non-Ischemic Gastrocnemius	267.76 ± 8.57	211.85 ± 8.75
7 Day Ischemic Gastrocnemius	224.57 ± 9.44	200.72 ± 14.4
14 Day Ischemic Gastrocnemius	290.61 ± 26.95	190.29 ± 22.9
Capillary Density, capillaries/mm^2 (CD)		
Non-Ischemic Gastrocnemius	417.81 ± 23.6	614.60 ± 57.5*
7 Day Ischemic Gastrocnemius	564.76 ± 40.5	918.54 ± 197.6*#
14 Day Ischemic Gastrocnemius	507.48 ± 54.2	1015.41 ± 174.9*#
Capillary Contacts (NCAF)		
Non-Ischemic Gastrocnemius	4.547 ± 0.220	4.280 ± 0.217
7 Day Ischemic Gastrocnemius	4.370 ± 0.584	5.170 ± 0.276*
14 Day Ischemic Gastrocnemius	6.220 ± 0.481*$	5.210 ± 0.255*#
Individual Capillary to Fiber Ratio (Cap/ Fib)		
Non-Ischemic Gastrocnemius	1.662 ± 0.089	1.555 ± 0.073
7 Day Ischemic Gastrocnemius	1.603 ± 0.233	1.896 ± 0.149*
14 Day Ischemic Gastrocnemius	2.248 ± 0.183*$	1.852 ± 0.097#
Share Factor (SF)		
Non-Ischemic Gastrocnemius	2.896 ± 0.014	2.933 ± 0.062
7 Day Ischemic Gastrocnemius	2.890 ± 0.038	2.938 ± 0.069
14 Day Ischemic Gastrocnemius	2.935 ± 0.023	3.014 ± 0.015

Figure 3.11. Comparison of fiber area (μm^2) (A) and capillary density (capillaries/mm^2) (B) in control gastrocnemius muscle from LCR and HCR. (*$p<0.05$ vs LCR value)

A.


5000
4000
3000
2000
1000
0
Area (μm2)
*
LCR
HCR

B.

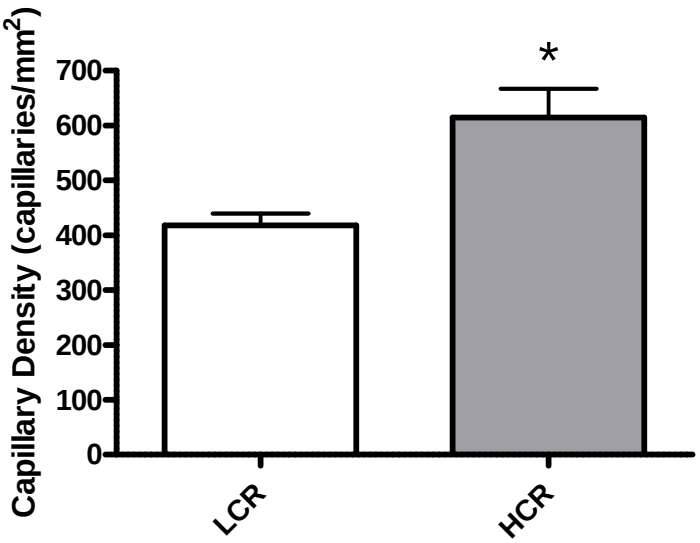
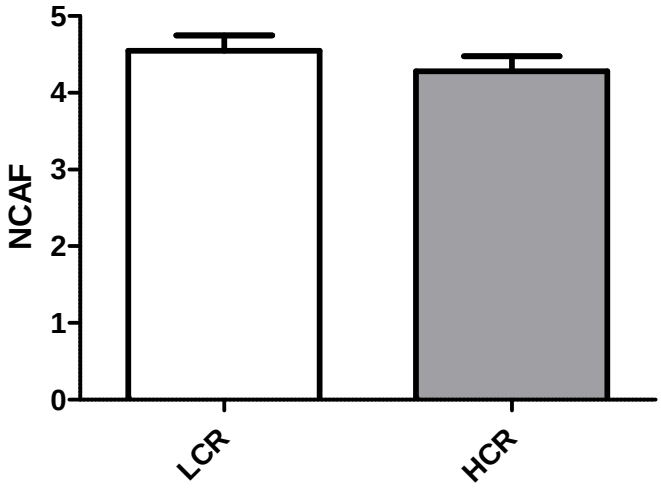

700
600
500
400
300
200
100
0
Capillary Density (capillaries/mm2)
*
LCR
HCR

Figure 3.12. Comparison of NCAF (A) and Cap/Fib ratio (B) in control gastrocnemius muscle from LCR and HCR animals. Neither NCAF nor Cap/fiber ratio demonstrated significant differences between the phenotypes under control conditions.

A.



B.

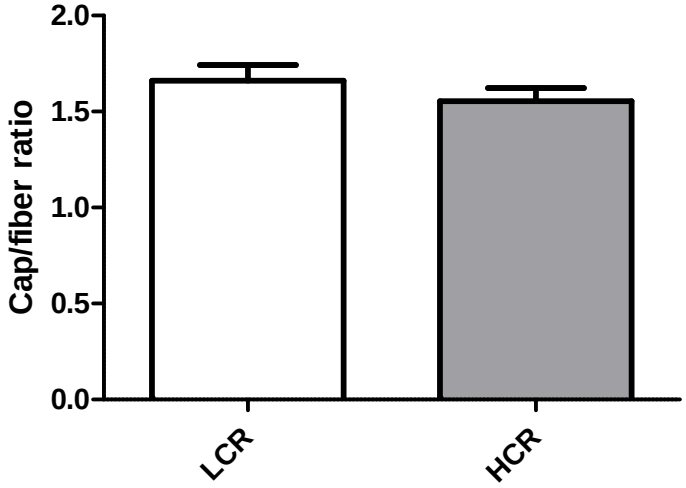


Figure 3.13. Comparison of the percentage of slow twitch fibers within control gastrocnemius muscle from LCR and HCR animals. (* $p<0.05$ vs. LCR).

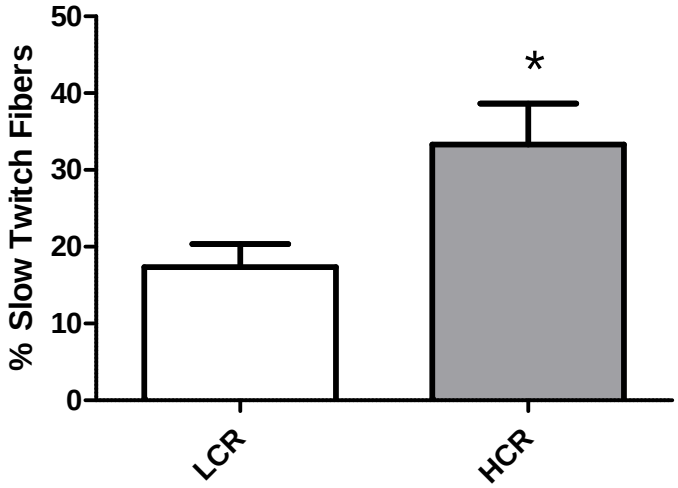
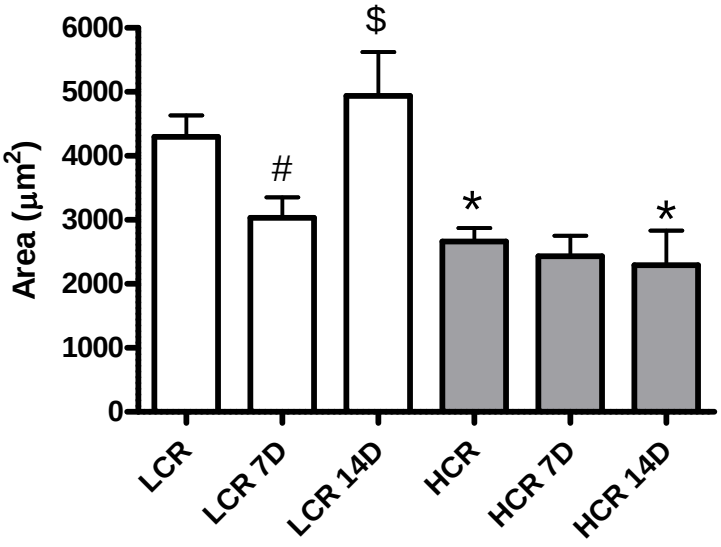

% Slow Twitch Fibers
50
40
30
20
10
0
*
LCR
HCR

Figure 3.14. Comparison of fiber area (A) and capillary density (CD) (B) in control, seven day (7D) and fourteen day (14D) ischemic gastrocnemius muscle samples from LCR and HCR animals. A: LCR fiber area was significantly less compared to HCR under control and 14D ischemic conditions (*, $p<0.05$). LCR significantly decreased fiber area between control and 7D ischemia (#, $p<0.05$). LCR significantly increased fiber area between 7D and 14D ischemic time points ($, $p<0.05$). B: LCR demonstrated significantly less capillary density than HCR under control, 7D and 14D ischemic time points (*, $p<0.05$). HCR significantly increased capillary density measurements from control at 7D and 14D ischemic time points (#, $p<0.05$).

A.


Area (µm²)
6000
5000
4000
3000
2000
1000
0
$
#
*
*
LCR
LCR 7D
LCR 14D
HCR
HCR 7D
HCR 14D

B.

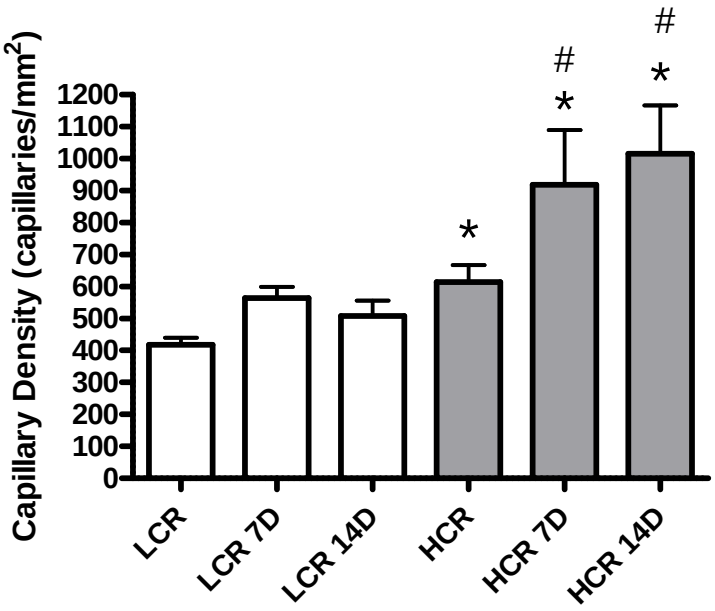
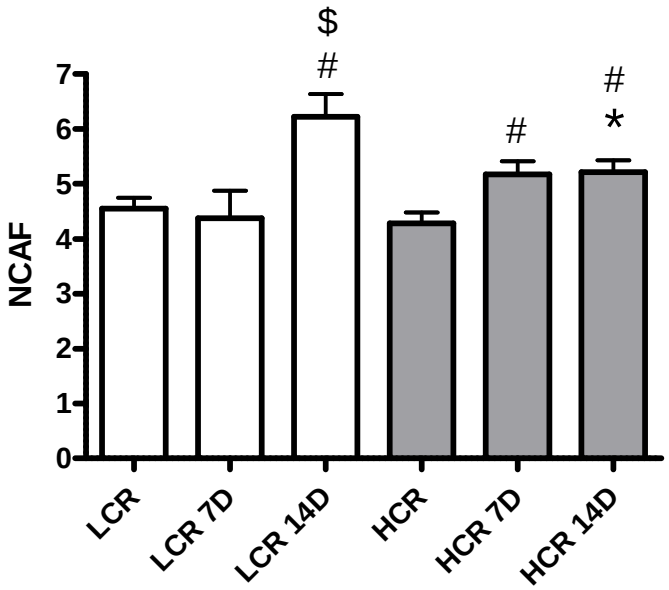

Capillary Density (capillaries/mm²)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
#
*
#
*
LCR
LCR 7D
LCR 14D
HCR
HCR 7D
HCR 14D

Figure 3.15. Comparison of NCAF (A) and Cap/Fib ratio (B) in control, seven day (7D) and (14D) ischemic gastrocnemius muscles from LCR and HCR animals. A: NCAF in LCR significantly differed from HCR at the 14D ischemic time point (*. $p<0.05$). LCR 14D ischemia significantly increased from control (#, $p<0.05$) and 7D ischemia ($, $p<0.05$). HCR 7D and 14D ischemia NCAF significantly increased above control NCAF (#, $p<0.05$). B: Cap/Fib ratio in LCR significantly differed from HCR at the 14D ischemic time point (*. $p<0.05$). LCR 14D ischemia Cap/Fib ratio significantly increased from control (#, $p<0.05$) and 7D ischemia Cap/fib ratio ($, $p<0.05$). HCR 7D ischemia Cap/Fib ratio significantly differed from control Cap/Fib ratio (#, $p<0.05$).

A.


$
#
#
*
#
NCAF
7
6
5
4
3
2
1
0
LCR
LCR 7D
LCR 14D
HCR
HCR 7D
HCR 14D

B.

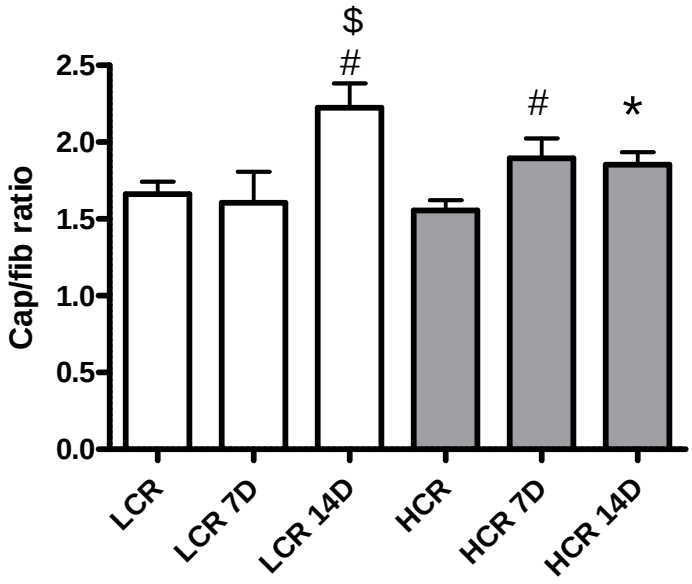
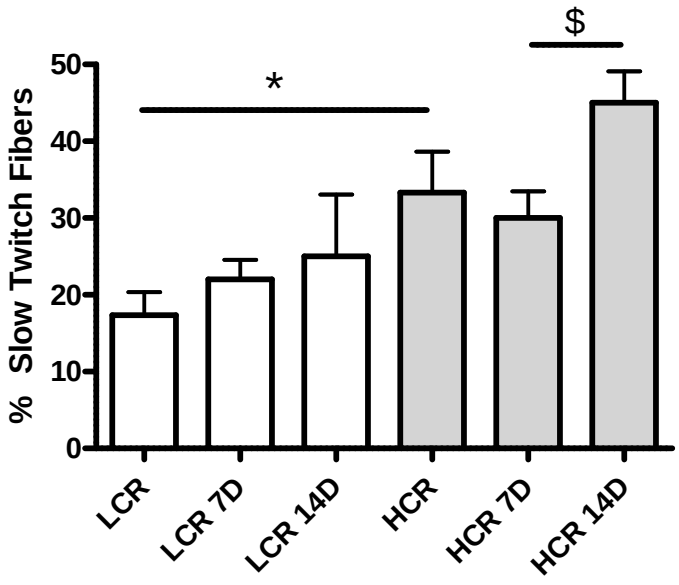

$
#
#
*
Cap/fib ratio
2.5
2.0
1.5
1.0
0.5
0.0
LCR
LCR 7D
LCR 14D
HCR
HCR 7D
HCR 14D

Figure 3.16. Comparison of the percentage of slow twitch fibers within control, 7D, and 14D ischemic gastrocnemius muscle from LCR and HCR animals. Percentage of LCR slow twitch fibers within control gastrocnemius muscle was significantly less compared to HCRs (*, $p<0.05$). HCR significantly increased the percentage of slow twitch fibers between 7D and 14D ischemic time points ($, $p<0.05$).


$
*
50
40
30
20
10
0
% Slow Twitch Fibers
LCR
LCR 7D
LCR 14D
HCR
HCR 7D
HCR 14D

Discussion

The development of intermittent claudication is a major decrement to quality of life in patients (67; 94; 100; 103). A mounting body of evidence suggests that exercise training can increase perfusion in ischemic tissue, improving quality of life and avoiding the necessity for surgical revascularization and other expensive therapies (67; 92; 100; 101). The majority of research on aerobic exercise training in PAOD patients has examined its effects upon vascular remodeling, including the mechanisms underlying changes in capillary density within the ischemic muscle (39; 61; 62; 80-82). However, many patients with intermittent claudication are unable to exercise or participate in exercise protocols due to pain in the ischemic limb or arthritic problems in joints, and exercise studies do not always provide definitive remodeling within tissue. Better understanding the genetic influences of intrinsic aerobic capacity and how endurance capacity affects the remodeling process within the vasculature in response to ischemic challenge, may help explain the variability in PAOD patients responses to aerobic exercise, and may provide new treatment options for individuals unable to participate in aerobic exercising protocols. The goal of this study was to investigate whether rats with low intrinsic running capacity have different vasculogenic responses following peripheral arterial occlusion than rats with high intrinsic running capacity.

The results of this study demonstrate that rats selectively bred, but not trained, for high endurance running capacity (HCR) differ in their response to ischemic stress in a model of PAOD compared to the low endurance running capacity (LCR) counterparts. These results provide strong evidence indicating that low endurance running capacity

confers increased risk for ischemic injury and is subject to delayed and less effective adaptive response to ischemic stress.

Intrinsic differences

LCR and HCR's inherently differ in capillary density, with HCR rats having significantly higher capillary density than LCR rats (Figure 3.11B). Under baseline conditions, the HCR strain are more primed for angiogenesis with a higher relative expression of mRNA for VEGF, eNOS, Ang2 and significantly less relative expression of Ang1 compared to the LCR strain (Figure 3.5 and 3.6). These results are consistent with those found by Lloyd *et al.* (62) who showed similarly increased amounts of angiogenic markers undergoing aerobic exercise training protocols, except that in the present studies, these were intrinsic differences predicated on aerobic phenotype. Similar to the actively exercised rats, the HCRs appear to have an underlying increased angiogenic potential compared to the LCR phenotype under basal conditions. As with capillary density, it is possible that the HCRs also have inherently more collateral vessels, allowing for redundant pathways to optimally sustain perfusion in peripheral tissues. The HCRs also appear to have a higher percentage of slow twitch fibers in their gastrocnemius muscle than their LCR counterpart (Figure 3.16). It is suggested by Wisloff *et al*. (106) and unpublished data from our lab that the arteriolar vessels are more responsive to dilating factors in the HCRs compared to the LCRs. These findings provide a potential explanation for the LCR strain's incapacity to endure an ischemic challenge, demonstrated by a significantly more pronounced drop in tissue perfusion, as measured by microspheres, (Figure 3.2) in response to femoral artery ligation.

LCR muscle cells have a significantly larger area compared to the HCR (Figure 3.11A). This finding is consistent with Howlett *et al.* (44) who found that mean cross-sectional area of the HCR fiber was 35% lower compared to the LCR. This increased intrinsic size difference in muscle area may be due to the LCR having a larger body frame (72) and hence requiring larger cells to support their body type. The HCR smaller fiber area is possibly due to its smaller body size (72). The smaller fiber area in combination with intrinsic higher capillary density would potentially be an advantageous phenotype for ischemic conditions because it provides for decreased diffusion distance for nutrients. It is these findings of smaller fiber area, more capillaries, and greater oxidative capacity in HCR rats that provides a potential mechanism to explain the inherent higher VO_{2max} observed in these animals without aerobic exercise training.

Increased capillary density (CD) (capillaries/mm^2) has been shown to strongly correlate with increased skeletal muscle oxygen conductance in this model (44). We now extend those observations by demonstrating that the HCRs may have inherently better protection for maintenance of tissue perfusion under ischemic conditions. This was functionally evident with the prolonged time to exhaustion with high frequency electrical stimulation (Figure 3.1). It appears that rats bred for higher endurance performance also select for smaller muscle fiber area and more capillaries which facilitates oxygen transport by decreasing the diffusion distance between the bloodstream and skeletal muscle. On the other hand, the larger fibers with less capillary density of the LCR phenotype may be less tolerant of ischemic conditions. As perfusion decreases, this can cause increased diffusion distance of nutrients imposing a more potent ischemic stress within the muscle fibers.

Acute Changes to Ischemic Stress

Microsphere perfusion indicates that remodeling occurs in the HCR animals in response to femoral artery occlusion (Figure 3.3). The LCRs appeared to be under a more potent ischemic stress due to the occlusion as shown by the large initial drop in tissue perfusion. The LCRs, despite this apparent increased ischemic pressure, do not demonstrate a measurable recovery in perfusion at seven days (Figure 3.3). The HCRs initially lose less perfusion with acute arterial occlusion, presumably associated with better ischemic tolerance, and also expand perfusion in the post-ischemic tissue, consistent with anatomic remodeling, altered arteriolar/collateral tone favoring dilation, or both. This flow preservation, suggesting an improved tolerance for ischemic stress by the HCR is further supported by the HCRs ability to maintain muscle fiber area (Figure 3.14A). Furthermore, despite less ischemic pressure and negligible changes in fiber area, HCR rats showed a significant increase in capillary density at seven days. This increase in capillary density appears to be an angiogenically driven event, as suggested by increased capillary contacts (NCAF) and capillary to fiber (Cap/Fib) ratio in HCR rats. However, the mRNA expression of angiogenic markers in the HCR did not significantly change. This raises the possibility that in the HCR rats, the baseline levels of expression of the typical angiogenic growth factors were sufficient to drive this angiogenesis, and the response occurred without need for additional increases. Alternatively, it is possible that the HCR rats use a different set of angiogenic factors in the response to ischemia. Conversely, the LCR animals did not generate a measurable change in perfusion following seven days of ischemic pressure (Figure 3.3). These animals responded to seven days of femoral artery ligation with a significant loss of

muscle fiber area (Figure 3.14A) suggesting an attempt by the tissue to decrease diffusion distance for a more efficient exchange of nutrients, or a simple atrophic loss of muscle mass. Despite this decrease in cell area, the LCR rats did not alter capillary density (CD), Cap/Fib ratio, or NCAF significantly from baseline levels. The LCR rats did not show significant changes in mRNA expression for any of the angiogenic factors from the baseline to the 7D ischemic time point, suggesting that the LCR rats initially respond to seven days of ischemia was a loss in fiber area and no angiogenic response.

Chronic ischemic stress adaptations

Following 14 days of femoral artery occlusion, the HCRs continued to maintain fiber area (Figure 3.14A) but did not significantly alter any angiogenic markers from seven day levels (Figure 3.9 and Figure 3.10), suggesting that after fourteen days of ischemia, angiogenesis was no longer an active pathway within this phenotype and vascular remodeling processes had been completed. This finding is consistent with Lloyd *et al*., who reported that ischemic induced angiogenesis typically alters mRNA within the 7-10 day window and return towards baseline levels near the 14 day time point (62). Of particular interest in the present study was that HCRs appeared to increase the percentage of slow twitch fibers with 14 days of ischemia. A fast to slow fiber switch is normally seen in response to aerobic exercise training and aging (75) but ischemia and unloading is typically associated with a slow to fast phenotypic shift. This finding suggests a more rapid return of spontaneous activity in the HCR rats. The HCR rats typically have increased cage activity and this cage activity may mimic aerobic exercise training. Therefore, this fiber type switch observed at 14D may be an aerobic exercise

training response and not a response related to the ischemic injury. The LCR rats also displayed a surprising finding, in that after an initial fiber loss at 7 days, muscle fiber area significantly increased again 14 days after femoral artery occlusion. This finding was unexpected, but also may reflect the recovery of spontaneous activity. LCRs do not exhibit any changes in body weight with femoral artery ligation. Thus, the possibility exists that these muscle cells at 7D ischemia decrease in fiber area in response to ischemic atrophy, but the decreased muscle mass was no longer sufficient to support the LCRs body weight, and increased mobility led to load dependent hypertrophy and re-expansion of the muscle fiber area, unrelated to the original occlusion. However, as a result of the hypertrophy, CD is maintained, and a relative ischemia may persist, leading to late/delayed angiogenesis, demonstrated by significant increases in NCAF and Cap/fib ratio, and significant increases in mRNA expression for VEGF, Flt-1, eNOS, and Ang2. Together, these findings suggest that a potent angiogenic drive remains inducible in the LCR phenotype. This angiogenic stimulus may be in response to signals associated with hypertrophy of the LCR cells, but not with the occlusive stimulus, or may be occurring at fourteen days as a synergistic action of the loading stimulus in combination with ischemic drive within the tissue.

This study suggests that LCR rats have an altered response to hind limb femoral artery occlusion compared to the HCR rats. This altered response is partially due to the phenotypic differences under baseline conditions, including significantly less capillary density, less angiogenic factor potential, larger fiber area, and a smaller percentage of slow twitch muscle fibers within the gastrocnemius muscle in the LCR animals. With onset of occlusion, the LCRs also appear to have an altered inducible response compared

to the HCR. These alterations include fiber area initially decreasing, followed by a rebound to a larger area, a potent angiogenic upregulation at 14D ischemia, and no significant changes in the percentage of slow twitch muscle fibers. These data suggest that low intrinsic aerobic capacity may have increased injury associated with ischemic disease and will generate an altered response that may require a combination ischemic challenge, and signaling from some other stress, such as increased mechanical loading of the muscle, to adequately stimulate vasculogenic responses.

CHAPTER 4: EFFECTS OF HIND LIMB ISCHEMIC CHALLENGE ON METABOLIC ADAPTATIONS IN LOW AND HIGH CAPACITY RUNNING RATS

Summary

Untrained low endurance running capacity (LCR) rats have been found to have a higher incident of risk factors for cardiovascular disease than their high endurance running capacity (HCR) counterparts. Previous work with this model suggests that the LCR phenotype may have an inability to tolerate a high fat diet, but it is unknown how this phenotype will metabolically adapt to stress imposed by unilateral hind limb femoral artery occlusion. We examined the levels of palmitate oxidation and citrate synthase activity under control conditions and following 7 and 14 days of unilateral hind limb femoral artery occlusion. Palmitate oxidation and citrate synthase activity of the LCR rats was less under control compared to the HCR counterparts. Due to their inherent advantage in palmitate oxidative capacity, HCR rats appear to have a delayed adaptive increase in red gastrocnemius palmitate oxidative capacity compared to the LCR phenotype. Therefore these data suggest that animals with inherently lower aerobic exercise capacity also have lower oxidative capacity that probably limits the ability to tolerate ischemic stress. Both strains maintain ischemia induced adaptations in oxidative capacity within red gastrocnemius muscle, but these changes are delayed suggesting differences in the regulation of changes in oxidative capacity.

Introduction

Peripheral artery occlusive disease (PAOD) is a form of ischemic disease, often with relatively limited treatment options. This disease is prominent as it affects 8-12 million Americans (67). PAOD pathophysiology is characterized by an impaired

perfusion of the lower extremities leading to pain with movement secondary to demand induced ischemia, and results in decreased quality of life (30; 92; 93; 103). Aerobic exercise training is an often employed treatment option for PAOD patients that has been associated with improved tissue perfusion and increased quality of life (92; 93). Aerobic exercise training also has been shown to be beneficial in other cardiovascular diseases, such as coronary heart disease and hypertension (11; 55; 58; 77; 79). The benefits of active exercise training most often are attributed to induced increases in capillarization leading to increased oxygen delivery to tissues (31; 58), increased usage of fat as an energetic substrate (1; 21; 22; 92; 93), and increased mitochondrial respiratory capacity and energy production (41; 60; 92; 93). However, active exercise training often produces a relatively heterogeneous response, despite well-controlled exercise protocols and well controlled study populations (2; 21; 30; 42; 60; 65; 92; 96). In PAOD patients, and in animal models of PAOD, aerobic exercise training research has focused primarily on the changes in capillarization and perfusion, or the supply component of demand ischemia (39; 61; 62; 80-82; 92; 93). The effects of decreased oxygen availability and the alterations that occur within skeletal muscle metabolic pathways of patient with PAOD are much less well studied. The adaptive changes in distal tissue that could modify the demand components of ischemia, such as substrate utilization, have been studied much less extensively. Research studies of ischemic stress on tissues outside of skeletal muscle suggest that adaptive mechanisms occur in metabolic enzymes, including citrate synthase expression and activity, and shifts in substrate preferences can occur. While the benefits of active exercise regimens in the setting of cardiovascular diseases, including PAOD, is well-established (65; 67; 96), a

better understanding of distal tissue responses, and the factors influencing distal tissue metabolism, could provide the basis for additional treatment options in PAOD patients who are not candidates for revascularization.

The capacity for aerobic exercise has two components. The genetic component, or intrinsic aerobic exercise capacity, is estimated to explain up to 70% of the variation seen in the population (9), and presumably explains the heterogeneous responses to active exercise training protocols. Active aerobic exercise, or the environmental element, is estimated to alter intrinsic aerobic exercise capacity, or VO_{2max}, but maximum gains are limited (10-20%) (26; 104), further highlighting the importance of the intrinsic component on the overall aerobic capacity. While responses to active exercise training have been characterized extensively, $VO_{2\ max}$ is multifactoral, and is impossible to model by single gene gain or loss of function models. Consequently, the contribution of the genetic, or intrinsic components of aerobic exercise capacity has been difficult to study effectively, and therefore, the importance of these intrinsic factors to the progression or attenuation of the pathophysiologic process such as PAOD are not well defined. Based on the relative lack of understanding of the influence of environmental compared to genetic influences conferring reduced risk for cardiovascular disease by intrinsic aerobic exercise capacity, Koch and Britton have used forced artificial selection based on the endurance treadmill running phenotype, to develop a rat model to investigate the relative role of intrinsic aerobic exercise capacity, independent of an active exercise training effect (51). Basic traits of these so-called high capacity and low capacity running strains (HCR and LCR) have now been characterized by other groups (83). Our groups also has reported on the relationship of this intrinsic background to alter the response to

superimposed, non-exercise external stress, such as high fat feeding (71), to determine the potential benefit of the intrinsic aerobic capacity on responses and adaptations to elements of chronic disease (51). Low endurance running capacity (LCR) rats display traits consistent with increased risk for cardiovascular and metabolic diseases in humans, such as insulin resistance (71) and endothelial dysfunction (unpublished data), while the high endurance running capacity (HCR) rats display characteristics consistent with decreased cardiovascular risk (106). However, whether these traits actually translate to functional improvement in response to a superimposed cardiovascular insult has not been demonstrated.

The definitive treatment option for PAOD is revascularization, either by surgical bypass or percutaneous methods (20; 66; 94; 103). However, many patients are not candidates for this treatment option (20; 66; 94). Therefore, understanding the distal tissue responses to ischemic stress becomes increasingly important as a basis for potential new treatment options in these individuals. Active exercise, in the absence of ischemia, causes distal tissue remodeling, demonstrated with fiber type switching (102), changes in mitochondrial number, and changes in enzyme expression that ultimately produce increased respiratory capacity (1; 21; 91). Similarly, ischemia alone, in the absence of active exercise, causes cardiac tissue to undergo phenotype switching of critical enzymes that regulate substrate utilization (73; 110). Skeletal muscle under ischemic stress alone has demonstrated it also retains remodeling capabilities (14; 15). Decreased oxygen availability induces skeletal muscle to alter fat oxidation for energy production (15; 87). Clearly, metabolic remodeling is a significant component of the tissue response to both active exercise and to ischemia, particularly with respect to fat metabolism. However, it

is not clear whether the intrinsic capacity for exercise influences the tissue metabolic remodeling in response to ischemia. Therefore, this study was designed to test the hypothesis that differences in intrinsic aerobic capacity, as established using the HCR and LCR rat strains, will alter the distal tissue remodeling of fat metabolism in response to peripheral arterial occlusion.

Results

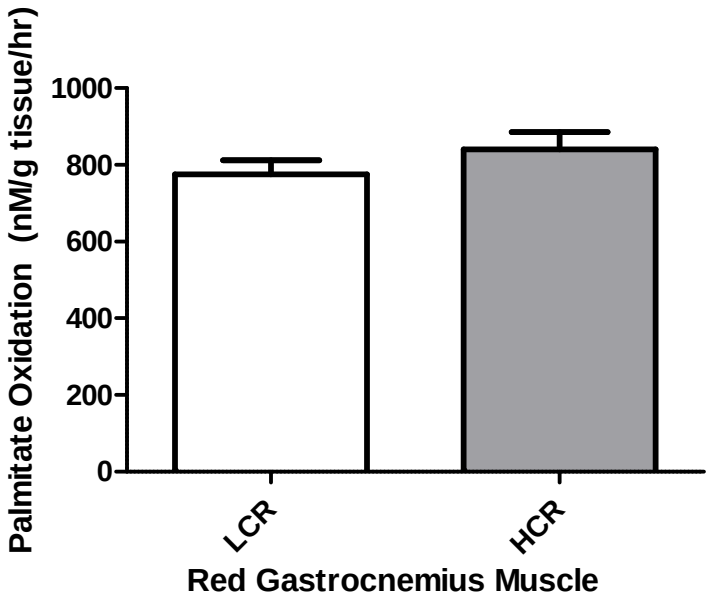
Measurements of fatty acid oxidation. Measurements of fatty acid oxidative (FAO) capacity under control conditions in each of the phenotypes are shown in Figure 4.1. Under control conditions, LCR red gastrocnemius muscle did not differ from the HCRs, while LCR mixed gastrocnemius FAO capacity was significantly less than HCR (*, $p < 0.05$, Figure 4.1B). This finding is consistent with control results previously reported our lab on FAO in response to high fat feeding using a cohort of generation 17 rats (71). In the present studies, when challenged with ischemia from a unilateral hind limb femoral artery occlusion, the LCR red gastrocnemius muscle generated an adaptive response by increasing FAO at both 7 and 14 days ($p < 0.05$, Figure 4.3A, 4.5A). The HCR phenotype only generated an increase from control in FAO in the red gastrocnemius tissue at fourteen days ($p < 0.05$, Figure 4.5B), but not seven days. Mixed gastrocnemius did not significantly change with ischemia in either phenotype and the differences observed at control disappeared.

Citrate synthase activity measurements. There were not measureable statistical differences in red gastrocnemius citrate synthase activity between the strains under control conditions (Figure 4.2A). Mixed gastrocnemius citrate synthase activity was

higher in the HCR animals under control conditions (Figure 4.2B, *, $p < 0.05$). Both strains significantly increased citrate synthase activity from control to 14 days ischemia (Figure 4.6A and B, #, $p<0.05$) in both the red and mixed gastrocnemius tissue.

Figure 4.1. Comparison of palmitate oxidation capacity in control red (A) and mixed (B) gastrocnemius muscles from LCR and HCR animals. A: No statistical differences were found between phenotypes in their control red gastrocnemius muscles. B. LCR control mixed gastrocnemius muscle palmitate oxidative capacity was significantly less than the HCR (*, $p<0.05$).

A.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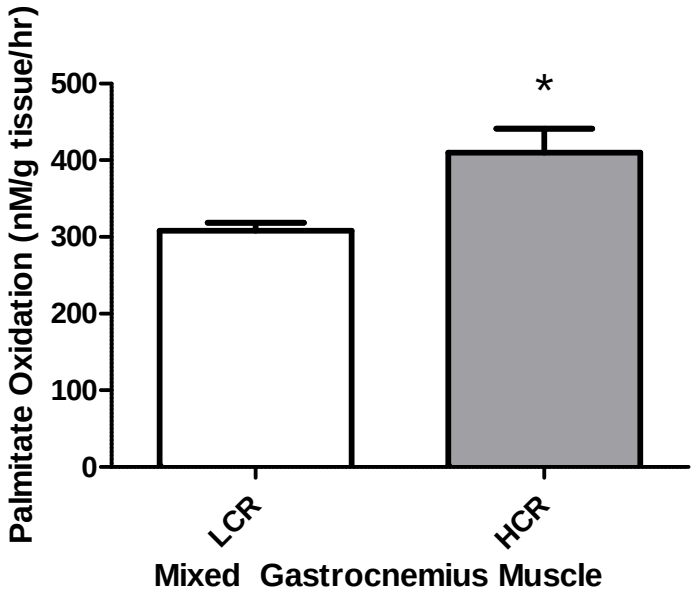
800
600
400
200
0
LCR
HCR
Red Gastrocnemius Muscle

B.


Palmitate Oxidation (nM/g tissue/hr)
500
400
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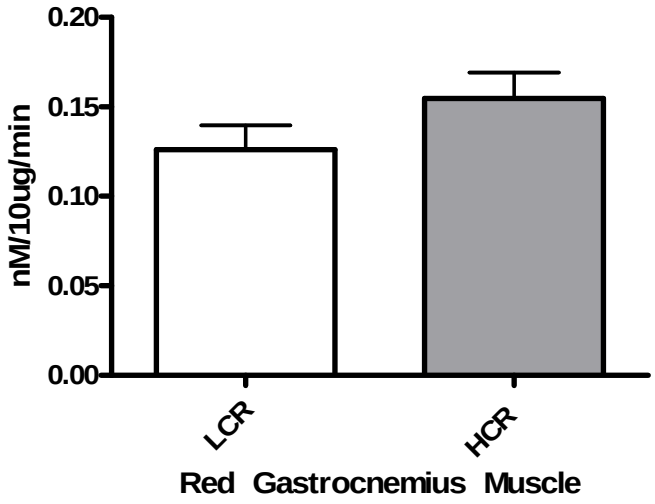
200
100
0
*
LCR
HCR
Mixed Gastrocnemius Muscle

Figure 4.2. Comparison of citrate synthase activity in control red (A) and mixed (B) gastrocnemius muscle from LCR and HCR animals. A: There was no statistical significance observed between the phenotypes for citrate synthase activity in control red gastrocnemius muscle. B: LCR control mixed gastrocnemius muscle citrate synthase activity was significantly less compared to HCR (*, $p<0.05$)

A.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LCR
HCR
Red Gastrocnemius Muscle

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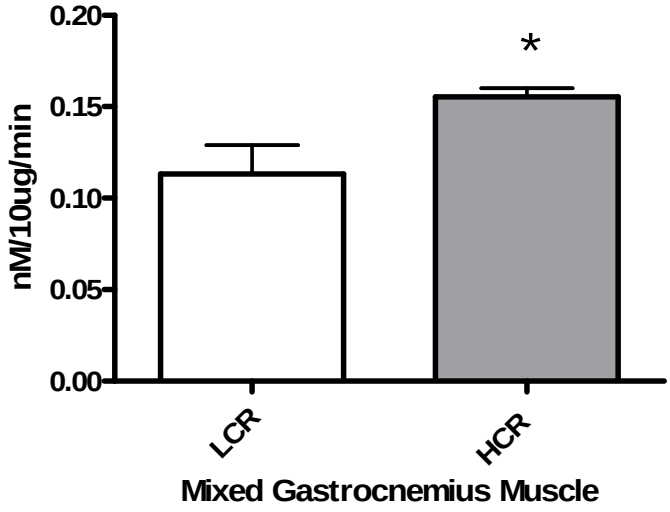
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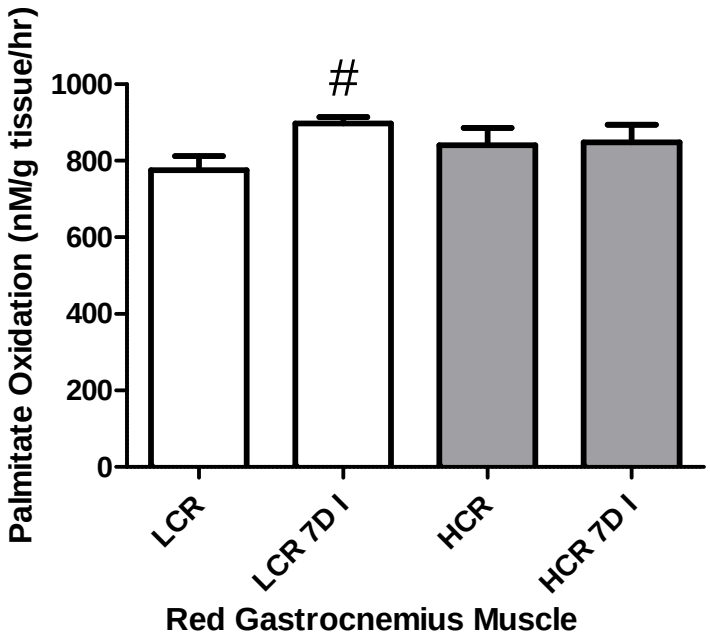
*
LCR
HCR
Mixed Gastrocnemius Muscle

Figure 4.3. Comparison of palmitate oxidation capacity between control and 7D ischemic gastrocnemius muscle from LCR and HCR animals, by fiber type. A: Red gastrocnemius muscle palmitate oxidation capacity increased significantly in LCR 7D from control (#, $p<0.05$), but no statistical differences were found between phenotypes. B. Mixed gastrocnemius muscle palmitate oxidation capacity was not significantly different in either phenotype after 7 days of ischemia.

A.


Palmitate Oxidation (nM/g tissue/hr)
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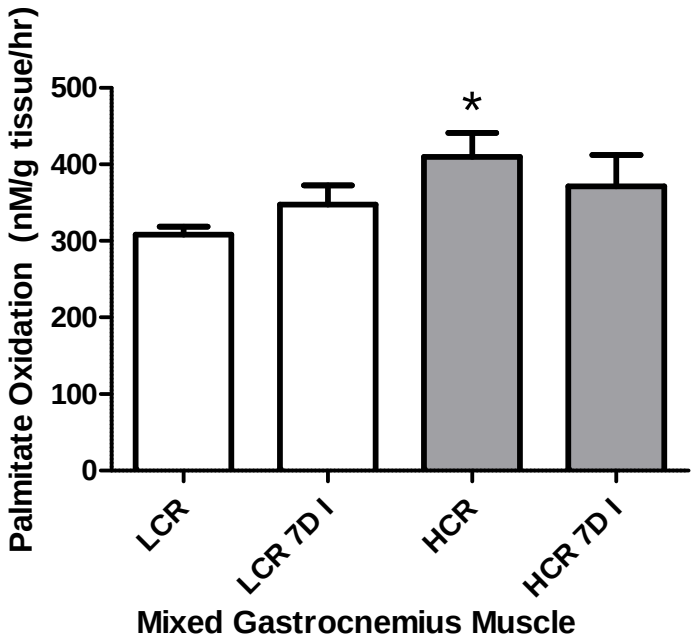
LCR
LCR 7D I
HCR
HCR 7D I
Red Gastrocnemius Muscle

B..


Palmitate Oxidation (nM/g tissue/hr)
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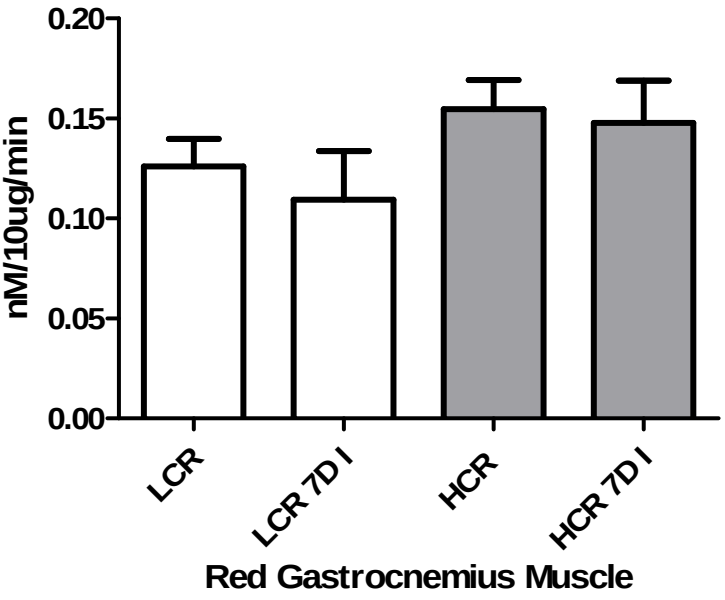
400
300
200
100
0
*
LCR
LCR 7D I
HCR
HCR 7D I
Mixed Gastrocnemius Muscle

Figure 4.4. Comparison of citrate synthase activity between control and 7D ischemic gastrocnemius muscle from LCR and HCR animals, by fiber type. A: Red gastrocnemius muscle citrate synthase activity was not significantly changed in either HCR or LCR animals after seven days of ischemia. B. Mixed gastrocnemius muscle citrate synthase activity was not significantly changed in either HCR or LCR animals following 7 days of ischemia.

A.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0.15
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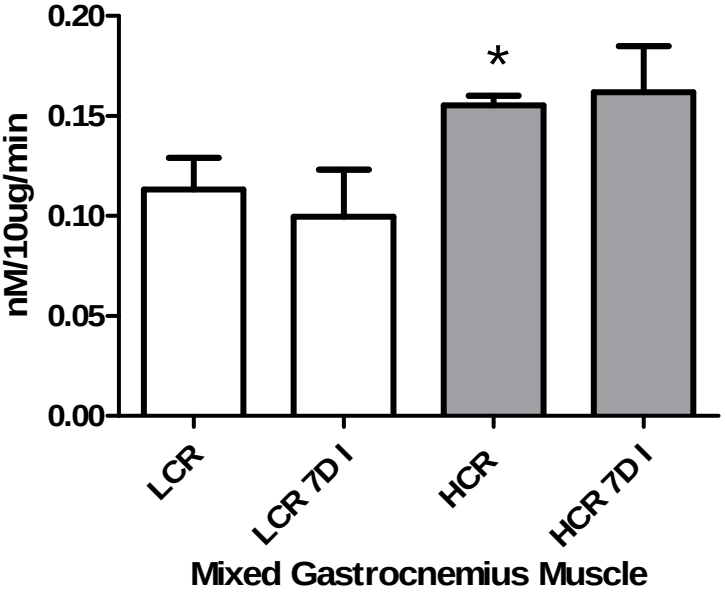
nM/10ug/min
LCR
LCR 7D I
HCR
HCR 7D I
Red Gastrocnemius Muscle

B.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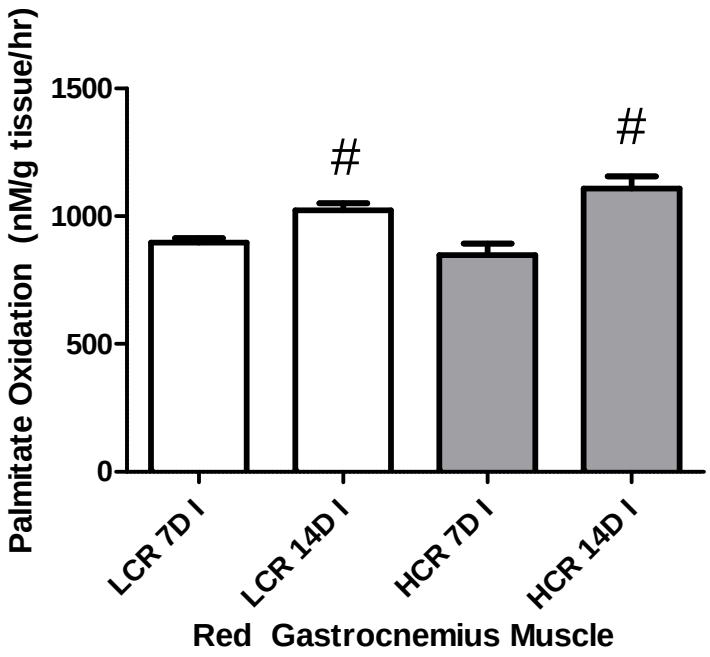
LCR
LCR 7D I
HCR
HCR 7D I
Mixed Gastrocnemius Muscle

Figure 4.5. Comparison of palmitate oxidation capacity between 7D and 14D ischemic gastrocnemius muscle from LCR and HCR animals, by fiber type. A: Red gastrocnemius muscle palmitate oxidation capacity increased significantly in both LCR and HCR phenotypes 14 days after ischemia, compared to the 7 day levels (#, $p<0.05$), but no statistical differences were found between phenotypes. B: Mixed gastrocnemius muscle palmitate oxidation capacity was not different in either phenotype at 14 following fourteen days of ischemia, relative to the seven day levels.

A.


Palmitate Oxidation (nM/g tissue/hr)
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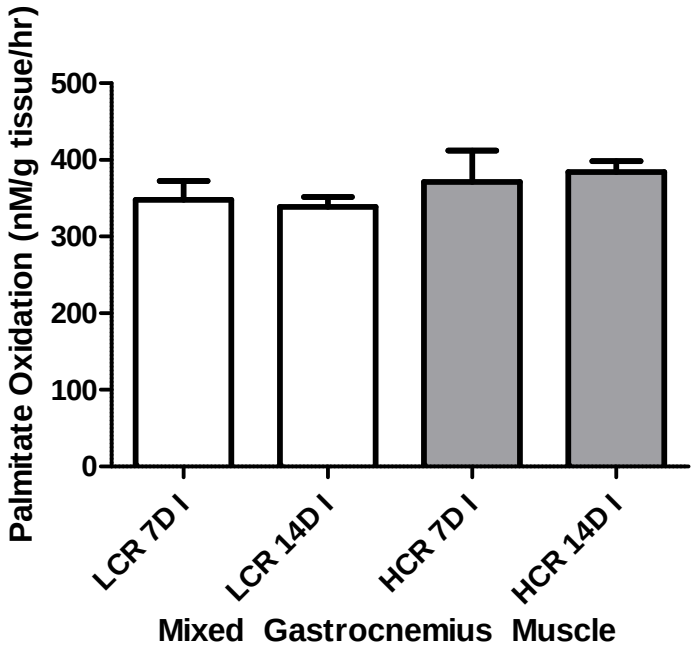
LCR 7D I
LCR 14D I
HCR 7D I
HCR 14D I
Red Gastrocnemius Muscle

B.


Palmitate Oxidation (nM/g tissue/hr)
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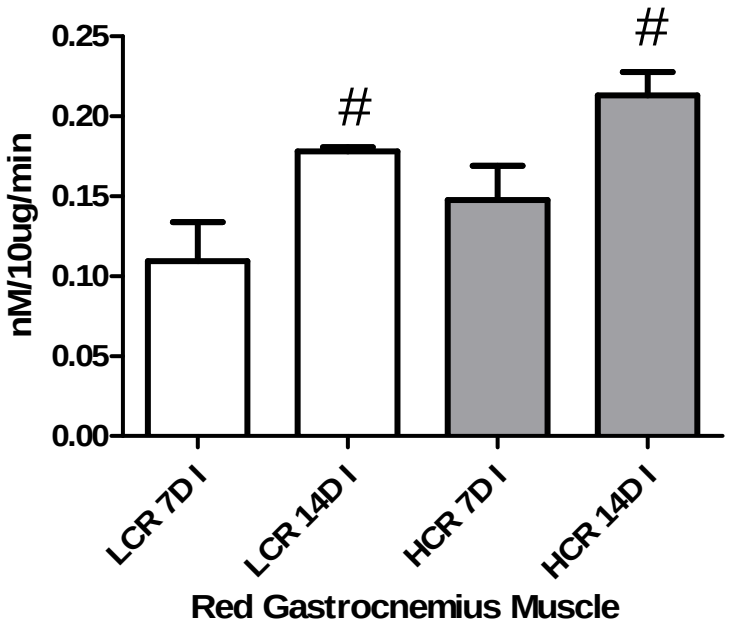
100
0
LCR 7D I
LCR 14D I
HCR 7D I
HCR 14D I
Mixed Gastrocnemius Muscle

Figure 4.6. Comparison of citrate synthase activity between 7D and 14D ischemic gastrocnemius muscle from LCR and HCR animals, by fiber type. A: Red gastrocnemius muscle citrate synthase activity was increased significantly in LCR and HCR animals 14 days after ischemia, compared to seven day values (#, $p<0.05$), but the phenotypes were not different. B: Mixed gastrocnemius muscle citrate synthase activity was increased only in the LCR animals 14 days after ischemia, relative to 7 day post-ischemic levels (#, $p<0.05$), but the phenotypes were not statistically different at 14 days.

A.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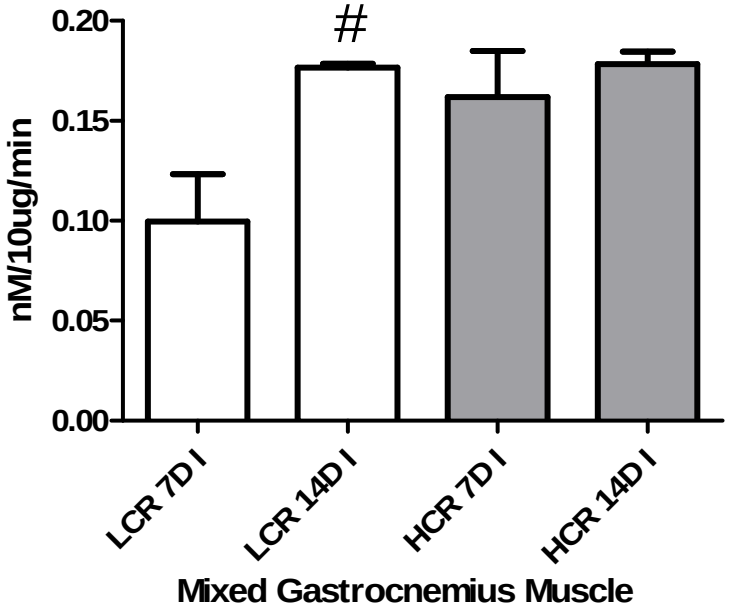
LCR 7D I
LCR 14D I
HCR 7D I
HCR 14D I
Red Gastrocnemius Muscle

B.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0.00
nM/10ug/min
#
LCR 7D I
LCR 14D I
HCR 7D I
HCR 14D I
Mixed Gastrocnemius Muscle

Discussion

Genetic selection based on an intrinsic aerobic capacity phenotype alone has shown differences at the level of skeletal muscle (44; 71; 106). Functional improvements following superimposed cardiovascular insult, as a function of intrinsic aerobic capacity have been very difficult to study accurately until the recent development of a suitable model. The creation of the HCR/LCR rat population and more recently, a similar mouse model has allowed for researchers to investigate if intrinsic aerobic exercise capacity confers an ability to adapt to stress and predisposes to disease (83). Previous studies have linked low rates of mitochondrial oxidative capacity in skeletal muscle of the low performance phenotype as a critical component behind the predisposition to insulin resistance, obesity, and metabolic complications (71). Thyfault *et al.* further expanded that low aerobic capacity was correlated with metabolic dysfunction within the liver (97). The present study utilized the model, with known differences in skeletal muscle oxidative capacity, to assess how these inherent differences would influence fatty acid oxidation following an ischemic insult mimicking PAOD.

Previously, Noland *et al.* (72) demonstrated inherent differences in fatty acid oxidation within the mixed gastrocnemius muscle for the HCR and LCR rats. The HCR rats showed a heightened oxidative capacity in the gastrocnemius compared to the LCRs, but these differences were tissue specific and did not extend into the liver or heart. Our data confirmed this finding of heighted oxidative capacity in the HCRs mixed gastrocnemius muscle compared to the LCRs, but further identified that even within skeletal muscle, these differences did not extend into a subset of red gastrocnemius muscle. A novel finding of this study was that the HCR's heightened oxidative capacity

within mixed gastrocnemius muscle was lost following both seven and fourteen days of ischemic challenge. Another interesting finding was that although the baseline palmitate oxidative capacity within red gastrocnemius muscle did not significantly differ between the phenotypes, following 7 and 14 days of ischemic challenge the LCR demonstrated a significant increase in oxidative capacity. The HCRs demonstrated a significant increase in palmitate oxidative capacity only at fourteen days. This finding suggests that skeletal muscles adapt to ischemia by increasing the palmitate oxidative capacity of their highly oxidative muscle fibers. Due to their inherent advantage in palmitate oxidative capacity, HCR rats appear to have a delayed adaptive increase in red gastrocnemius palmitate oxidative capacity compared to the LCR phenotype.

Citrate synthase activity has routinely been used to assess mitochondrial volume density (41; 42; 60). This enzyme also participates in the pathway of oxidative capacity leading to energy production within muscle cells. Utilizing citrate synthase activity as a marker for mitochondrial volume/density in this model suggests that the HCR phenotype, under baseline conditions, may have significantly more mitochondrial volume/density than the LCR phenotype in the mixed gastrocnemius tissue. This difference does not appear to extend to the red gastrocnemius tissue. For both phenotypes, ischemia triggers increases in citrate synthase activity only at fourteen days in the mixed gastrocnemius muscle. This differs from active exercise inductions in citrate synthase activity that can be induced with a single bout of an endurance exercise event (60). Thus, ischemic stress appears to trigger a delayed increased in citrate synthase activity when compared to active exercise and the increase in citrate synthase activity induced by ischemic challenge is influenced by intrinsic exercise capacity. This finding suggests

there are other potential regulators of fatty acid oxidative capacity and citrate synthase activity with exercise training stimuli are not present under ischemic conditions.

It has been established previously that there is a shift in the predominant fuel within skeletal muscle from fat to carbohydrates under ischemic or exercise conditions (17). The ability of skeletal muscle to preserve fat utilization and spare glycogen is thought to be advantageous. Increased aerobic capacity facilitates fatty acid oxidation through increased vascular supply, metabolic enzymes, and mitochondria number. The higher baseline palmitate oxidative capacity and citrate synthase activity of the HCR rats should provide additional functional reserve and fatigue resistance in skeletal muscles stressed by decreased vascular supply. Our data suggest that the HCR rats did not need higher palmitate oxidative capacity and citrate synthase activity as an adaptive response to ischemia in the first seven days after ischemic occlusion. Conversely, having lower palmitate oxidative capacity and citrate synthase activity, as seen with the LCR phenotype likely was disadvantageous in first seven days after ischemia, requiring an immediate metabolic adaptive response to sustain tissue viability, due to lack of intrinsic reserve capability.

In summary, these data suggest that animals with inherently lower aerobic exercise capacity also have lower oxidative capacity that probably limits the ability to tolerate ischemic stress. Alternatively, the higher aerobic exercise capacity with higher oxidative capacity provides short-term protection against ischemic stress. Both strains maintain ischemia induced adaptations in oxidative capacity within red gastrocnemius muscle, but these changes are delayed in comparison to those triggered by active exercise,

suggesting differences in the regulation of changes in oxidative capacity triggered by ischemia versus active aerobic exercise.

CHAPTER 5: CONCLUSIONS

Aerobic exercise capacity influences an individual's quality of life and susceptibility to disease. A mounting body of evidence suggests that individuals with low aerobic capacity have increased morbidity and mortality and are at higher risk for cardiovascular diseases. Furthermore, a high aerobic capacity appears to have protective effects against metabolic disorders and cardiovascular disease. The extent to which the protection against metabolic and cardiovascular diseases is attributable to the intrinsic aerobic capacity, versus active exercise training, is not well understood. Therefore, the goal of this dissertation was to examine the effects of inherent differences in aerobic capacity on response to ischemic stress. **Our hypothesis was that the low aerobic endurance running capacity (LCR) phenotype will show altered vascular and metabolic adaptive responses to peripheral arterial occlusion when compared to the high aerobic endurance running capacity (HCR) phenotype.**

The results indicate that the hypothesis was correct: the LCR animals did differ from the HCR animals in both the vascular and the metabolic adaptive responses to peripheral arterial occlusion. The different vascular responses were first demonstrated when control and seven day time point were compared. The HCR rat's adaptive responses primarily occurred in this time frame, while the vast majority of changes in the LCR occurred within the seven to fourteen day time frame. In addition, it is speculated that the eventual response that did occur in the LCR may have been due to skeletal muscle signals arising from an overload response from the initial loss of muscle fiber area that rebounded by the fourteen day time point, rather than a direct response to tissue ischemia signals alone. With respect to the metabolic adaptive responses within the

muscle fibers, these data also supports the hypothesis that there was a different response between the phenotypes. The LCR animals increased palmitate oxidative capacity of the red gastrocnemius muscle at seven days while the HCR's were not changed at this time point. Taken together, these data demonstrates that intrinsic exercise capacity influences the adaptive responses following ischemic injury and perhaps the progression of ischemic disease.

There are three possible explanations for the differences in the LCR and HCR responses to peripheral arterial occlusion. The first explanation is that because of their genetic endowment, the HCR rats have less need to adapt to arterial occlusion that the LCR rats. Thus, our experimental manipulation of unilateral hind limb femoral artery occlusion does not stress the HCR rats to the same extent as this manipulation stresses the LCR rats. Supporting evidence for such a postulate can be seen in the perfusion data obtained with microspheres (Figure 3.2). The LCR rats endured a more significant loss of perfusion after femoral artery occlusion, supporting the explanation that this same manipulation induced a different injury within the phenotypes due to the genetic endowment. Although each muscle in each phenotype was paced to exhaustion following occlusion to normalize the initial ischemic stresses, it is likely that any signals arising from that stress were more completely eliminated as the HCRs recovered to a higher post-ischemic flow. In addition, the present study has shown that HCR rats have higher capillary density (Figure 3.11), significantly higher expression of pro-angiogenic growth factors (Figure 3.5, Figure 3.6), a higher percentage of slow twitch muscle fibers (Figure 3.13) and increased oxidative capacity, with both increased palmitate oxidative capacity (Figure 4.1) and citrate synthase activity (Figure 4.2) within the mixed

gastrocnemius muscle compared to the LCR rats. Because of these inherent advantages, it is likely that arterial occlusion produced less ischemic stress in the HCR animals than the LCRs, and consequently induced less robust changes in angiogenic markers NCAF and Cap/Fib ratio (Figure 3.12 and Figure 3.15). This depressed ischemic drive in the HCRs did not induce significant changes with angiogenic growth factors (Figure 3.7-Figure 3.10). With respect to metabolic endpoints, the ischemic stimulus appeared to be better tolerated within the HCR so that there were no significant changes in oxidative capacity between the control and seven day time point (Figure 4.3 and Figure 4.4). Taken together, there is a strong argument that the differences between the phenotypes in their adaptive responses to peripheral arterial occlusion is determined by the inherent differences that exist due to the genetic endowment.

A second possible explanation for the differences in adaption between the phenotypes is that the two groups adapt in the same way, but the LCR response is attenuated due to poor intrinsic aerobic capacity. This scenario is supported by the data showing that both the LCR and HCR animals responded with increases in NCAF and Cap/Fib ratio (Figures 3.12 and Figure 3.15), but the LCR response did not occur until the fourteen day time point (Figure 3.15) whereas the HCR group demonstrated increases in these values at the seven day time point (Figure 3.12). In the metabolic adaptive responses, both phenotypes responded with increased palmitate oxidative capacity and citrate synthase activity in the red gastrocnemius muscle at the fourteen day time point (Figure 4.5 and Figure 4.6), but the LCR also had to increase palmitate oxidation at an earlier (Figure 4.3) time point. These data support the explanation that the adaptive

responses employed by both phenotypes are similar but the timing of the response is the component that is influenced by inherent aerobic exercise capacity.

A third possible explanation is that the HCR and LCR phenotypes utilize different mechanism to adapt to the peripheral arterial occlusion. This conclusion is supported by the fact that the HCR rats changed capillary density but the LCR rats did not (Figure 3.14). Furthermore, the HCR phenotype adapted to ischemia in the gastrocnemius muscle by increasing the percentage of slow twitch fibers (Figure 3.16), as if there rats were undergoing exercise training, but the LCR muscle does not demonstrate significant fiber type switching under these circumstances. The LCR animals increased NCAF and Cap/ Fib ratio at fourteen days after occlusion (Figure 3.15), but this increase may be due to an overload stimulus and not the ischemic stimulus. These data support the explanation that difference in adaptive responses of the two phenotypes in their response to peripheral artery occlusion may be due to different mechanism and pathways. Therefore, intrinsic aerobic capacity may respond to the same stimulus with different mechanistic adaptive responses determined in part by the same gene profiles that establish intrinsic aerobic running capacity.

The present study has established that intrinsic aerobic capacity influences skeletal muscle capillary density, oxidative capacity, and muscle fiber type composition. We have also shown that intrinsic aerobic capacity influences adaptive responses to peripheral arterial occlusion. The high intrinsic aerobic capacity phenotype responds by changing capillary density in the gastrocnemius muscle within seven days. This phenotype makes no significant changes in oxidative capacity within the seven day time period, but eventually increases the percentage of slow twitch fibers in the muscle.

Therefore, it may be suggested that this phenotype relies primarily on early vascular adaptive changes to respond to ischemic injury, and with chronic stimuli, can expand adaptive responses with manipulation of muscle fiber composition and oxidative capacity.

In contrast, the low intrinsic aerobic capacity phenotype adapts to peripheral arterial occlusion by increasing production of angiogenic growth factors and skeletal muscle oxidative capacity. However, this phenotype's vascular adaptive responses appear to be delayed and may be induced by overload stimuli rather than ischemic stimuli. Therefore, it appears that this phenotype is more dependent on changes in oxidative capacity until the loss of muscle fiber area reaches threshold to stimulate a hypertrophic response. It is then this hypertrophic response that induces, or at least augments, angiogenic changes within skeletal muscle leading to increased NCAF and Cap/Fib ratio but this change in muscle fiber area prevents significant changes in capillary density.

Future Directions

Research has shown that active aerobic exercise can also increase capillary density and oxidative capacity within skeletal muscle. One future avenue of research might be to determine the relative effect of superimposing active aerobic exercise training on low and high intrinsic aerobic capacity phenotypes to assess the impact of active exercise on each phenotype's adaptive responses to occlusive ischemia. The results may shed light on how much of the adaptive response is intrinsically determined and whether or not the response is alterable. Another direction might be to explore the interaction between multiple environmental stresses, such as both active such as insulin

resistance and occlusive ischemia, to determine the extent to which the phenotypic differences are altered similarly by multiple forms of tissue stress.

There are numerous global ideas for future directions but based upon the current data, it appears there are more unanswered questions associated with the metabolic responses compared to the vascular responses between the phenotypes. Due to this circumstance, more focused research on the intrinsic differences as well as the adaptive metabolic responses to ischemia may better explain the changes in palmitate oxidation and citrate synthase activity observed between the phenotypes.

This study demonstrates that intrinsic aerobic exercise capacity influences the response to ischemic stress. The findings with palmitate oxidative capacity and citrate synthase activity demonstrate the complexity of skeletal muscle metabolism. Citrate synthase, an enzyme in the TCA cycle, has been studied routinely to assess oxidative capacity and mitochondrial volume. The present studies demonstrate that changes in citrate synthase activity do not correlate with changes in palmitate oxidation, at least in post-ischemic skeletal muscle. There are other possible regulators, in addition to citrate synthase, that may explain changes in lipid oxidation, and there are additional enzymes that may expand our knowledge of changes in glucose utilization. Regulation of lipid oxidation appears to be regulated by peroxisome proliferation activated receptors (PPAR) (68), in particular, PPARα and PPAR gamma coactivator-1 alpha (PGC-1α). Both of these factors are potential candidates for critical regulators of skeletal muscle lipid metabolism, due to their influence to induce transcription of pyruvate dehydrogenase kinase-4 (PDK4), carnitine palmitoyltransferase-1 (CPT-1), and uncoupling protein 3 (UCP3)(27; 56; 68). These three enzymes are associated with increased fatty acid beta

oxidation (56; 68). With respect to ischemia, when tissue should decrease reliance on fatty acid oxidation, it is predicted that the tissues would decrease PPARα and PGC-1α influences. However, in the kidney, another tissue that relies primarily on fatty acid oxidation for energy, pretreatment with PPARα and PGC-1α restored expression and activity of fatty acid oxidation enzymes, which accounted for protective affects against acute renal failure (78). These data support the suggestion that ischemia should reduce expression of PPARα and PGC-1α (78) but also indicates that maintenance or supplemental therapy with these ligands can potentially provide protection against ischemic injury. In addition, PGC-1α has emerged as a critical mediator in other signaling pathways including those driving mitochondrial biogenesis, angiogenesis, and fiber composition (3). It appears that PGC-1 may have a more vital role in muscle adaption to aerobic exercise training and pathological conditions such as cachexia, dystrophy, and PAOD (3).

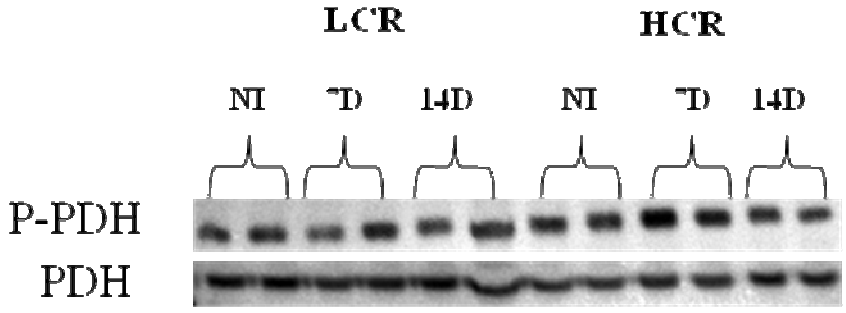
PDK4 has been shown to play a critical role in the partitioning of substrates toward lipid or carbohydrate oxidation (76). This enzyme performs this regulatory role with its ability to phosphorylate pyruvate dehydrogenase (PDH), the complex enzyme that catalyzes the conversion of pyruvate to acetyl-CoA(76). Upon PDH phosphorylation, this enzyme is less active and glucose oxidation decreases within the tissue and an increased reliance on fatty acid oxidation. Therefore, an increase in PDK4 would be associated with an increase in fatty acid oxidation and less reliance on glucose oxidation (76). In relation to ischemia, it would be suggested that this enzyme in addition to PPARα and PGC-1α should decrease to suppress the reliance on fatty acid oxidation.

PDH is the final step that commits nutrient carbons to enter the tricarboxylic acid (TCA) cycle or fatty acid synthesis (16). This enzyme is carefully regulated through phosphorylation and dephosphorylation (27; 59; 76). With phosphorylation (PPDH), PDH activity decreases, causing decreased pyruvate conversion to acetyl-CoA. Within the muscle, P-PDH is associated with a substrate preference for fatty acid utilization (27; 59; 76). This enzyme has primarily been studied in relation to high fat diets and obesity. Little is understood in skeletal muscle about the changes in this enzyme with ischemia. In the heart, myocardial substrate metabolism during ischemia is dependent upon the severity of ischemia. A very severe reduction of blood flow can decrease substrate flux through PDH. When perfusion is only partially reduced, or the ischemia is less severe, there is an increase in the rate of glycolysis and lactate uptake and a corresponding decrease in fatty acid oxidation (16). It has been demonstrated that keeping PDH activity high and reducing the pyruvate conversion to lactate during ischemia is beneficial for cardiac cells as it reduces the acidification of the intracellular compartment (16). As preliminary data for future studies, and to determine other potential stimuli for citrate synthase activity, we were interested in exploring whether changes in PDH would identify whether ischemic skeletal muscle was shifting substrate preference towards increased glucose oxidation, and differed by phenotype. P-PDH and PDH protein expression were compared by western blot in control, 7D and 14D ischemic gastrocnemius muscle from LCR and HCR animals (Figure 5.1). However, these data did not demonstrate a significant difference in PDH activity between phenotypes nor between ischemic time points. These data suggest that there is not a difference between the phenotypes in their ability to regulate PDH enzyme activity and thus substrate

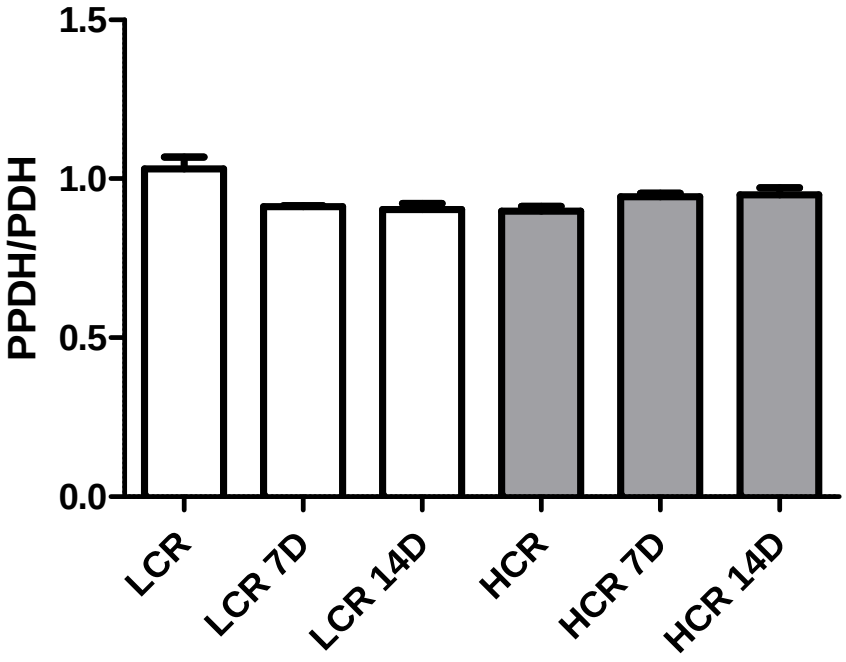
preference. However, there is a potential trend that the LCR animals, under control conditions, may rely more on lipid oxidation than the HCR, as evidence by an increased P-PDH/PDH. This data does suggest that more studies are necessary to more completely determine the role phenotypic determinants in other critical oxidative enzymes and whether there is a difference in substrate preference. Ultimately, once more complete characterization is completed, systematic analysis of the specific genes that might explain the phenotypic responses to ischemia might be worthwhile, but more likely, studies such as the present serve to highlight the highly integrated responses to complex stressors, and that phenotypic profiling might be a more reliable means by which to capture the overall characteristics of these multifactoral responses.

Figure 5.1. Comparison of P-PDH and PDH protein expression in LCR and HCR control, 7D and 14D ischemic gastrocnemius muscle. A. Representative blots of PPHD and PDH. B. P-PDH/PDH ratio.

A.


LCR
HCR
NI
7D
14D
NI
7D
14D
P-PDH
PDH

B.


PPDH/PDH
1.5
1.0
0.5
0.0
LCR
LCR 7D
LCR 14D
HCR
HCR 7D
HCR 14D

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APPENDIX A: RESEARCH STATEMENTS

Responsible Conduct of Research

The ECU Research Ethics Oversight Committee oversees all research compliance activities of the University under the direction of the Vice Chancellor for Research, Economic Development, and Community Engagement. The committee reviews, approves, and resolves conflicts of interest issues and reviews and investigates cases of scientific misconduct for individual faculty and the University. A copy of policy regarding scientific ethics and misconduct (Part VII section VI of the ECU Faculty Manual) is given to each faculty member upon employment. This policy is updated regularly on the ECU web site. The University's "Policy and Procedures on ethics in Research and Creative Activity" constitutes institutional efforts to promote responsibility by faculty, staff, post doctoral students, and students: "to seek honestly and to promulgate ethically the truth in all phases of work." The Policy provides, among other things, a list of principles to which the University subscribes in its research activities, and procedures for reporting, investigating, and determining penalties for unethical research activities (Part 7 (VI)).

The Bioethics Center, a joint program of BSOM and PCMH, provides educational opportunities for faculty, students, physicians, and hospital staff and supports the efforts of hospital committees charged to address ethical issues, including those dealing with research. The center sponsors bioethics conferences, workshops, and lectures and is staffed by faculty of the Department of Medical Humanities. The University's "Principles and Policy for the Protection of Human Subjects of Research" establishes "responsible for protecting the rights and welfare of individuals who act as subjects for research conducted by its {principle investigators]." The Policy provides a Statement of Ethical

Principles and implements these Principles through the University Medical Center Institutional Review Board (Part 7(IV)).

The University and Medical Center Institutional Review Board (UMCIRB) reviews and approves of any research, including clinical trials, involving the use of human subjects. The board protects the rights and welfare of human subjects engaged in research at BSOM, PCMH and its affiliates, and ECU, and in research conducted elsewhere by representatives of the University in connection with their responsibilities.

The University's Policy on: "Animal Care and Use in Research and Instruction" becomes another instance of institutional efforts to ensure that "animals used in research and teaching will receive humane treatment at all times," and to comply fully with applicable federal laws and regulations. The Policy provides specific responsibilities required by faculty members who conduct or supervise the conduct of animal experimentation. The Policy, moreover, establishes the Animal Care and Use Committee with the authority formally to monitor the care and use of vertebrate animals (Part 7(V)).

Statement on the Care and Use of Animals in Research

All animal research at East Carolina University meets the requirements and guidelines of the NIH Officer of Animal Care and Use. The animal facilities at East Carolina University are managed under the direction of the Department of Comparative Medicine and are AALAC and IACUC accredited.

APPENDIX B: ANIMAL CARE AND USE COMMITTEE PROTOCOL APPROVAL

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

February 8, 2008

Robert Lust, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Lust:

Your Animal Use Protocol entitled, "Cardiovascular Consequences of Forced Artificial Selection Based on Aerobic Running Capacity," (AUP #Q250) was reviewed by this institution's Animal Care and Use Committee on 2/8/08. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure