Central and Peripheral Adaptations to Exercise in Cardiovascular Disease

by

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Heart failure (HF), directly affected by hypertension, is a leading cause of mortality in Canada, and manifests with myocardial dysfunction. Current drug therapies have merit, however their efficacy is limited as evident by the 50% five-year survival rate for HF. Therefore, non-pharmacological treatments are required to manage hypertension and prevent HF. This thesis is an investigation into the molecular effects of exercise training in models of HF.

Endurance training (ET) has long been known to exert hypotensive effects and improve the quality of life of individuals afflicted with HF. Recently, high intensity interval training (HIIT) has been touted as a time efficient alternative in this patient population. However, clinical evidence on the safety and efficacy of HIIT in patients with compromised cardiac function remains scarce.

This thesis demonstrated in hypertensive rats, ET increased mitochondrial content (~25%; P<0.05), shifted fibre type to a slow/oxidative phenotype (increased IIA, decreased IIX and IIB) and increased capillarization and endothelial nitric oxide synthase (eNOS) content. In contrast, HIIT did not uniformly increase mitochondrial content, was not as robust at inducing fast-to-slow fibre type transitions, and reduced capillarization and eNOS protein with increased hypoxia inducible factor 1 alpha (HIF1α).
Within the heart, ET decreased fibrosis by ~40%, promoted a 20% increase in the left ventricular capillary/fibre ratio, an increase in eNOS protein, and a decrease in HIF1α. HIIT did not decrease fibrosis, increased left ventricular mass by 20%, and increased brain natriuretic peptide 50% in the absence of concomitant angiogenesis. Altogether, the skeletal and cardiac muscle data indicated ET and HIIT had divergent effects in hypertensive rats.

In addition to hypertension, congenital heart defects represent a cause of HF. Therefore, individuals with complex adult congenital heart disease embarked on 6 months of ET. All subjects increased their exercise capacity and tolerance supporting the use of ET in humans with complex cardiovascular disease.

In summary, while the current thesis illustrates the beneficial effects of ET on skeletal and cardiac muscles, as well as in complex patients, there remains a critical need for additional research on the effects of HIIT in settings of compromised cardiovascular function.
This thesis is based on the following publications:


I would like to acknowledge the work completed by these collaborators:

**Darin Bloemberg**: Produced the slides for immunofluorescent and histochemical analysis, performed the skeletal muscle MHC and SDH analyses.

**Mayne da Silva**: Treadmill exercised the animals.
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<td>Angiotensin converting enzyme</td>
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<td>Left ventricular hypertrophy</td>
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CHAPTER ONE
REVIEW OF THE LITERATURE
1.1 INTRODUCTION

The Greek philosopher Plato stated that “Lack of activity destroys the good condition of every human being, while movement and methodical physical exercise save it and preserve it”, illustrating that the irrefutable importance of exercise has long been understood. The ability of the respiratory, cardiovascular, and skeletal muscle systems to respond to the increased demand for oxygen uptake during exercise in order to produce adenosine triphosphate (ATP) represents arguably the most important indicator of mortality and morbidity in the population. Men and women alike with moderate to high levels of exercise capacity benefit from reduced risk of all-cause mortality (24, 25, 128, 171, 211, 217, 283). Based on substantial data, health organizations across the planet have recommended increased physical activity and exercise training to improve overall health and for the treatment and prevention of disease (22, 102, 136, 205, 297, 330). This chapter will focus on the recent and important historical literature that has expanded our understanding of the diverse effects of exercise, with a particular emphasis on skeletal and cardiac muscle.

1.2 EPIDEMIOLOGICAL REVIEW OF THE EFFECTS OF CARDIORESPIRATORY FITNESS AND EXERCISE TRAINING IN HEALTH AND DISEASE

1.2.1 Effects of Cardiorespiratory Fitness and Exercise Training in Health

Epidemiologic data demonstrates an inverse association between cardiorespiratory fitness (CRF) level and all-cause and cardiovascular (CV) mortality and morbidity. The protective effect of exercise training on all-cause mortality is independent of age, obesity status, smoking status, and alcohol intake (179). Perhaps the most convincing evidence of
the importance of CRF comes from the Aerobics Centre Longitudinal Study (n=25,341 men and 7,080 women) where the men and women with the highest exercise capacity had a 45% and 53% lower risk for all-cause mortality and a 47% and 70% lower risk for of cardiovascular disease (CVD) mortality (25). In the Lipid Research Clinics Prevalence study (n=2,994), Mora and colleagues observed that low levels of CRF (defined as ≤ median value) were associated with almost a 2-fold increase in CV mortality risk in asymptomatic women after 20 years of follow-up (211). A meta-analysis (170) observed that a 1 MET (1 metabolic equivalent = 3.5 mL O₂ uptake /kg/min) increase in CRF was associated with 13% and 15% reductions in all-cause and CVD mortality, respectively, indicating the importance of maintaining or improving CRF through exercise training in both health and disease.

1.2.2 Effects of Cardiorespiratory Fitness and Exercise Training in Disease

The beneficial effect of exercise training in the presence of disease has been well documented (1, 45, 92, 112, 182). This chapter, however, will focus on chronic heart failure (HF) and a leading precursor for the development of HF, hypertension.

1.2.2.1 Hypertension

The main effect of exercise training on hypertension is a reduction in systolic and diastolic blood pressure. A recent meta-analysis reported a blood pressure (BP) reduction in healthy individuals of 2.1 mmHg for systolic BP (SBP) and 1.7 mmHg in diastolic BP (DBP), along with reduction of 8.3 mmHg in SBP and 5.2 mmHg in DBP in hypertensive individuals (62). The Osaka Gas Company questionnaire showed that low-intensity walking had profound effects on the susceptibility for hypertension, and concluded that every 10 minute increase in daily walking decreased the risk of hypertension 12% (137).
Evenson and colleagues (88) demonstrated that higher levels of fitness were associated with a lower risk of all-cause and CVD mortality among both hypertensive and normotensive women and men. Reductions in BP may also reduce the prevalence and severity of left ventricular hypertrophy (LVH) caused by long-term hypertension. The development of hypertrophy is discussed in further detail in Section 1.4.2.1. Briefly, hypertension leads to a pathological hypertrophic response in the left ventricle (LV), and this eventually leads to the inability of the heart to function appropriately. LVH is a leading cause of HF and significantly elevates the risk of sudden cardiac death.

1.2.2.2 Heart Failure

Exercise was introduced as a feasible clinical intervention in HF patients in the early 1990’s, in contrast to the long standing advice given by physicians to HF patients to reduce their physical activity (52). It was thought that exercise-induced symptoms in HF and hemodynamic overload could be avoided with reduced levels of exertion. While Coats and colleagues demonstrated no correlation between left ventricular ejection fraction (LVEF) and exercise capacity, a clear relationship between skeletal muscle mass and exercise capacity in HF patients exists (49). The “muscle hypothesis of chronic heart failure” (52), drew attention to peripheral factors amenable to exercise training interventions (51) such as, impaired endothelium-dependent vasodilation, and the profoundly negative structural, metabolic, and functional alterations in skeletal muscle as a result of HF (5, 58, 247, 332). Since then, exercise training has been shown to be effective at reducing symptoms of exercise intolerance (e.g., muscle fatigue, dyspnea) (53), improving quality of life (15), enhancing endothelium-dependent vasodilation (132) de novo angiogenesis (286), and increasing oxidative capacity of skeletal muscle (246) in
Reduced HF-related mortality as a result of exercise training has yet to be demonstrated in a randomized control trial (RCT), however, the largest RCT that included exercise training demonstrated only a ~60% adherence rate to the training prescription (93). However, a meta-analysis (246) has demonstrated a reduction in mortality as a result of exercise training through structured cardiac rehabilitation programs in HF patients.

Altogether, there is overwhelming evidence for the beneficial effects of exercise training, however the mechanisms responsible for these responses remain only partially elucidated, particularly in the context of disease. Nevertheless, adaptations within peripheral tissues (i.e., skeletal muscle) and the LV likely contribute to these responses. The following sections will outline the metabolic and structural adaptations, with a particular emphasis on the molecular responses that occur as a result of exercise training in these tissues.

1.3 Metabolic and Structural Adaptations to Skeletal Muscle as a Result of Exercise Training

Skeletal muscle exhibits superb plasticity in response to changes in functional demands. Chronic endurance exercise leads to a variety of physiological and biochemical adaptations in skeletal muscle, including mitochondrial biogenesis, angiogenesis, and fiber type transformation. These adaptive changes are the basis for the improvement of physical performance and other health benefits.
1.3.1 Major Pathways for the Generation of ATP in Skeletal Muscle

ATP is the currency for energy provision in skeletal muscle. While at rest, the skeletal muscle requires very little ATP provision, however during strenuous exercise the energy expenditure may increase 100 fold over resting values (121, 149). During exercise, the majority of ATP is produced aerobically in the mitochondria through oxidative phosphorylation. During transitions from rest to exercise, or from a lower to a higher power output, and power outputs requiring rapid energy production, ATP can also be produced via substrate phosphorylation occurring mainly in the cytoplasm. The breakdown of phosphocreatine and glycolytic pathways are the most notable. While under most conditions, the majority of daily energy provision comes from the oxidative system (32), aerobic and anaerobic pathways work together to maintain ATP homeostasis.

Skeletal muscle is equipped to maintain homeostatic levels of ATP through the metabolic breakdown of fuel sources, namely carbohydrate (CHO) and free fatty acids (FFA). Under resting conditions both CHO and FFA are oxidized to provide the energy required for basal metabolic processes in skeletal muscle, and there is a reciprocal relationship between the utilization of CHO and FFA. There are two main sources of CHO and FFA available to skeletal muscle, one is endogenous, skeletal muscle glycogen and triacylglycerol (TAG), while the exogenous sources (liver glycogen and adipose tissue) must be transported to and into the muscle cell. During exercise, the predominate fuel source depends largely on the intensity of the exercise stimulus, such that at lower intensities fat metabolism dominates, with the maximum rate of fatty acid oxidation occurring at ~65% of peak oxygen uptake, and CHO dominates thereafter (Figure 1.1;
However, once a ‘steady state’ at a given aerobic exercise intensity is reached, there can be reciprocal shifts in the proportion of CHO and FFA that are oxidized (147). The interaction between CHO and FFA oxidation is dependent on the intracellular and extracellular metabolic environments (310).

Figure 1.1. Contribution of carbohydrate and fat at various exercise intensities. Increasing absolute and relative contribution from carbohydrate stores with increasing exercise intensity. Increasing absolute and decreasing relative contribution from fat stores until ~65%Wmax, after which absolute contribution from fat decreases (333).
1.3.2 Regulation of Metabolism and Fuel Interactions

There are two main pathways responsible for maintaining ATP homeostasis during exercise:

1. Aerobic respiration (nicotinamide adenine dinucleotide (NADH) + O₂ + ADP)
2. Anaerobic ATP provision (substrate phosphorylation; glycolysis & high energy phosphate system (PCr))

Each system plays a role, dependent mainly on exercise intensity, and the ATP requirements are met through a balance of both.

Aerobic respiration occurs in the mitochondria where the electron transport chain harnesses electrochemical energy to produce ATP + H₂O from NADH + O₂ + ADP. During ATP generation, CHO and FFA converge at the mitochondria as acetyl-CoA, and there are several regulatory rate-limiting processes that are externally regulated that dictate whether the acetyl-CoA is derived primarily from the breakdown of CHO or FFA (Figure 1.2). Although the regulation of metabolism has many components, Ca²⁺ activation of phosphorylase (PHOS) and pyruvate dehydrogenase (PDH) represents a strong activating signal, while the concentration of free ADP (ADPᵢ) and free adenosine monophosphate (AMPᵢ) in the cell act as potent feedback regulators responsible for activating rate-limiting enzymes in glycolysis. Specifically, increases in ADPᵢ and AMPᵢ will allosterically interact with PHOSa to increase the sensitivity to substrates, attenuate ATP inhibition of phosphofructokinase (PFK) and decrease the rate of phosphorylation of pyruvate dehydrogenase (PDH), all of which increase enzymatic flux rates and the facilitation of acetyl accumulation within the mitochondria.
Figure 1.2. Major points of regulation of rate limiting enzymes in glycolysis.

Increases in the level of Ca\textsuperscript{2+} and ADP\textsubscript{f} are proportional to the exercise intensity (150), such that at higher levels of intensity the breakdown of CHO provides the majority of the substrate for ATP generation in the mitochondria. Training-induced increases in fatty acid oxidation at an absolute power output are largely attributed to a reduction in ADP\textsubscript{f} as a result of the induction in mitochondrial biogenesis.

Important points of regulation of exogenous fat metabolism include the transport of proteins into the skeletal muscle, and the eventual transport into the mitochondria by
the carnitine shuttle. The abundance of transporters on the plasma membrane influences the rate of FFA entering the cell and the rate-limiting enzyme responsible for the transport of proteins into the mitochondria is carnitine palmitoyltransferase-1 (CPT-1). An increase in fatty acid transport into mitochondria ultimately increases nicotinamide adenine dinucleotide (NADH) and acetyl-CoA content within the matrix, resulting in a reduction in PDH flux and a shift towards fatty acid oxidation at rest and low power outputs. In contrast, at higher power outputs glycolytic flux is high and there are several factors that reduce the reliance on fat metabolism (for review, see (310)):

- decreased delivery of FFAs, because of a reduction in the release from adipose tissue at higher intensities of exercise, most likely due to reduced adipose tissue blood flow;
- reduced IMTG hydrolysis due to AMPK phosphorylation of hormone sensitive lipase (HSL) that inhibits the phosphorylation (activation) by epinephrine and Ca\(^{2+}\);
- impairment in CPT-1 flux, due to depletion of free carnitine because of the acetylcarnitine formation that results from an increased flux through PDH;
- reductions in CPT I activity at higher exercise intensities due to decreases in pH from 7 to 6.8.

Ultimately, the main bioenergetics systems provide the ATP necessary during exertion through an elegant balance, allowing each to provide energy for activities that are best suited to their metabolic properties.
1.3.3 Skeletal Muscle Metabolic Adaptations to Exercise Training

Research in the late 1960’s and early 1970’s demonstrated the remarkable plasticity of rat (144, 146) and human (140, 279) skeletal muscle and the ability of exercise training to stimulate increases in mitochondrial volume per unit muscle mass. Despite this knowledge, it was not until the late 1990’s that research shed light on the molecular signals responsible for the proliferation of mitochondria in response to exercise training. A major breakthrough in the understanding of how transcription factors control mitochondrial biogenesis was the discovery of the nuclear encoded transcriptional co-activator, peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) (256). Since this seminal report a family of transcriptional co-activators (PGC-1α, PCG-1β and PPAR related co-factor (PRC)) and a number of transcription factors, such as nuclear respiratory factors (NRFs), PPARs and estrogen related receptors (ERRs), have been identified to mediate exercise-induced adaptations. It is now widely recognized the mRNA and protein expressions of these factors are dynamically regulated in response to a variety of signaling pathways (e.g., AMP activated protein kinase (AMPK), calcium/calmodulin kinase (CaMK)) involved in cellular growth, differentiation and metabolism (221, 287).

The DNA within the mitochondria (mtDNA) transcribes 22 transfer RNA, 2 ribosomal RNA and 13 subunits of the electron transport chain. The remaining mitochondrial proteome (~1600 proteins) is transcribed within the nucleus, translated within the cytosol and transported into the mitochondria. Therefore, in order for mitochondria to proliferate, a coordinated signaling cascade must occur, as both mitochondrial and nuclear genomes must be activated proportionately, as complexes I, II,
IV and V of the electron transport chain are comprised of both nuclear and mitochondrial encoded proteins. The expression of the mitochondrial transcription factor A (Tfam), a nuclear encoded protein, is essential in this coordination (337) as Tfam binds to the D-loop of mtDNA (319) and regulates mitochondrial transcription (298), mtDNA stabilization and replication (163).

In response to a single bout of exercise the molecular mechanisms responsible for mitochondrial biogenesis are activated (214, 241, 248). This process appears to be regulated by CaMK activated signaling and p-38 mitogen activated protein kinase (p38 MAPK) (355) and by changes in the energy status of the cell (increases in AMP) through the activation of AMPK (159). The resultant activation of PGC-1α promotes its translocation to the nucleus (188, 355) and the mitochondrial matrix (278, 309) where it interacts with Tfam to regulate both the expression of nuclear and mitochondrial encoded genomes. Therefore, PGC-1α activation appears to be sufficient to coordinate nuclear and mitochondrial genomes to promote exercise-induced mitochondrial biogenesis. The finding that forced overexpression of PGC-1α in resting rat skeletal muscle results in increased mitochondrial content further supports this (17). However, PGC-1α is not required for these events, as genetic ablation of PGC-1α does not prevent exercise-induced mitochondrial biogenesis. This is likely because of compensatory activation of PGC-1β as complete ablation of both PGC-1 isoforms prevents mitochondrial exercise responses. Therefore, chronic exercise is thought to induce mitochondrial biogenesis through repeated transient increases in mRNAs, a process regulated by PGC-1 (241). The induction of mitochondrial biogenesis enables oxidative phosphorylation to occur in the presence of reduced ADP concentrations at submaximal intensities, allowing for a greater
reliance on FFA breakdown to contribute ATP generation at a given power output. The allosteric activation of PHOS, PFK and PDH is therefore attenuated post-training due to the lower cytosolic levels of ADP$_f$ and in conjunction with the greater availability and transport of FFA post-training, allows for a greater reliance on FFA and a decrease flux through glycolysis. The relationship between lower ADP$_f$ levels required to drive mitochondrial respiration to the same extent post-training is depicted in Figure 1.3.

![Figure 1.3. The effect of mitochondrial proliferation on the regulation of mitochondrial respiration rates. ADP, adenosine diphosphate; ATP, adenosine triphosphate; NADH, nicotinamide adenine dinucleotide (reduced), P$_i$, inorganic phosphate. (148).](image)

1.3.4 Structural Effects of Exercise Training on Skeletal Muscle

1.3.4.1 Fibre Type Composition

In addition to training-induced adaptations in ATP formation, chronic exercise has the ability to alter maximal rates of ATP hydrolysis through alterations in myosin heavy chain (MHC) isoform expression. This coordination ensures that ATP demand and utilization are optimal after training. Mammalian skeletal muscle contains a continuum of
different fibre types that demonstrate diverse structural properties and functional capabilities. Traditionally, muscle fibres are classified by myofibrillar actomyosin ATPase histochemistry analyses of myosin heavy chain (MHC) isoforms. Based on the expression of the predominant MHC isoforms, rodent muscle is comprised of type I, IIA, IIX, and IIB fibres (324), while humans have three fibre types (I, IIA, and IIX) (86). Hybrid fibres may also be present in mammalian skeletal muscle (type I/IIA, IIA/IIX, IIXB) (26). Technically, fibre typing a muscle only specifies the MHC composition, which directly influences the maximal shortening velocity. However, shortening velocity is largely correlated with rates of ATP hydrolysis, and therefore fibre type can influence the overall metabolic phenotype of a muscle. Nevertheless, both chronic low-frequency stimulation and cross-innervation studies suggest that alterations in energy flux drive metabolic adaptations (as described above) in concert with changes in muscle fibre type composition.

It is well characterized that human type I slow-twitch, oxidative fibres are slow in force generation (354) and have an oxidative phenotype (high oxidative enzyme expression, mitochondrial content, and capillary density) (254). Type IIA fast-twitch, oxidative fibres are fast in force generation but have similar oxidative profiles to the type I fibres. Type IIX fibres are fast-twitch with a glycolytic metabolic profile (high glycolytic enzyme expression, lower mitochondrial content and capillary density (254). Type IIB fibres are an even more fast-twitch, glycolytic phenotype than type IIX fibres (270, 271, 324). Endurance exercise promotes adaptations in skeletal muscle that shift fibres toward a more oxidative phenotype expression. Specifically, endurance exercise promotes fibre type transformation along a spectrum from type IIB/IIX to IIA (91),
however there is very little evidence that type II fibres become type I following training in rodents.

The coordinated increase in the expression of metabolic and fibre type genes suggest similar signals and/or transcription control. Indeed, as with mitochondrial biogenesis, PGC-1α appears to mediate these responses, as skeletal muscle-specific PGC-1α overexpression leads to an increased percentage of slow-twitch fibres (185) and improves exercise capacity in rodents (40). However, recent evidence, based on combined PGC-1α/β deficient skeletal muscle, indicates that the PGC-1 co-activators are not necessary for fibre type determination (356, 361), such that PGC-1α may influence the maintenance of slow-twitch, type I fibres, but is not required for exercise-induced fibre type transformation. In addition, recent evidence demonstrates the relationship between PGC-1α content and fibre type differs between species (mouse, rat and human), and the highest PGC-1α content does not uniformly correspond to type I fibres (123).

The signals influencing the transcriptional control of fibre type appear to involve Ca\(^{2+}\). Elevated cytosolic Ca\(^{2+}\) activates the Ca\(^{2+}\)/calmodulin-dependent serine/threonine protein phosphatase, calcineurin (CnA) and CaMK pathway. CaMK contributes to exercise-induced genetic reprogramming in adult skeletal muscle. Several studies demonstrate that CnA and CaMK both stimulate the activities of transcriptional factors, myocyte enhancer factor 2 (MEF2) and nuclear factor of activated T-cells (NFAT) (200, 208, 357). The mechanism by which CaMK activates MEF2 works through class II histone deacetylases (HDAC4, HDAC5, and HDAC9). CaMK interacts with HDACs to inhibit MEF2, resulting in repression of the target genes by preventing the formation of MEF2-HDAC complexes, and induces nuclear export of HDAC4 and HDAC5 through
phosphorylation. Deletion of the *Hdac5* and *Hdac9* genes increases type I and IIA fibres in soleus and plantaris muscles, whereas muscle-specific expression of a non-phosphorylatable HDAC prevents exercise-induced fibre type transformations (253). Deletion of the *Mef2c* and *Mef2d* genes resulted in reduced percentage of type I fibres in soleus muscle, and skeletal muscle-specific overexpression of a constitutively active MEF2C chimera protein led to increased type I fibres and oxidative phenotype (253). These findings support the view that CaMK activates fibre type transformation through MEF2 due to inhibition of HDACs, however, CaMK is not required for exercise-induced fibre type transformation (2). The activation of CnA also promotes the expression of slow-twitch muscle, through dephosphorylation and activation of the NFAT (48). While both CaMK and CnA are activated by Ca\(^{2+}\), in contrast to CaMK, CnA appears to be required in fibre type adaptations with exercise, as deletion of the *CnAα* or *CnAβ* genes or pharmacological (e.g, by cyclosporine A) inhibition of the CnA-NFAT pathway results in a reduction of type I fibres within skeletal muscles (200, 208, 220, 229).

In addition to Ca\(^{2+}\) mediated signaling, metabolic cues sensed by AMPK, also play a functional role in skeletal muscle fibre type transformations. Muscle-specific expression of a dominant-negative form of AMPK\(\alpha2\) blocked voluntary running-induced IIB-IID/X/IIA fibre type transformation (272). Conversely, muscle-specific expression of an active mutant of AMPK\(\gamma1\) led to a marked increase in type IIA/X fibres in triceps muscle (272), indicating that AMPK is functionally important for exercise-induced fibre type transformation. However, these genetic models have an impaired exercise tolerance as a result of AMPK activity ablation, which dictates that these animals exercise at a much lower exercise intensity. Therefore, it remains unclear if AMPK has a direct role in...
fibre type changes, or if the observed impairment in fibre type changes in these genetic models of compromised AMPK signaling, result from attenuated Ca$^{2+}$ signaling as a result of the lower power output.

1.3.4.2 Angiogenesis

Training induced adaptations result in a coordinated increase in the oxidative capacity and fatigue resistance within skeletal muscle. No less important to these responses is an increased ability to perfuse active skeletal muscle with oxygen rich blood containing carbohydrates and fatty acids, a process influenced by the muscle capillary density. Some of the earliest work investigating the adaptive responses of skeletal muscle to exercise training demonstrated an increase in the capillary density of the trained muscle (27, 42). The formation of new capillaries from pre-existing capillaries, or angiogenesis, is effective at improving exchange between blood and tissue. The resultant increase in capillary beds allows for a more coordinated metabolic response to exercise, such that blood flow is more closely matched to tissues with increased metabolic demand. The enhancement of the skeletal muscle capillary network allows for i) increased surface area for diffusion, ii) shortened diffusion distance within the muscle, and iii) increased length of time for diffusive exchange between blood and tissue (160). Collectively these changes promote improved O$_2$ and substrate delivery along with enhanced removal of carbon dioxide (CO$_2$) and metabolic intermediates.

Exercise-induced angiogenesis is mediated by the coordination of a combination of metabolic stress, growth factors, hypoxia and physical stimuli, such as shear and mechanical stresses. Vascular endothelial growth factor (VEGF) is a potent mitogen of endothelial cells (82) that has been implicated in the angiogenic response to exercise.
(225) under the regulation of PGC-1α. The mechanism by which PGC-1α stimulates VEGF transcription involves co-activation of the nuclear receptor ERRα (7). ATP turnover, as occurs with acute or chronic exercise stimuli, activates signaling molecules (e.g., p38 MAPK and AMPK) that promote angiogenesis through the ERRα-VEGF signaling cascade via the activation of PGC-1α (7). VEGF binds to two primary receptors on the endothelial cell: VEGFR1, a fms-like tyrosine kinase (Flt-1) receptor, and VEGFR2. VEGF-induced activation of VEGFR2 stimulates endothelial cell proliferation, migration, and differentiation in most cells (21, 115, 339, 358). Activation of VEGFR2 elicits a potent signaling cascade, which stimulates gene expression for a variety of genes, including endothelial nitric oxide synthase (eNOS) (299). In addition, VEGFR2 activation leads to nitric oxide (NO) production through eNOS via mobilization of intracellular Ca^{2+} stores (66). NO production is critical for VEGF signaling, since inhibiting NO production diminishes normal angiogenic responses to stimuli (155, 227). Up-regulation of VEGF mRNA occurs in rat muscle following contractions (3, 133) or with a single exercise bout (34) as well as during exercise in both health and disease (113, 129, 267). Increases in VEGF mRNA after exercise coincide with increases in VEGF protein (3, 225) and chronic training is associated with a reduced VEGF expression in skeletal muscle as adaptations progress (268).

Hypoxia also exerts its effect on angiogenesis primarily through an up-regulation of VEGF, and is one of the most potent angiogenic stimuli. The promoter region of VEGF contains an HRE (hypoxic-response element) (304) that binds the transcription factor HIF-1α (hypoxia-inducible factor 1α) under hypoxic conditions, resulting in increased VEGF transcription when P_{O2} is low (94). Lowering the P_{O2} of endothelial
cells, as occurs during conditions of repeated contractions or exercise, stimulates cell proliferation, whereas returning $P_{O_2}$ to a high value reduces VEGF expression and lessens endothelial cell activation (304). HIF-1α mRNA and protein are elevated during exercise in both rodents and humans (190, 196). Chronic exposure to hypoxia in humans decreases resting mRNA levels of VEGF and its receptors (223) and attenuates their increase during exercise (224). However, this effect does not eliminate the increase in capillary density with training (223). These data clearly demonstrate the complex relationship between muscle $P_{O_2}$ during exercise, the up-regulation of VEGF, and the development of angiogenesis and implicates stimuli other than low $P_{O_2}$ in exercise-related angiogenesis.

Physical stresses, such as elevated shear stress and mechanical stretch, can initiate signals that remodel vasculature through matrix metalloproteinases (MMPs). VEGF is elevated in muscle with treatments of hyperemia, overload, and contractions, however there are distinctions in the responses of the MMPs. Up-regulation of VEGF activates membrane type-1 (MT1)-MMP (102). MMP-2 activity and MT1-MMP mRNA were elevated by muscle overload but not with muscle hyperemia (81). The inhibition of MMP activity eliminates angiogenesis typically induced by muscle stimulation (38). Ultimately, evidence exists that physical stress induces angiogenesis, however, studies isolating the distinct stimuli experienced by the cell from shear or mechanical stress are difficult to construct.

In summary, a sophisticated signaling-transcription network within individual muscle fibres mediates exercise-induced skeletal muscle adaptation (Figure 1.4). While it is likely that parallel pathways exist (e.g., P38 activation through AMPK), this figure represents a simplified version of the present knowledge regarding regulation of exercise-
induced adaptations. Oxidative and metabolic stresses induced by exercise/contractile activity stimulate PGC-1α activity and expression, which in turn promotes mitochondrial biogenesis through interactions with transcription factors (NRF1, NRF2, and Tfam) on nuclear-encoded and mitochondria-encoded genes. Multiple regulatory factors sense Ca$^{2+}$ (CnA and CaMK) and metabolic stress (AMPK and PKD1) converging on transcriptional factors (NFAT and MEF2) and repressors (HDACs) in mediating endurance exercise-induced slow-twitch muscle gene expression and fibre type transformation. PGC-1α also promotes angiogenesis through an interaction with ERRα in activating the gene that encodes VEGF.

**Figure 1.4.** Simplified representation of the current understanding of the signaling and molecular mechanisms underlying endurance exercise-induced adaptations in skeletal muscle.

1.4 **Metabolic and Structural Effects of Exercise Training In Cardiac Muscle**

1.4.1 **Regulation of Fuel Usage in the Heart**

Per gram of tissue, the heart consumes more energy than any other organ of the body. It is estimated that the human heart consumes up to 5kg per day of ATP (316). To
do this, the heart requires an uninterrupted supply of a variety of substrates (fatty acids, glucose, lactate, amino acids and ketone bodies) delivered by the blood stream, and has thus been termed a “metabolic omnivore” (316). Heart muscle is a highly oxidative tissue that produces more than 90% of its energy from oxidative phosphorylation, demonstrated by the fact that mitochondria occupy ~30% of cardiomyocyte volume (317). It has been demonstrated that during maximal exercise, the heart uses more than 90% of its oxidative capacity, indicating a strict matching between supply and demand for ATP exists (210). Substrate selection for oxidation is influenced by availability and is generally governed by Randle’s “glucose-fatty acid cycle” (257). Substrate selection also occurs at a transcriptional, translational or post-translational level through the regulation of specific enzymes in metabolic pathways, much like in skeletal muscle (see Sections 1.3.2 & 1.3.3).

1.4.2 Cardiac Muscle Adaptations to Exercise Training

1.4.2.1 Metabolic Adaptations to Exercise Training in Cardiac Muscle

As previously discussed in detail, the metabolic effects of exercise training in skeletal muscle are well established, however, far less is known regarding the effect of exercise training on cardiac muscle metabolism. While there is some suggestion that exercise training can improve myocardial energy metabolism in states of disease (114, 341), direct evidence for the effects of exercise training on cardiac muscle are sparse. Nevertheless, the maximal capacity of the heart to pump blood is increased following training. This partially manifests as a result of increased blood volume, and therefore preload-induced increases in stroke volume, as well as cardiac hypertrophy and peak systolic pressure. As a result, there is reduction in heart rate at rest and during exercise.
Approximately 50% of the ATP consumed in a beating heart occurs during isovolumetric contraction, and is not directly linked to the pumping of blood. A reduction in heart rate therefore improves the overall metabolic efficiency of the heart. However, in contrast to skeletal muscle, in healthy hearts the cardiac muscle does not appreciably change following training, as hypertrophy is not associated with increased capillarization or mitochondrial content, suggesting metabolism is optimized before the commencement of a training regime.

However, in conditions of compromised cardiovascular performance, exercise training may alter the biochemical properties of the heart, as exercise training has been shown to improve the oxidative capacity and restore deficiencies in energy transfer in experimental HF (168). An increase in either left ventricular (LV) mass or mitochondrial content may explain the finding that regular exercise increases glycolysis and oxidative metabolism in the cardiac muscles of rodents with compromised cardiac performance. (56, 166, 313). However, an increased capacity for fatty acid utilization within the heart is not a uniform finding following exercise training (157, 323). Thus, whether the heart metabolically adapts to exercise training awaits further investigation.

1.4.2.2 Structural Effects of Exercise Training on Cardiac Muscle

Exercise-induced cardiac hypertrophy is a physiological adaptation, defined as concentric or eccentric, and is associated with normal cardiac structure and normal or improved cardiac function. Physiological cardiac enlargement in athletes has been reported since the late 1890s, and several aspects of athletes’ heart have been intensively investigated in humans (for review, see (255)) as well as in animals models (342).
Generally the athlete’s heart has been described as “the complex structural, functional, and electrical cardiac remodeling induced by long-term exercise training” (255).

Importantly, exercise training-induced cardiac hypertrophy includes a balanced increase of LV and left atrial diameters, cardiac mass, and LV wall thicknesses affected by myocyte hypertrophy and angiogenesis (84, 89, 235). Early increases in capillary density as a result of training has been demonstrated in many species, and as exercise training progresses, angiogenesis match increases in hypertrophy, such that exercise does not affect capillary density in healthy hearts.

1.4.3 Effects of Disease on Cardiac Muscle

HF is globally defined as the inability of the heart to pump blood. Historically, HF has been classified as systolic (inability of the heart to pump blood) or diastolic (inability of the heart to relax, and therefore fill). In the last few years, a consensus has evolved to replace the terms systolic and diastolic HF with HF with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF), respectively (73). However, there is still ongoing debate regarding whether HFrEF and HFpEF are different diseases (30) or the same disease at different stages (72). Therefore, for the purpose of this review the term “HF” will be used as a global inability of the heart to function as required to pump blood to the systemic circulation.

There are several causes of HF, including myocardial infarction, hypertension and congenital abnormalities. While many pathophysiological aspects of HF remain obscure, the following factors have been implicated in the cause and/or progression of HF (295):

- metabolic disturbance or neurohumoral dysfunction at the
level of the heart;
• systolic or diastolic dysfunction at the level of the left and right ventricles;
• peripheral abnormalities, such as endothelial dysfunction or progressive loss of myocytes;
• inability of the cardiac cells to produce sufficient energy (ATP);
• disruptions in excitation-contraction coupling or failure of the sarcomeric machinery;
• dysfunctional genes or gene expression.

Due to the multifactorial nature of HF, its pathophysiology has been described from many different viewpoints, however, this review will focus on the metabolic disturbances and structural changes that have been demonstrated in HF.

1.4.3.1 Metabolic Effects of Disease on Cardiac Muscle

A generalized metabolic myopathy has been described in relation to HF as there are profound effects on metabolism in both skeletal and cardiac muscle (334). The metabolic characterization of the failing heart includes (334):

• an early switch in substrate utilization from fatty acids to glucose,
• decreased oxidative capacity and energy production (reduced mitochondrial biogenesis),
• decreased energy transfer by the phosphotransfer kinases,
• altered energy utilization, and
• decreased efficiency of energy consumption.

In HF, the main myocardial substrate switches from FFA to glucose with a down-regulation of enzymes involved in fatty acid oxidation (258, 277). It has been suggested
that the switch between FFA and CHO aids in the efficiency of ATP production in the failing heart (318), however, the timing of the shift is not a consistent finding, and it is not always associated with improved function (103, 312, 335). However, down-regulation of PGC-1α and reduced oxidation of FFA precede contractile dysfunction, suggesting that mitochondrial dysfunction plays an important role in the pathogenesis of HF. Given the decrease in mitochondrial content associated with HF, exercise training represents a plausible lifestyle intervention, although the effects of exercise on mitochondrial content in the failing heart, or with hypertension, remains to be determined.

1.4.3.2 Structural Effects of Disease on Cardiac Muscle

Hypertension remains a leading cause of diastolic dysfunction (154), which independently increases the risk of developing overt HF (301), and is present in almost all patients with HF. Over time, increased afterload leads to diastolic dysfunction mainly through myocyte hypertrophy and interstitial fibrosis (20). Hypertrophy, at least initially, is a physiological adaptation to normalize wall stress (165). Pathological hypertrophy is associated with the loss of cardiomyocytes (apoptosis and necrosis), development of fibrosis, cardiac dysfunction and increased risk of HF and sudden death (130, 226, 346).

An important humoral system affecting the development of hypertrophy is the renin-angiotensin-aldosterone system (RAAS). Briefly, in the RAAS, angiotensinogen produced in the liver is converted into angiotensin I by renin produced in the kidneys. When circulating pressure is low, as in HF, the activity of renin will increase the production of angiotensin I. Angiotensin I is converted into angiotensin II by angiotensin-
converting enzyme (ACE) in endothelial cells in the lung. Angiotensin II effectively increases blood pressure by inducing peripheral vasoconstriction and stimulating aldosterone production. Angiotensin II and aldosterone have direct effects on the myocardium, whereby they induce hypertrophy and increase interstitial fibrosis (10). As a result, long-term activation of the RAAS has been implicated in inducing diastolic dysfunction and eventual HF.

An increase in fibrosis is a hallmark finding in diastolic dysfunction and HF. Fibroblasts are a unique cellular component of the heart that adapt to stress by proliferation. Stressors can include acute insults, such as myocardial infarctions, or longstanding hypertension and HF. Research on the molecular basis of fibroblast function is limited by the lack of a fibroblast specific marker, thereby making it difficult to study tissue specific changes in response to diseases such as HF. Compounds implicated in the formation of fibrosis in response to stress include histone deacytłases (HDACs) (100, 289), endothelin-1 (350), fibroblast growth stimulating factor-2 (FGF-2) (236), among others (for review, see (98)). Ultimately, however, cardiac remodeling is accomplished by the coordinated efforts of several cell types, including myocytes, fibroblasts, immune cells, and vascular cells, and is beyond the scope of the current thesis.

The interactions between morphological remodeling and metabolic interactions during the development of HF are incompletely understood, and therefore so are the effects of exercise on the LV of these individuals.
1.5 **Adult Congenital Heart Disease**

While not prevalent within the population, congenital abnormalities compromise cardiac performance and represent extreme HF. Congenital heart disease (CHD) is the most common birth defect, representing 1% of all congenital abnormalities present at birth (143). Patients affected by CHD represent a unique clinical population, whose life expectancy has changed dramatically in the last 50 years due to advancements in surgical and postoperative management. More than 90% of patients will reach adulthood and currently in North America there are more adults living with CHD than children. The most recent population estimates of adult CHD (ACHD) patients in Canada and the United States are 100,000 and 850,000 respectively (194, 343).

1.5.1 **Exercise Capacity in Adult Congenital Heart Disease**

A reduced exercise capacity in ACHD patients, with a broad range of cardiac conditions, including those who report being asymptomatic, is an increasingly common finding (Figure 1.5) (74, 76, 80, 81, 96, 262). As with many diseases, most commonly HF, there are various factors impacting exercise tolerance in ACHD (97).

- Hemodynamic factors (residual lesions/shunts)
- Pulmonary factors (post-operative sequelae/status after residual infections
- Locomotor factors (paresis, scoliosis),
- Low habitual level of physical activity/absence of exercise training.

Congenital patients represent one of the most extreme models of inactivity, and therefore present an ideal population to study exercise interventions.
1.5.2.1 Exercise Capacity in Common Cardiac Lesions

Atrial and Ventricular Septal Defects

An atrial septal defect (ASD) causes a right volume overload resulting in increased pulmonary pressures and in some cases results in pulmonary hypertension in large untreated ASDs. In patients with un-operated ASDs the increase in cardiac output (Q) during exercise along with the maximal achievable HR are lower than normal influencing exercise tolerance (239). In un-operated adult patients exercise capacity generally improves after undergoing percutaneous ASD closure (107).

A ventricular septal defect (VSD) represents communication between the left and right ventricles anywhere along the interventricular septum (13). They are often associated with LV volume overload and resultant LV dilatation. There is a higher
pulmonary to systemic flow ratio in patients with VSD, at both rest and during exercise, and this fraction decreases with increasing exercise intensity. Maximal oxygen uptake is reduced by ~10% when compared to aged matched controls (80), however lower than normal O$_2$ uptake is positively correlated with low levels of daily physical activity in these patients (265). Patients who underwent corrective surgery early in life (<1 year of age) demonstrate near normal exercise capacity despite abnormal exercise hemodynamics (203, 264).

*Valvular and Obstructive Anomalies*

Congenital aortic stenosis (AS) results from abnormal development of the aortic valve commissures and often is associated with a bicuspid aortic valve. The severity of AS is classified by the mean aortic gradient (≤20mmHg mild; 21-49mmHg moderate; ≥50mmHg severe). Patients with AS demonstrate reduced exercise capacity attributed to the inability of Q to increase adequately and are susceptible to sudden cardiac death (142).

Coarctation of the aorta (COA) occurs when a localized area of stenosis or a longer hypoplastic segment of the aorta is present (142, 158). Normal exercise capacity is observed in patients with “successful” repair for COA (125, 262), however in many cases an elevated peak blood pressure response to exercise is observed (125, 158, 178).

*Cyanotic Congenital Heart Disease*

Three of the more prominent forms of cyanotic CHD are tetralogy of Fallot (TOF), transposition of the great arteries (TGA), and Fontan circulation.
The anatomic features of TOF consists of:

- Pulmonary artery stenosis
- Ventricular septal defect
- Overriding aorta
- Right ventricular hypertrophy

Maximal oxygen uptake in patients with TOF is consistently lower than normal (~81%) (265, 275, 347). Both an inadequate ventilatory response to exercise along with chronotropic incompetence contribute to abnormal exercise capacity in TOF patients (275).

In TGA the right ventricle (RV) gives rise to the aorta, and the LV gives rise to the pulmonary artery. The majority of adults currently living with TGA have undergone a Senning or Mustard procedure to link the separate circulations that exist in this defect. These interventions insert a baffle in the atria to redirect systemic venous blood to the LV and pulmonary artery and arterial blood to the RV thus rendering the RV as the systemic ventricle. By adulthood, many TGA patients will have; diastolic dysfunction due to the limitation the baffle puts on ventricular filling; right ventricular dysfunction due to hypertrophy; impaired chronotropic response; and atrial arrhythmias, all of which contribute to diminished exercise capacity (231).

A number of CHD conditions include a single functioning ventricle, and survival depends on creating a communication between the caval veins and the pulmonary artery, thus bypassing the RV (Fontan procedure). Patients who have undergone a Fontan procedure have a significantly reduced exercise capacity (105, 134, 263, 265), roughly ~37% of normal controls (79). Maximal oxygen uptake is generally in the range of 15-
35mL/kg/min due to the reduced Q and HR response, lower oxygen saturation caused by a right-to-left due to shunting and an abnormal ventilatory response to exercise.

1.5.2 Exercise Training and Adult Congenital Heart Disease

Although clinical exercise testing in ACHD is commonplace, the results are often used to predict morbidity and mortality, the need for surgical intervention, and activity restriction, but are rarely used to guide exercise training. Previous research on ACHD has largely been focused on medical issues such as the hemodynamic effects of medications, surgical and other invasive procedures, and quality of life. However, one of the main concerns in both adult and pediatric CHD patients and their parents is regarding the appropriateness and safety of exercise training (237). Current guidelines are surrounded around the appropriateness of participation in sport, not daily exercise, and exercise counseling and advice is suboptimal, even in specialist clinics (135). Many adult patients report a moderate to high level of anxiety related to exercise, often times associated with overly restrictive advice regarding physical exertion in childhood. However, these same patients report a desire to gain knowledge and willingness to participate in exercise programs. To date, there are no specific guidelines related to exercise rehabilitation or training programs specific to ACHD patients. However, it has been suggested that utilizing guidelines in place for patients with HF is useful due to the similarities in the manifestation of many ACHD defects and HF.

1.5.2.1 Prognostic Value of Exercise Capacity in Adult Congenital Heart Disease

As with acquired heart disease (193), formal assessment of exercise capacity is a valuable prognostic tool (75, 109). Maximal oxygen uptake (or the bodies maximal
ability to produce ATP aerobically in mL/kg/min) is routinely used to assess the need for interventions and low oxygen uptake correlates with an increased incidence of both morbidity and mortality in ACHD (74). Cardiac transplant, for example, is recommended when O$_2$ uptake is <15mL/kg/min (41). Patients who have undergone repair for TOF have a 48% greater risk of 5-year mortality when O$_2$ uptake <36% of predicted norms (108).

Despite the evidence that low exercise capacity clearly has a detrimental impact on morbidity and mortality, there remains significant work to be done before exercise training is a commonplace clinical recommendation in ACHD. To date no general recommendations are made for the ACHD patients in regards to exercise, and individualized evaluations of each patient is recommended prior to commencing exercise training of any kind (112). This is sound and prudent advice, given the complexity and elevated risk of arrhythmias and HF (186, 338) in this patient population. However, this requires exercise professionals to have a sound understanding of how various physiological systems (cardiovascular, respiratory, metabolic, hormonal, etc.) respond to exercise, acutely and chronically, and the ability to translate these responses in situations of compromised function and/or structural abnormalities treated with pharmaceutical interventions that may impact on all, or some of the systems mentioned above, at rest and during exercise.

1.6 Effects of Intensity

Given the well-documented benefits of exercise on health, there is a renewed interest in identifying the most optimal type of exercise. In the literature two broad types
of ‘aerobic-type’ exercise have been studied, moderate-intensity continuous and high-intensity intermittent training (HIIT). Recently, HIIT has been described as a time efficient alternative to traditional endurance exercise, as only 3-10 min of active exercise is required (116). However, this is somewhat misleading, as the training programme still requires ~30 min to complete given the active recovery between intervals, and this is the equivalent to the minimum time recommended for moderate-intensity exercise (330). Moderate-intensity intermittent exercise has been used in a clinical setting for decades and is known to have a plethora of benefits (see sections 1.2.1 & 1.2.2). Therefore, it is really the intensity of exercise that primarily differentiates current ET and HIIT models. HIIT is characterized by brief, repeated bursts of intense exercise separated by periods of rest or low-intensity exercise (‘active recovery’). One of the most common models employed in HIIT studies is the Wingate Test, which consists of 30 s of “all-out” cycling against a high resistance, consisting of 4 to 6 repetitions interspersed by 4 min of recovery (116). Many studies have transitioned to a model consisting of 10 x 1 min cycling efforts at an intensity eliciting 85%–90% of HR_max interspersed with 1 min recovery periods.

The metabolic effects of ET and HIIT in young, healthy subjects appear to be equivalent. Acutely, both types of exercise similarly activate signaling pathways, including p38 MAPK, CaMKII and AMPK (12, 111). Chronically, ET and HIIT result in comparable increases in skeletal muscle mitochondrial content, maximal activities of oxidative enzymes, the expression of plasma membrane transport proteins, glycogen content, and 24 hour post exercise energy expenditure (38, 39, 70, 138, 305). However, in contrast to ET, the effects of HIIT on central parameters is ‘limited or equivocal’ (307),
as demonstrated in 3 recent meta-analyses (11, 120, 348). Many of the studies investigating the effects of HIIT have used young, health subjects. It is evident more research is required to elucidate the effects of HIIT on central parameters in older, and clinical populations.

While the evidence on the effects of HIIT in clinical populations is sparse, a study by Wisloff and colleagues (352) reported in patients with post-infarction HF, that HIIT was superior to moderate ET, with regards to LV remodeling, aerobic capacity, endothelial function, and quality of life. However, it is important to note, while infarctions affecting a large portion of the LV are undoubtedly a basis for HF, the etiology, disease progression, and molecular fingerprint is fundamentally different than hypertrophic (hypertensive) related HF. Therefore, while ET is known to decrease blood pressure in the setting of hypertension (112) the physiological effects of HIIT remain to be determined in the most common cause of HF. This is further emphasized in studies in diseased rodents, which have demonstrated negative adaptations within the heart as a result of exercise training (47, 68, 153). Furthermore, HIIT does not uniformly increase stroke volume (120, 198). As a result, it has been suggested that an ‘intensity threshold’ exists in pathological conditions, such that higher intensities of exercise in the presence of high blood pressure elicit detrimental adaptations. However, low-intensity exercise in a model of hypertension has also been shown to be insufficient to induce mitochondrial biogenesis (47). It is clear, that while the effects of ET have been demonstrated in many clinical populations (for review see (112)), the impact of HIIT in hypertension and HF remain an area where further large-scale studies are required.
Of the limited studies that do exist in common causes of HF, the data is difficult to interpret and inconsistent. HIIT was superior to ET stable CAD patients, however, the ET was lower than what is generally clinically used (273), likely explaining why others have found no difference between HIIT and ET in a cardiac rehabilitation setting (67, 331). Smaller scale studies have demonstrated HIIT is effective in overweight and obese, but otherwise healthy, individuals (118, 119) and those with type II diabetes mellitus (117).

Significant knowledge gaps exist on the effects of HIIT on cardiac morphology and disease progression in hypertension induced HF. While, there is potential benefit from HIIT, studies examining its effects in HF remain sparse, with only 200 combined HF patients studied to date (251). Despite the knowledge gaps identified (104, 112, 251) on the use of HIIT in clinical populations, many recommend HIIT in the setting of cardiovascular diseases (112, 162, 204). There is acknowledgement however regarding risk in low fitness populations: “it may also be prudent to include a preconditioning phase of training.....(moderate intensity endurance exercise) prior to initiating HIIT” (116). This is an essential message, as higher baseline CRF reduces risk associated with exercise-induced ischemic events (329).

In summary, while exercise has extensive effects in health and disease on both cardiac and skeletal muscle, the overall impact of an exercise intensity (HIIT) which promotes a significant elevation in the factors responsible for the adaptations beyond which is seen as a result of ET remains to be fully elucidated.
CHAPTER TWO

AIMS OF THE THESIS
Currently, heart disease is a leading cause of mortality in Canadians. Alarmingly, hypertension, a leading cause for the development of HF, occurs in 20% of Canadians and the incidence is increasing in all age groups. Thus, the rising burden of hypertension is recognized as a clinical problem that urgently needs addressing before irreversible hypertension is established. Lifestyle interventions that improve blood pressure control may be particularly beneficial as therapeutic options. While exercise training is known to decrease blood pressure and improve cardiovascular fitness, it remains to be determined what intensity of training is optimal in clinical populations.

While ET is the prominent intensity of exercise within clinical settings, HIIT has been suggested to be a time-efficient alternative to improve fitness and decrease all-cause-mortality in individuals with cardiovascular disease. This paradigm shift in intensity of training has been prefaced with the observations that in healthy young individuals ET and HIIT elicit similar adaptations in skeletal muscle. In addition, there is one study suggesting HIIT is more advantageous than ET at improving whole body oxygen uptake post-infarction, suggesting that HIIT is safe and effective in some cardiovascular disease settings. However, startlingly, there is very little clinical evidence to support the use of HIIT in individuals with hypertension, or the effects of HIIT on the biochemical/molecular adaptations within cardiac muscle following exercise training.
Therefore, the purpose of the current thesis was to determine the efficacy of ET and HIIT in a pre-clinical animal model of HF with overt hypertension, and to apply this knowledge to exercise prescription in ACHD patients. Specifically it was hypothesized that:

1- Exercise-induced increases in skeletal muscle oxidative capacity would be similar following ET and HIIT in Dahl-salt sensitive rats.

2- Both ET and HIIT would similarly prevent pathological remodeling and the induction of HF in Dahl-salt sensitive rats.

3- Both ET and HIIT would improve whole body oxygen uptake and exercise tolerance in ACHD patients of moderate to great complexity.
CHAPTER THREE

HIGH INTENSITY INTERVAL AND ENDURANCE TRAINING ARE ASSOCIATED WITH DIVERGENT SKELETAL MUSCLE ADAPTATIONS IN A RODENT MODEL OF HYPERTENSION
3.1 Abstract

Skeletal muscle is extremely adaptable to a variety of metabolic challenges, as both traditional moderate intensity endurance (ET) and high intensity interval training (HIIT) increase the oxidative potential in a coordinated manner. While these responses have been clearly demonstrated in healthy individuals, it remains to be determined if both exercise intensities produce similar responses in the context of hypertension, one of the most prevalent and costly diseases worldwide. Therefore, in the current study we utilized the Dahl sodium sensitive rat, a rodent model of hypertension, to determine the molecular responses to 4 weeks of either ET or HIIT in the red (RG) and white gastrocnemius (WG) muscles. In the RG, both ET and HIIT increased the content of electron transport chain proteins and increased succinate dehydrogenase (SDH) content in type I fibres. While both intensities of exercise shifted fibre type in RG (increased IIA, decreased IIX), only HIIT was associated with a reduction in eNOS protein and an increase in HIF1α protein. In the WG, both ET and HIIT increased markers of the electron transport chain, however, HIIT decreased SDH content in a fibre-specific manner. While ET lead to an increase type IIA, decrease in IIB fibres and increased capillarization, in contrast HIIT increased the percentage of IIB fibres, decreased capillary to fibre ratios, decreased eNOS, and increased HIF1α protein. Altogether, these data show that unlike in healthy animals, ET and HIIT have divergent effects in the skeletal muscle of hypertensive rats. This suggests ET may be optimal at improving the oxidative capacity of skeletal muscle in animals with hypertension.
3.2 Introduction

Hypertension remains one of the greatest contributors to cardiac-related mortality (177) and the development of heart failure (HF) worldwide (8, 177). While directly affected by hypertension, HF remains a complex disorder that manifests with myocardial dysfunction (reduced ability of the heart to pump blood) and results in skeletal muscle metabolic abnormalities (52, 192). The underlying pathology of heart failure continues to be elucidated, however, alterations in energy metabolism have been repeatedly implicated in the disease progression (59, 83, 151, 336). HF is also associated with a derangement in whole body oxidative potential, as both cardiac and skeletal muscle display reductions in mitochondrial content and capillarization (37, 52, 58, 247). The metabolic disturbances at the level of the skeletal muscle are implicated in the prevalence of exercise intolerance in HF patients; however, they are amenable to exercise training (112, 247). Therefore, elucidating which exercise intensities improve the oxidative capacity of skeletal muscle is essential for clinical care of individuals with hypertension, as they are at elevated risk for the development of HF and exercise intolerance.

Skeletal muscle has a remarkable ability to adapt to exercise training, such that a single bout of exercise is sufficient to induce the activation of transcription factors involved in the regulation of the metabolic profile (241). Mitochondrial biogenesis (involving both proliferation and alterations in mitochondria) results from the cumulative effects of repeated transient up-regulation of the mRNAs of factors involved in mitochondrial and metabolic adaptations (241). Coordinated responses such as increases in mitochondrial content, elevated maximal activities of oxidative enzyme, and higher expression of plasma membrane transporters, all facilitate a higher oxidative potential of
skeletal muscle (50, 145, 146, 242, 243, 325). In healthy individuals these adaptations appear to occur independent of the intensity of training, as both high intensity interval training (HIIT) and moderate intensity endurance training (ET) induce these responses (38, 39, 110, 111, 120, 238, 240, 305, 320). These data suggest that HIIT may provide an equally effective and time-efficient alternative to ET.

While the molecular adaptations to exercise within skeletal muscle appear to be independent of exercise intensity in healthy individuals, this remains to be determined in the presence of hypertension or compromised cardiac performance. The clinical relevance of HIIT remains debatable, especially within the context of hypertension and HF, as unlike ET, HIIT does not uniformly increase stroke volume (120, 198). In rodent models of hypertension, extreme or exhaustive exercise has also been associated with negative adaptations in the heart (16, 68, 152, 291). As a result, it has been suggested that an intensity threshold exists in pathological conditions, such that higher intensities of exercise in the presence of high blood pressure elicit detrimental adaptations. However, low-intensity exercise in a model of hypertension has also been shown to be insufficient to induce mitochondrial biogenesis (47), highlighting the necessity for additional research examining the effectiveness of exercise training protocols that involve different fibre recruitment patterns in the presence of hypertension.

Therefore, it remains to be determined if both ET and HIIT increase the oxidative potential of skeletal muscle in the presence of high blood pressure. The present study aimed to determine if ET and HIIT improved the oxidative potential of skeletal muscle in Dahl sodium sensitive (Dahl/SS) rats. We hypothesized that both ET and HIIT would increase markers of whole muscle mitochondrial content, shift fibre composition towards
a slower, more fatigue-resistant fibre type, and improve the skeletal muscle capillary to fibre ratio similarly in Dahl/SS rats, providing support for the use of HIIT in the presence of hypertension.
3.3 METHODS

3.3.1 Animals and Experimental Design

The Dahl/SS rat develops hypertension, HF, and an increased risk of mortality on a high-sodium diet within 8 weeks, and follows the most prevalent known human progression of hypertension-induced HF (9, 269). Given the rapid progression towards HF, these animals are ideal for elucidating the optimal intensity of exercise as a lifestyle intervention to improve the oxidative potential of skeletal muscle.

We examined the effects of ET and HIIT in male Dahl/SS rats when compared to sedentary (SED) animals. Dahl/SS rats (8 weeks of age; n=18) and were fed high sodium chow to induce the hypertensive phenotype. The diet containing 8% sodium chloride was purchased through Research Diets (New Brunswick, NJ, USA). The animals were randomly assigned to 3 experimental conditions: SED (n=6), classical ET (n=6) or HIIT (n=6). The high sodium diets and exercise interventions commenced at the same time point (e.g., week 1). The high sodium diets and exercise interventions commenced at the same time point (e.g., week 1). Animals were housed 1 per cage in a temperature-regulated room on a 12:12 hr light-dark cycle with water available ad libitum. This study was approved by the University of Guelph Animal Care Committee, and conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health.

3.3.2 Treadmill Exercise

All rats were familiarized with a rodent treadmill (Columbus Instruments, Columbus, OH, USA) on at least 3 occasions (10 m/min, 0% grade, 10-15 min) before
randomization. Briefly, at the same time as the initiation of the HS diet, both the ET and HIIT animals trained 5 days/week for 4 weeks at a progressively more challenging intensity. The ET animals trained beginning at 10-15 m/min at 0% grade and progressed to 20 m/min at 10% grade for 45 min at week 4. HIIT animals began by alternating between active rest (2 min at 10 m/min, 0% grade) and high intensity (1 min at 20 m/min, 10% grade) for 30 min and progressed to intervals of 2 min active rest and high intensity (10 m/min, 10% grade and 20 m/min, 15% grade) for 45 minutes at week 4. Forty-eight hours after the last exercise bout, animals were anaesthetized with pentobarbital (100 mg/kg body weight) and the red and white gastrocnemius were removed with one sample rapidly frozen in liquid nitrogen, stored at -80 ºC, and a second sample embedded in optimum cutting temperature compound (OCT; Fisher Scientific, Ottawa, ON, CA) for histochemical analysis.

3.3.3 Arterial Blood Pressure and Heart Rate

Systolic and diastolic blood pressures were measured in conscious, restrained rats using a CODA™ 2 tail-cuff system (Kent Scientific, Torrington, CT, USA) in a dark temperature-controlled room (22°C) in the morning. Rats were acclimatized on a minimum of three occasions prior to the study. On measurement days, rats were subjected to 15 acclimation measurements in a restraint holder, and pressure and heart rate were averaged from the last 10 measurement cycles.

3.3.4 Western blotting

Whole-muscle homogenates (n=18) were separated by electrophoresis using SDS-PAGE, transferred to polyvinylidene difluoride membranes, and quantified, as previously
reported (28). Proteins were separated on a 6%, 7.5%, 10% or 12% resolving gel as required to optimize for MW separation, and transferred to a polyvinylidene difluoride membrane (Roche, Laval, QC, CA). The following commercially available antibodies were used: total oxidative phosphorylation antibody cocktail (OXPHOS, Abcam, Cambridge, MA, USA, ab110413, 1:500), (eNOS, Abcam, ab5589 1:1000), vascular endothelial growth factor (VEGF, Abcam 1:1000, ab46154), vascular endothelial growth factor receptor 2 (VEGFR2, Abcam, ab39256, 1:1000), hypoxia inducible factor 1 alpha (HIF1α, Abcam, ab463, 1:1000), alpha tubulin (Abcam, ab40742, 1:5000). All samples were detected from the same Western blot by cutting gels and transferring onto a single membrane to limit variability. Equal loading of protein was verified using Ponceau staining. Bands were visualized using enhanced chemiluminescence (Western Lightning Plus-ECL, PerkinElmer, Woodbridge, ON, CA), and quantified by densiometry (Alpha Innotech Fluorchem HD2, Fisher Scientific, Ottawa, ON, CA).

3.3.5 Citrate Synthase

Citrate synthase (CS) activity was assayed in homogenates after lysing the mitochondria with 0.04% Triton X-100 and repeated freeze-thawing. CS activity was determined spectrophotometrically at 37°C at 412 nm as previously reported (311).

3.3.6 Histochemistry

Red (RG) and white (WG) gastrocnemius portions embedded in OCT compound were cut into 10 μm cross sections with a cryostat (Thermo Fisher Scientific, Ottawa, ON CA) maintained at -20°C. Cross sections were analyzed for fibre type composition and fibre type-specific cross sectional area (CSA) using immunofluorescent detection of
myosin heavy chains (MHC), as previously described (26). This technique allows for the identification of type I (blue), type IIA (green), type IIX (unstained), type IIB (red), and hybrid fibre types. Fibre type composition was quantified by counting all representative fibres within each cross section, and CSA was calculated by outlining all fibres from 10 separate regions of each cross section (>50 per type per muscle per animal). Imaging was performed with an Axio Observer Z1 fluorescent microscope and associated AxioVision software (Carl Zeiss).

Succinate dehydrogenase (SDH) histochemical activity staining was determined as a general indicator of oxidative potential (26). Images were acquired with a PixeLink digital camera connected to a Nikon microscope and quantified with ImageJ analysis software (National Institutes of Health). Individual images were assembled into composite panoramic images and matched to corresponding panoramic images attained during MHC analysis. SDH staining intensity was analyzed in individual fibre types after subtracting the background.

Capillary to fibre ratio quantification was adapted from previous work (43). Briefly, sections were fixed in 10% formalin buffered solution for 10 min, permeabilized with 0.5% TritonX-100 for 10 min, and then blocked in 10% goat serum for 30 min. Sections were incubated overnight in 1.5% goat serum with the appropriate primary antibodies specific for the endothelium (collagen IV, 1:50) and sarcolemma (dystrophin, 1:200) (Developmental Studies Hybridoma Bank, Iowa City, IA, USA). After three 5 min washes in PBS, sections were incubated for 1 hour in 3% goat serum with the appropriate fluorescent secondary antibodies (Life Technologies, Burlington, ON, CA). Nuclear counterstaining was also performed by incubating slides for 5 min in 4',6-diamidino-2-
phenylindole (DAPI) prior to visualization. Capillarization was quantified manually with longitudinal fibres excluded from analysis.

3.3.7 Statistical Analysis

Data, with the exception of fibre type, are represented as a percentage of SED. Fibre types are represented as a percentage of total fibres within SED, ET and HIIT. Values were analyzed for significance when compared to SED using a non-paired t-test with the $\alpha$-value set to $p<0.05$. 
3.4 Results

3.4.1. Systolic and diastolic blood pressure

We first aimed to ensure that the high sodium diet elicited hypertension. All animals displayed both systolic (SED: 189±3, ET: 189±4, HIIT: 192±1 mmHg) and diastolic (SED: 149±1, ET: 151±2, HIIT: 150±2 mmHg) hypertension, versus Dahl/SS rats fed low sodium chow (269). However, 4 weeks of ET or HIIT did not alter (P>0.05) blood pressure.

3.4.2 Markers of mitochondrial content

We next aimed to characterize the effects of ET and HIIT on the oxidative capacity of the skeletal muscle in this model of hypertension. ET and HIIT increased (P<0.05; ~25%) the protein content of various markers of the electron transport chain (i.e., OXPHOS) in the RG (Figure 3.1A, B). In the WG, ET and HIIT increased (P<0.05) the protein content of the OXPHOS subunits (Figure 3.1C,D).
Figure 3.1. Mitochondrial content in whole muscle homogenate in red and white gastrocnemius. A. Density quantifications of oxidative phosphorylation (OXPHOS) proteins in red (RG; A) and white (WG; C) gastrocnemius of SED, endurance training (ET), and high intensity interval training (HIIT) groups. Representative western blot of OXPHOS proteins in RG (B) and WG (D); α-tubulin is presented as a loading control. * vs. SED; P<0.05. Data are means ± SEM.

In addition, while ET and HIIT did not alter the activity of CS in the RG (Figure 3.2A), both exercise intensities increased SDH content in type I muscle fibres specifically, which represent ~45% of the fibres within the RG (Table 3.1). However, in contrast to the RG, both ET and HIIT decreased (P<0.05) CS in the WG (Figure 3.2B) by ~25%, while SDH content was decreased in type IIB fibres following HIIT (Table 3.1).
Figure 3.2. Citrate synthase activity in red and white gastrocnemius following endurance training (ET) and high intensity interval training (HIIT). A. Citrate synthase (CS) activity in red gastrocnemius. Absolute values µmol/min/g wwt in RG as follows: SED 60.6 ± 1.8, ET 61.6 ± 3.2 and, HIIT 59.4 ± 1.4. B. CS activity in white gastrocnemius demonstrating a reduction in activity in both ET and HIIT, * vs. SED; P<0.05. Absolute values µmol/min/g wwt in WG as follows: SED 26.5 ± 2.2, ET 19 ± 0.8 and, HIIT 22.8 ± 1.03. Data are means ± SEM.
Table 3.1. Fibre Type, CSA and SDH in SED, ET and HIIT rats

<table>
<thead>
<tr>
<th>fibre Type</th>
<th>Type I</th>
<th>Type IIA</th>
<th>Type IIAX</th>
<th>Type IIX</th>
<th>Type IIXB</th>
<th>Type IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibre count %population</td>
<td>359 ± 38</td>
<td>45.0 ± 3.5</td>
<td>28.1 ± 1.4</td>
<td>7.6 ± 1.7</td>
<td>14.8 ± 2.3</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>SED CSA (µm)</td>
<td>539 ± 40</td>
<td>3759.6 ± 95.9</td>
<td>2464.2 ± 169</td>
<td>2385.5 ± 81.8</td>
<td>2594.5 ± 142.1</td>
<td>- -</td>
</tr>
<tr>
<td>SDH (AU)</td>
<td>20.7 ± 1</td>
<td>35.5 ± 1.1</td>
<td>- -</td>
<td>28 ± 1.1</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>%population</td>
<td>301 ± 15</td>
<td>44.9 ± 2.4</td>
<td>33.4 ± 1.5*</td>
<td>16.7 ± 2.2*</td>
<td>4.9 ± 0.6*</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>ET CSA (µm)</td>
<td>524 ± 69</td>
<td>3627.4 ± 207</td>
<td>2316.4 ± 87.3</td>
<td>2601.4 ± 89.7</td>
<td>2471.4 ± 67.5</td>
<td>- -</td>
</tr>
<tr>
<td>SDH (AU)</td>
<td>24.3 ± 0.3*</td>
<td>37.1 ± 0.4</td>
<td>- -</td>
<td>27.5 ± 0.4</td>
<td>- -</td>
<td>- -</td>
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<tr>
<td>%population</td>
<td>274 ± 23</td>
<td>50.3 ± 2</td>
<td>34.5 ± 2.2*</td>
<td>7.4 ± 1.6</td>
<td>7.8 ± 1.4*</td>
<td>0.0 ± 0.0</td>
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<tr>
<td>HIIT CSA (µm)</td>
<td>566 ± 6.4</td>
<td>3955.4 ± 174.9</td>
<td>2696.5 ± 149*</td>
<td>2746.0 ± 200</td>
<td>2555.0 ± 210</td>
<td>- -</td>
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<tr>
<td>SDH (AU)</td>
<td>25.1 ± 0.8*</td>
<td>36.9 ± 0.7</td>
<td>- -</td>
<td>26.2 ± 1.1</td>
<td>- -</td>
<td>- -</td>
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</table>

<table>
<thead>
<tr>
<th>fibre Type</th>
<th>Type I</th>
<th>Type IIA</th>
<th>Type IIAX</th>
<th>Type IIX</th>
<th>Type IIXB</th>
<th>Type IIB</th>
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<tbody>
<tr>
<td>Fibre count %population</td>
<td>211 ± 8</td>
<td>0.0 ± 0.0</td>
<td>1.0 ± 0.5</td>
<td>0.0 ± 0.0</td>
<td>5.4 ± 1.3</td>
<td>12.1 ± 2.2</td>
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<tr>
<td>SED CSA (µm)</td>
<td>491 ± 7.4</td>
<td>- -</td>
<td>1635.4 ± 79.5</td>
<td>- -</td>
<td>1773.9 ± 80.1</td>
<td>2575.8 ± 114.4</td>
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<td>SDH (AU)</td>
<td>- -</td>
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<td>- -</td>
<td>32.0 ± 1</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>%population</td>
<td>238 ± 16</td>
<td>0.0 ± 0.0</td>
<td>2.6 ± 1.6</td>
<td>2.1 ± 2.1</td>
<td>15.7 ± 3.8*</td>
<td>5.7 ± 1.4</td>
</tr>
<tr>
<td>ET CSA (µm)</td>
<td>474 ± 8.6</td>
<td>- -</td>
<td>1308.6 ± 28.7*</td>
<td>- -</td>
<td>1981.9 ± 135.9</td>
<td>3009 ± 180.1*</td>
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<td>SDH (AU)</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>29.6 ± 1.3</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>%population</td>
<td>235 ± 17</td>
<td>0.0 ± 0.0</td>
<td>1.5 ± 1</td>
<td>0.0 ± 0.0</td>
<td>7.3 ± 1.6</td>
<td>6.4 ± 1</td>
</tr>
<tr>
<td>HIIT CSA (µm)</td>
<td>493 ± 7.7</td>
<td>- -</td>
<td>1159.2 ± 241</td>
<td>- -</td>
<td>1802.7 ± 88.5</td>
<td>2197.4 ± 130*</td>
</tr>
<tr>
<td>SDH (AU)</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>30.9 ± 1.5</td>
<td>- -</td>
<td>- -</td>
</tr>
</tbody>
</table>

Values are means SEM, n=6 per group. SED, sedentary; ET, endurance training, HIIT; high intensity interval training; CSA cross sectional area; SDH, succinate dehydrogenase.
3.4.3 Fibre type composition

Given the variable responses in the oxidative capacity of muscle to exercise training in the RG and WG, we next examined MHC content within each muscle type. Within the RG, both ET and HIIT resulted in an increase in the percentage of type IIA and decrease in IIX fibres, when compared to SED, however, only ET resulted in a 2-fold increase in IIAAX intermediate fibres (Figure 3.3A and Table 3.1). Within the WG, ET and HIIT had divergent effects on fibre type. Specifically, ET resulted in a reduction IIB and IIXB fibres and a 3-fold increase in the percentage of IIX fibres (Figure 3.3B and Table 3.1). In contrast, HIIT resulted in a reduction in IIXB and an increase in IIB fibres (Figure 3.3B and Table 3.1).
Figure 3.3. Effects of endurance training (ET) and high intensity interval training (HIIT) on skeletal muscle fibre type composition. A. Composite fluorescent microscopy images of red gastrocnemius (RG): sedentary (SED; top), ET (middle), HIIT (bottom). B. Serial cross-section of RG showing SDH activity staining: SED (top), ET (middle), HIIT (bottom). C. Composite fluorescent microscopy images of white gastrocnemius (WG): SED (top), ET (middle), HIIT (bottom). D. Serial cross-section of WG showing SDH activity staining: SED (top), ET (middle), HIIT (bottom) * vs. SED; P<0.05. Data are means ± SEM.
3.4.4 Capillarization and angiogenic factors

Given the divergent effects of ET and HIIT on the oxidative capacity and fibre type profile of skeletal muscle, we next characterized the effect of these training regimes on capillary to fibre ratio. The capillary to fibre ratio was decreased in the RG following both ET and HIIT (Figure 3.4A, B). However, this was only associated with a reduction in eNOS (Figure 3.5B), and an increase in HIF1α protein contents (Figure 3.5C) following HIIT, suggesting hypoxia occurred only in the HIIT animals. In addition, in the WG, ET increased the capillary to fibre ratio (Figure 3.4C, D), while HIIT decreased the capillary to fibre ratio (Figure 3.4C, D) and increased the protein content of HIF1α (Figure 3.5G); once again suggesting HIIT induced hypoxia. ET and HIIT did not alter the protein contents of VEGF or its receptor, VEGFR2, in either the RG or WG (Figure 3.5D, E, H, I).

**Figure 3.4. Red and white gastrocnemius capillary to fibre ratio.** Composite fluorescent images of red (RG; A) and white (WG; C) gastrocnemius: sedentary (SED; left), endurance training (ET; middle), high intensity interval training (HIIT; right) in both. B. * vs. SED; P<0.05. * vs. SED; P<0.05. Data are means ± SEM.
Figure 3.5. Western blot analysis of eNOS, HIF1α, VEGF, and VEGFR2 in red and white gastrocnemius. A. Representative blots; α-tubulin is presented as a loading control. B-E. Density quantifications in RG of eNOS protein (B), HIF1α protein (C), VEGF protein (D), and VEGFR2 protein (E) in sedentary (SED), endurance training (ET), and high intensity interval training (HIIT). F-I. Density quantifications in WG of eNOS protein (F), HIF1α protein (G), VEGF protein (H), and VEGFR2 protein (I) in SED, ET and HIIT. * vs. SED; P<0.05. Data are means ± SEM.
3.5 DISCUSSION

In the current study we provide evidence that in a model of hypertension, both ET and HIIT increased markers of OXPHOS protein content and SDH content in type I fibres, and induced a transition towards a slower, fatigue-resistant fibre type in the RG. However, HIIT induced a reduction in eNOS protein and an increase in HIF1α protein. In the WG, ET and HIIT both increased markers of the electron transport chain. However, while ET shifted the WG towards an a fatigue-resistant fibre type, and promoted angiogenesis, HIIT shifted the WG towards a fast, fatigable fibre type, decreased capillary to fibre ratios, decreased eNOS, and increased HIF1α protein. Altogether, these data demonstrated that unlike in healthy animals, ET and HIIT have divergent effects in the skeletal muscle of hypertensive rats, and suggest that ET may be optimal at improving the overall oxidative capacity of skeletal muscle in animals with hypertension.

3.5.1 Markers of mitochondrial content

Skeletal muscle mitochondrial proliferation is a well-known adaptation to exercise training (145, 146). In the current study, both ET and HIIT increased the protein content of various electron transport chain subunits in the RG, suggesting both intensities of exercise increase the oxidative potential of skeletal muscle. These adaptations were more robust in type I fibres, as only type I fibres in the RG displayed an increase in SDH activity. These data support previous work in healthy humans, which have shown that both ET and HIIT increase various markers of mitochondrial content, and have a similar response in type I and II fibres (294, 300). It was surprising that neither ET nor HIIT increased CS activity in the current study in the RG. However, since SDH only increased in type I fibres, which represent ~45% of the fibres in the RG, it is likely that small CS
adaptations within type I fibres would be undetectable at the mixed homogenate level. Altogether, ET and HIIT displayed similar mitochondrial responses in the RG.

Both ET and HIIT decreased CS activity and SDH content in type IIX and IIB fibres in the WG. This finding was unexpected, however, these data clearly suggest that typical exercise adaptations are compromised in a model of disease. Nevertheless, previous work has demonstrated inconsistencies in the response of CS activity after exercise training in spontaneously-hypertensive rats (124, 201, 290). This is further supported by evidence that the transcriptional control of CS may differ from other mitochondrial proteins, such that the promoter region of CS lacks binding domains for PPARs or NRFs (172). While both ET and HIIT increased markers of the electron transport chain, these adaptations suggest the absence of a coordinated increase in the oxidative capacity within the WG following both ET and HIIT in a model of hypertension.

3.5.2 Fibre type composition in response to exercise

Fibre type transitions occur under conditions of overloading such as exercise training (243, 244). In general, endurance exercise training promotes a slower, fatigue-resistant fibre type (242). This transition follows a sequential order from type IIB to IIX to IIA to I, along with the appearance of hybrid fibres. In the current study, both ET and HIIT promoted a significantly higher percentage of type IIA fibres and fewer IIX. However, only ET promoted a shift towards IIAX hybrid fibre. In contrast, within the WG, ET induced an increase in IIX fibres, while HIIT increased type IIB fibres. These fibre transitions suggest that in a hypertensive model, ET promotes a slower, fatigue-resistant fibre type, while HIIT promotes a faster, fatigable fibre type. While it is well
known that recruitment plays a role in exercise-related fibre transitions (242), HIIT resulted in adaptations in WG electron transport chain proteins, suggesting wide spread recruitment was likely. Although the etiology of the divergent responses in fibre type transitions remains unclear, it is possible that during exercise the HIIT animals are unable to maintain the Q required by the exercising skeletal muscle. Regardless of the mechanism, the current data suggest that ET is the more appropriate form of training in the presence of hypertension, a finding that may also extend to other pathological situations associated with impaired skeletal muscle oxidative capacity.

3.5.3 Capillarization, eNOS, HIF1α content

A key determinant of the increased matching of metabolic demand and oxygen (O₂) delivery to the skeletal muscle post training is due to proliferation in capillary beds within the trained muscle (50). During exercise training, active muscle experiences repetitive oxygen stress (a fall in intracellular partial pressure of O₂), such that exercise training promotes angiogenesis (266). The resultant increase in capillary beds allows for a more coordinated metabolic response to exercise, such that blood flow is more closely matched to tissues with increased metabolic demand. Surprisingly, within the RG both ET and HIIT animals experienced a reduction in capillary to fibre ratio when compared to SED animals. This decrease in capillarization was only accompanied by reduced eNOS and an increase in HIF1α protein in the HIIT animals. These data suggest hypoxia may have occurred, or been more prominent, in the HIIT animals. HIF1α suppresses mitochondrial biogenesis and is considered the primary transcriptional factor responsible for adaptations to transient changes in Po₂, such as repetitive exercise training (296, 340). Chronic exercise training in healthy subjects results in lower HIF1α protein content (190).
Our data demonstrate that ET increased the capillary to fibre ratio in the WG, while HIIT decreased the capillary to fibre ratio, stimulating an increase in HIF1α protein content. Once again this suggests that HIIT potentially induced a greater hypoxic stimuli in the contracting muscle. In contrast, previous reports in healthy rodents and humans have shown that capillary to fibre ratio and eNOS content are increased as a result of both ET and HIIT (55, 238). However, in a rodent model of type II diabetes, ET preferentially increased the capillarization of oxidative muscles supporting the current interpretation that ET and HIIT have divergent effects in pathological conditions (195).

In conclusion, our data provide evidence that in hypertensive Dahl/SS rats, ET increased the percentage of IIAX fibres and expression of oxidative proteins in RG, and preferentially increased IIX fibres and capillary to fibre ratio in WG. Conversely, while HIIT increased the percentage of IIX fibres and oxidative proteins, it was associated with a reduction in eNOS content in RG, an increase in IIB fibres, and reduced capillarization in the WG.

3.5.4 Perspectives and Significance

Altogether, the current data suggest that in animals with hypertension, ET may represent the optimal stimulus for coordinated adaptations resulting in enhanced skeletal muscle oxidative capacity. “The mechanism by which HIIT elicited negative responses in skeletal muscle remains unknown, but may be explained by a local redox imbalance, as the larger fluctuations in metabolites during HIIT (e.g. IMP) would be anticipated to increase ROS to a greater degree. ROS is required for many physiological signaling processes, and stimulates increases in the expression of HIF1α (218), as observed following HIIT. However, disproportionate levels of ROS decreases the downstream
activation of VEGR2 (35), impacting VEGF-mediated angiogenesis, and potentially explaining the reduction in capillarization following HIIT. While this mechanism remains speculative, exercise training in the presence of existing oxidative stress (e.g., aging) is not associated with the beneficial effects of exercise (327, 328). This data suggests that exercise-mediated increases in ROS can be detrimental in situations with oxidative stress/diminished antioxidant defenses. Therefore, future studies should investigate the effects of ET and HIIT in hypertensive humans, and the associated changes in the oxidative state of the skeletal muscle.”
CHAPTER FOUR

HIGH INTENSITY INTERVAL AND ENDURANCE TRAINING HAVE
OPPOSING EFFECTS ON MARKERS OF HEART FAILURE AND CARDIAC
REMODELING IN HYPERTENSIVE RATS
4.1 Abstract

There has been re-emerging interest and significant work dedicated to investigating the metabolic effects of high intensity interval training (HIIT) in recent years. HIIT is considered to be a time efficient alternative to classic endurance training (ET) that elicits similar metabolic responses in skeletal muscle. However, there is a lack of information on the impact of HIIT on cardiac muscle in disease. Therefore, we determined the efficacy of ET and HIIT to alter cardiac muscle characteristics involved in the development of diastolic dysfunction, such as ventricular hypertrophy, fibrosis and angiogenesis, in a well-established rodent model of hypertension-induced heart failure before the development of overt heart failure. ET decreased left ventricle fibrosis by ~40% (P < 0.05), and promoted a 20% (P<0.05) increase in the left ventricular capillary/fibre ratio, an increase in eNOS protein (P<0.05), and a decrease in hypoxia inducible factor 1 alpha protein content (P<0.05). In contrast, HIIT did not decrease existing fibrosis, and HIIT animals displayed a 20% increase in left ventricular mass (P<0.05) and a 20% decrease in cross sectional area (P<0.05). HIIT also increased brain natriuretic peptide by 50% (P<0.05), in the absence of concomitant angiogenesis, strongly suggesting pathological cardiac remodeling. The current data support the longstanding belief in the effectiveness of ET in hypertension. However, HIIT promoted a pathological adaptation in the left ventricle in the presence of hypertension, highlighting the need for further research on the widespread effects of HIIT in the presence of disease.
4.2 Introduction

Low aerobic capacity represents the greatest predictor of all-cause mortality, and is a clinically relevant parameter that is improved with chronic endurance exercise training (23). The beneficial effects of endurance exercise training are multifaceted, affecting genetic programs in skeletal muscle that result in mitochondrial biogenesis, increased skeletal muscle capillarization, improved vascular compliance, and increased stroke volume and $Q$ (27, 50, 145, 281). As a result, chronic exercise training is a well-known primary and secondary intervention for various pathologies, including but not limited to heart failure (HF), hypertension, diabetes, as well as slowing the progression of aging (31, 249, 261, 349).

While exercise training prevents disease and recovers the health of individuals, there is debate in the literature regarding the optimal intensity of exercise. Two types of exercise have largely been employed to elicit functional improvements in aerobic capacity, including classic endurance training (ET) and high-intensity interval training (HIIT). Acutely, in healthy subjects both types of exercise similarly activate signaling pathways, including p38 MAPK, CaMKII and AMPK (111). Chronically, ET and HIIT result in comparable increases in skeletal muscle mitochondrial content, maximal activities of oxidative enzymes (e.g., citrate synthase, cytochrome c oxidase) the expression of plasma membrane transport proteins, glycogen content, and 24 hour post exercise energy expenditure (38, 39, 70, 138, 305). These data suggest that HIIT may represent a time efficient clinically relevant tool to improve aerobic fitness in healthy individuals (116).
However, despite the wealth of data highlighting similar molecular and metabolic responses in skeletal muscle following different exercise training intensities in healthy individuals, intensity specific adaptations in the presence of disease, specifically within the diseased heart, remain to be elucidated. While ET increases capillarization and mitochondrial content, decreases fibrosis and prevents pathological hypertrophy in rodent models of HF, (173, 207, 212) the effect of HIIT on these molecular adaptations is unknown. Therefore, it remains to be determined if HIIT represents an optimal secondary prevention strategy in individuals with existing cardiovascular disease.

The ongoing debate regarding the effectiveness of ET and HIIT is further exemplified by the fact that while ET consistently increases stroke volume and decreases heart rate (HR), decreasing the energetic demands of a beating heart in healthy individuals there is some evidence Q may not always increase as a result of HIIT (120, 191). The inconsistencies of the effects of HIIT in healthy populations makes the investigation of the use of HIIT in the presence of disease even more relevant, as the molecular signals and consequent adaptations are likely to be altered in disease states.

The effects of differing exercise intensities on diseased cardiac muscle are incompletely understood, therefore, we aimed to determine if ET and HIIT were comparable at altering various molecular responses in a rodent model of hypertension that is associated with the development of HF. Dahl/SS rats were chosen as a model of hypertension because of the rapid increase in blood pressure, pathological cardiac remodeling and mortality displayed after commencing a high sodium (HS) diet (269). We hypothesized that both ET and HIIT would decrease left ventricular fibrosis, cross
sectional area, and molecular markers of heart failure, and increase left ventricular capillarization similarly in Dahl/SS rats, providing clinical support for the use of HIIT.
4.3 METHODS

4.3.1 Animals and experimental design

We aimed to determine if ET and HIIT had similar effects in preventing/exacerbating various molecular markers of HF. Male Dahl/SS rats were purchased from Charles River Laboratories (Saint-Constant, QC, CA) (8 weeks of age; n=24). Dahl/SS rats fed a HS diet rapidly develop hypertension and display increased wall thickness and ventricular mass at 3 weeks, start to lose body weight after 5 weeks, with mortality beginning at 7 weeks (269). We therefore chose to examine Dahl/SS rats after consuming a HS diet for 4 weeks when the only clinically relevant marker of disease was hypertension.

Rats were randomly assigned to receive a low sodium diet (LS, 0.3% NaCl chow, n=6) or a HS diet (8% NaCl chow, n=18) to induce the hypertensive phenotype. Diets were purchased through Research Diets (New Brunswick, NJ, USA), and were matched for caloric content and macronutrient composition. All animals fed the LS diet remained sedentary (LS-SED), while animals fed the HS diet were subdivided into three groups: sedentary (HS-SED, n=6), classical endurance training (HS-ET, n=6) and high intensity interval training (HS-HIIT, n=6). The HS diets and exercise interventions commenced at the same time point (e.g., week 1). Animals were housed 1 per cage in a temperature-regulated room on a 12:12 hr light-dark cycle with water available ad libitum. This study was approved by the University of Guelph Animal Care Committee, and conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health.
4.3.2 Treadmill Exercise

All rats were familiarized with a rodent treadmill (Columbus Instruments, Columbus, OH, USA) on at least 3 occasions (10 m/min, 0% grade, 10-15 min) before randomization. Exercise protocols were developed based on the American Physiological Society’s resource on exercise in animals, as well as from previous experience training rodents in our institution (174, 308). The ET group trained 5 days/week for 4 weeks at progressively more challenging “moderate continuous” intensities. The HIIT groups also trained for 5 days/week for 4 weeks, however at progressively more challenging “intermittent high” intensities. Detailed protocols and average work done for both ET and HIIT can be found in Table 4.1. The average work intensity (Joules/min) (234) was ~50% higher in HIIT versus ET over the entire 4-week training intervention. Forty-eight hours after the last exercise bout, animals were anaesthetized with pentobarbital (100 mg/kg body weight) and the heart rapidly removed. After weighing the heart, a small sample from the LV was embedded in OCT for histochemical analysis, while the remainder of the LV was rapidly frozen in liquid nitrogen and stored at -80 ºC. The tibia was also removed, dissected and measured for length.

Table 4.1. Exercise Protocols and Work for HS-ET and HS-HIIT rats at weeks 1-4 of training.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Week-1</th>
<th></th>
<th>Week-2</th>
<th></th>
<th>Week-3</th>
<th></th>
<th>Week-4</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HS-ET</td>
<td>HS-HIIT</td>
<td>HS-ET</td>
<td>HS-HIIT</td>
<td>HS-ET</td>
<td>HS-HIIT</td>
<td>HS-ET</td>
<td>HS-HIIT</td>
</tr>
<tr>
<td>Average Speed</td>
<td>m/min</td>
<td>15</td>
<td>20</td>
<td>18</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Grade (%)</td>
<td></td>
<td>0</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Exercise Time</td>
<td>min/day</td>
<td>30</td>
<td>8</td>
<td>45</td>
<td>15</td>
<td>45</td>
<td>23</td>
<td>45</td>
</tr>
<tr>
<td>Average Work</td>
<td>Joules/min</td>
<td>0.3</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>9</td>
</tr>
</tbody>
</table>

High sodium endurance training (HS-ET) and HS- high intensity interval training (HS-HIIT).

Total exercise time for both ET and HIIT remained consistent weekly; e.g., week 1 30 min; weeks 2-4 45 min.
4.3.3 Hemodynamics

Mean arterial blood pressure (MAP) was measured in conscious, restrained rats using a CODA® 2 tail-cuff system (Kent Scientific, Torrington, CT, USA) in a dark temperature-controlled room (22°C) in the morning. Rats were acclimatized on a minimum of three occasions prior to the study. On measurement days, conscious rats underwent 25 blood pressure measurements and MAP was averaged over the last 10 readings. HR was continuously monitored, with the corresponding last 10 measurement cycles averaged to calculate HR.

4.3.4 Western blotting

Cardiac muscle (25-50 mg) was homogenized as previously reported (269) and 5µg of homogenate loaded for SDS-PAGE. Proteins were separated on a 6%, 7.5%, 10% or 12% resolving gel as required to optimize for molecular weight separation, and transferred to polyvinylidene difluoride membrane (Roche, Laval, QC, CA). The following commercially available antibodies were used: total OXPHOS antibody cocktail (Abcam, Cambridge, MA, USA, ab110413, 1:500,), eNOS (Abcam, ab5589, 1:1000), VEGF (Abcam, ab46154, 1:1000), HIF1α (Abcam, ab463, 1:1000), alpha tubulin (Abcam, ab40742, 1:5000), muscle RING finger protein-1 (MuRF1; Santa Cruz Biotechnology, Dallas, TX, USA, sc-32920, 1:500), Muscle atrophy F-box (MAFbx; Santa Cruz, sc33782, 1:500), forkhead transcription factor-3a, Serine residue 253 (FOXO3a Ser253; Abcam, ab47285, 1:500), atrial natriuretic peptide (ANP; Abcam, ab180649, 1:500), BNP (Abcam, ab19645, 1:500) and beta-myosin heavy chain (β-MHC; Abcam, ab172967, 1:2000). All samples were detected from the same Western blot by
cutting gels and transferring onto a single membrane to limit variability. Equal loading of protein was verified using Ponceau staining as well as constant alpha tubulin. All blots were quantified using enhanced chemiluminescence (Perkin Elmer, Woodbridge, ON, CA) and quantified by densitometry (Alpha Innotech Fluorchem HD2, Fisher Scientific, Ottawa, ON, CA).

4.3.5 Citrate synthase activity

Citrate synthase (CS) activity was assayed in homogenates after lysing the mitochondria with 0.04% Triton X-100 and repeated freeze-thawing. CS activity was determined spectrophotometrically at 37°C and 412 nm as previously reported (311).

4.3.6 Histochemistry

LV tissue harvested from the subendocardial region of the lateral LV wall (the LV free wall) was embedded in OCT was cut into 10 µm cross sections with a cryostat (Thermo Fisher Scientific, Ottawa, ON, CA) maintained at -20°C. Capillary to fibre ratio quantification and cross sectional area (CSA) measurements were adapted from previous work (26, 43). Briefly, sections were fixed in 10% formalin buffered solution for 10 min, permeabilized with 0.5% TritonX-100 for 10 min, and then blocked in 10% goat serum for 30 min. Sections were incubated overnight in 1.5% goat serum with the appropriate primary antibodies specific for the endothelium (collagen IV, 1:50) and sarcolemma (dystrophin, 1:200) (Developmental Studies Hybridoma Bank, Iowa City, IA, USA). After three 5 min washes in PBS, sections were incubated for 1 hour in 3% goat serum with the appropriate fluorescent secondary antibodies (Life Technologies, Burlington, ON, CA). Nuclear counterstaining was also performed by incubating slides for 5 min in
4',6-diamidino-2-phenylindole (DAPI) prior to visualization. Capillaries were manually counted from 10 separate regions of each cross section (>50 fibres/cross section) and longitudinal fibres were discounted from analysis.

CSA was calculated by outlining all fibres from 10 separate regions of each cross section (>50 per type per muscle per animal). All imaging was performed with an Axio Observer Z1 fluorescent microscope and associated AxioVision software (Carl Zeiss).

To quantify LV fibrosis, sections were stained using picrosirius red as previously reported (14). Sections were imaged using an Olympus FSX 100 light microscope and images were acquired in Cell Sense software (Olympus, Tokyo, Japan). Using standard light microscopy, picrosirius red staining reveals collagen as red and cardiac fibers and cytoplasm as yellow. To quantify fibrosis, Cell Sense software was used to threshold images, which isolated total area of red (fibrosis) and total area of yellow (cytoplasm/fiber). Fibrosis was expressed as a percent of total tissue area. For each animal, fibrosis was determined by averaging 5 different locations within the LV.

4.3.7 Statistical Analysis

A one-way ANOVA, followed by a Newman-Keuls Multiple Comparison post-hoc analysis was used to determine the effects ET and HIIT in the setting of HS. A $p<0.05$ was considered statistically significant. All pre- and post-intervention MAP and HR measures were analyzed for significance using a paired t-test with the $\alpha$-value set to $P<0.05$. 
4.4 RESULTS

4.4.1 Animal phenotype and characterization of hypertension

We first aimed to characterize the phenotype of the hypertensive rats. There were no differences between groups of animals in MAP, HR or body weight prior to commencing the 4-week intervention (Table 4.2). Similar to previous reports, the LS group displayed mild hypertension (69, 245), while the consumption of the HS diet further increased (P<0.05) resting MAP ~10mmHg (Table 2), confirming the induction of overt hypertension in these animals.

Resting MAP was not altered by either 4 weeks of ET or HIIT (Table 2). A loss of body weight represents an early clinical marker of HF in these animals, however body weight was unaffected by either the HS diet or exercise training (Table 2), confirming the absence of pronounced clinical symptoms of late stage HF. HR and tibia length remained consistent between groups (Table 2).
Table 4.2. Morphometrics and hemodynamics of LS-SED, HS-SED, HS-ET and HS-HIIT rats before and after 4 weeks of training.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th></th>
<th></th>
<th></th>
<th>Baseline</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LS-SED</td>
<td>HS-SED</td>
<td>HS-ET</td>
<td>HS-HIIT</td>
<td>LS-SED</td>
<td>HS-SED</td>
<td>HS-ET</td>
<td>HS-HIIT</td>
<td></td>
</tr>
<tr>
<td>Mean Arterial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pressure (mmHg)</td>
<td>134 ± 2</td>
<td>135 ± 18</td>
<td>129 ± 2</td>
<td>137 ± 3</td>
<td>154 ± 3*†</td>
<td>163 ± 1*</td>
<td>165 ± 3*</td>
<td>165 ± 2*</td>
<td></td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>398 ± 15</td>
<td>417 ± 16</td>
<td>410 ± 10</td>
<td>420 ± 5</td>
<td>396 ± 14</td>
<td>358 ± 11*</td>
<td>364 ± 8*</td>
<td>369 ± 11*</td>
<td></td>
</tr>
<tr>
<td>Body weight (BW, g)</td>
<td>235 ± 7</td>
<td>245 ± 4</td>
<td>235 ± 6</td>
<td>245 ± 4</td>
<td>324 ± 6*</td>
<td>320 ± 5*</td>
<td>305 ± 7*</td>
<td>316 ± 4*</td>
<td></td>
</tr>
<tr>
<td>Heart weight (HW, g)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.91 ± 0.02</td>
<td>0.95 ± 0.01</td>
<td>0.94 ± 0.03</td>
<td>1.14± 0.03†</td>
</tr>
<tr>
<td>Tibia length (cm)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.15 ± 0.02</td>
<td>4.15 ± 0.02</td>
<td>4.2 ± 0.03</td>
<td>4.13 ± 0.02</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.8 ± 0.08</td>
<td>2.9 ± 0.08</td>
<td>3.1 ± 0.09</td>
<td>3.6 ± 0.1</td>
</tr>
</tbody>
</table>

Low sodium sedentary (LS-SED), high sodium SED (HS-SED), HS endurance training (HS-ET) and HS high intensity interval training (HS-HIIT). * vs. baseline; † vs. all other groups; P<0.05. Data are means ± SEM.
As mitochondrial content is reduced in end stage HF, we investigated the content of 5 OXPHOS proteins and CS activity. After 4 weeks of HS, OXPHOS protein content and CS activity were not altered, suggesting the absence of reductions in mitochondrial content (Figure 4.1). Altogether, these data confirmed that after 4 weeks of consuming HS, the Dahl/SS rat begins to display hypertension, as opposed to end stage HF, and therefore represents an ideal model/age to determine the effect of exercise intensity on the molecular adaptations in the heart in the early stages of HF progression.

**Figure 4.1. Mitochondrial content and citrate synthase (CS) activity in the left ventricle (LV).** A. Representative western blot of LV OXPHOS proteins in low sodium sedentary (LS-SED), high sodium SED (HS-SED), HS endurance training (HS-ET) and HS high intensity interval training (HS-HIIT) B-E. Density quantifications of LV OXPHOS in LS-SED, HS-SED, HS-ET and HS-HIIT, demonstrating no change in mitochondrial content in both ET and HIIT. F. CS activity as expressed per gram wet weight. HS had no effect on CS activity. Data are means ± SEM.
4.4.2 Effect of training on fibrosis

We examined myocardial fibrosis to determine if ET and HIIT were effective at reducing the development of fibrosis in Dahl/SS rats. While the consumption of HS did not further increase fibrosis as compared to LS, ET training reduced (P<0.05) fibrosis by ~40% (Figure 4.2). In contrast to ET, HIIT did not ameliorate the development of fibrosis (Figure 4.2). These data suggested that ET preferentially protects the heart from the development fibrosis in response to hypertension.

Figure 4.2. Left ventricular (LV) fibrosis in response to high sodium (HS) with endurance training (ET) and high intensity interval training (HIIT). A. Composite wide field microscopy images of LV; with picrosirius red stain for fibrosis; low sodium-SED (LS-SED; top left), HS-SED (top right), HS-ET (bottom left) and HS-HIIT (bottom right). B. Quantification (% area) of fibrosis; HS-ET demonstrates significantly less % area of fibrosis vs. all other groups: * vs. LS-SED, HS-SED and HS-HIIT, P<0.05 C. Data are means ± SEM.
4.4.3 The effects of training on hypertrophy and markers of HF

Consumption of a HS diet by Dahl/SS rats induces cardiac hypertrophy after 11 weeks (269), however in the current study, 4 weeks of HS was not sufficient to induce significant hypertrophy in the sedentary group (Table 4.2 and Fig. 4.3A). Also, ET training did not affect heart weight. Surprisingly however, HIIT increased heart weight by ~20% (Table 4.2 and Fig. 4.3A) demonstrating that HIIT initiated the development of cardiac hypertrophy. Similar to cardiac weight, HS and ET did not affect CSA (Fig. 4.3B and C). In contrast, HIIT resulted in an ~20% reduction in CSA (Fig. 4.3B and C), indicative of alterations in cardiac fibres and potential for pathological remodeling.
Figure 4.3. Effect of high sodium (HS) with endurance training (ET) and high intensity interval training (HIIT) on heart weight and cardiac fibre cross-sectional area. A. Heart weight normalized to tibia length (HW/TL) in low sodium (LS), HS-ET and HS-HIIT. HS-HIIT demonstrates a significant increase in HW/TL when compared to all other groups: * vs. LS-SED, P<0.05. B. Composite wide field microscopy images of left ventricle; LS-SED (top left), HS-SED (top right), HS-ET (bottom left) and HS-HIIT (bottom right). C. Left ventricle cardiac fibre cross-sectional area in LS-SED, HS-SED, HS-ET and HS-HIIT. HS-HIIT demonstrates a decrease in cross-sectional area when compared to HS-SED and HS-ET: * vs. HS-SED, vs. † HS-ET; P<0.05. Data are means ± SEM.
To further investigate the potential that HIIT exacerbated the development of HF, we determined the protein expression of BNP, ANP and β-MHC (Fig. 4.4). HIIT animals demonstrated ~50% higher BNP content (P<0.05) when compared to LS-SED, HS-SED and HS-ET suggesting a progression towards HF (Figure 4.4B). ANP content was unchanged in response to HS or exercise (Fig. 4C). β-MHC content remained unchanged in all groups (Figure 4.4D).

**Figure 4.4. Western blot analysis of brain natriuretic peptide (BNP), atrial natriuretic peptide (ANP) and β-myosin heavy chain (β-MHC).** A. Representative blots; α-tubulin is presented as a loading control. B. Density quantifications of BNP of low sodium sedentary (LS-SED), high sodium SED (HS-SED), HS endurance training (HS-ET) and HS high intensity interval training (HS-HIIT), demonstrating an decrease in protein as a result of HS-HIIT; • vs. LS-SED, HS-SED and HS-ET; P<0.05. C. Density quantifications of ANP protein. D. Density quantifications of protein. Data are means ± SEM.
Molecular regulators of muscle hypertrophy and atrophy have also been linked to the transition from hypertrophy to HF (99, 351), however, we examined several proteins associated with atrophy, and show that FOXO3A, MuRF1 and MAFbx were not altered in any condition (Figure 4.5A-D).

Figure 4.5. Western blot analysis for FOXO3a (Ser253), MuRF1 and MAFbx. A. Representative blots; α-tubulin is presented as a loading control. B. Density quantifications of FOXO3a (Ser253) protein in LV of low sodium sedentary (LS-SED), high sodium SED (HS-SED), HS endurance training (HS-ET) and HS high intensity interval training (HS-HIIT). C. Density quantifications of MAFbx. D. Density quantifications of MuRF1 protein. Data are means ± SEM.

Altogether, these data suggest that while ET did not elicit a hypertrophic response, HIIT induced cardiac remodeling associated with hypertrophy in the presence of elevated
BNP levels indicating that the remodeling that occurred in response to HIIT was pathological.

4.4.4 Effects of training on cardiac capillarization

While HS did not reduce capillary/fibre ratios at this early stage of disease progression, animals that underwent ET had a higher (P<0.05) capillary/fibre ratio by ~20%, while in contrast HIIT had no effect (Figure 4.6A and B). To further support these data, we examined the protein expression of known angiogenic factors (Figure 4.7). Specifically, we found that ET increased (P<0.05) the protein content of eNOS within the heart (Figure 4.7B). In addition, HIF1α, a protein responsive to hypoxia, was elevated in both HS-SED and HS-HIIT groups (Figure 4.7C). In contrast, ET training, and the resulting angiogenesis, may have contributed to the decreased expression of HIF1α because ET reduces myocardial oxygen demand, and therefore would lower hypoxic stimuli (Figure 4.7C). VEGF protein was not altered in any group (Figure 4.7D). Taken together, the current data indicated that ET, but not HIIT, induced angiogenesis within the LV of Dahl/SS rats.
Figure 4.6. Left ventricular (LV) capillary to fibre ratio. A. Composite wide field microscopy images of LV; of low sodium sedentary (LS-SED-top left), high sodium SED (HS-SED-top right), HS endurance training (HS-ET-bottom left) and HS high intensity interval training (HS-HIIT-bottom right) B. LV capillary to fibre ratio demonstrating a significant increase in response to HS-ET; * vs. LS-SED, HS-SED and HS-HIIT; P<0.05. Data are means ± SEM.
Figure 4.7. Western blot analysis of eNOS, HIF1α, and VEGF. A. Representative blots; α-tubulin is presented as a loading control. B. Density quantifications of eNOS protein in LV of low sodium sedentary (LS-SED), high sodium SED (HS-SED), HS endurance training (HS-ET) and HS high intensity interval training (HS-HIIT), demonstrating a significant increase as a result of ET, * vs. all other groups; P<0.05 C. Density quantifications of HIF1α, demonstrating a significant increase in response to both HS and HS-HIIT; * vs. LS-SED; ‡ vs. HS-ET. D. Density quantifications of VEGF protein. Data are means ± SEM.
4.5 DISCUSSION

The current data provides evidence, that before overt HF develops in Dahl/SS rats fed a HS diet, classical ET: i) ameliorated fibrosis, and ii) induced coronary angiogenesis. In stark contrast, HIIT induced: i) LV hypertrophy, ii) a reduction in cardiac fibre CSA, and iii) increased the expression of BNP, a protein associated with HF, without altering fibrosis or angiogenesis. Altogether, the current data suggested that ET and HIIT induce divergent signals in the heart, and the potential that HIIT exacerbated the development towards HF in hypertensive Dahl/SS rats.

4.5.1 Left ventricular fibrosis

Cardiac remodeling, and in particular an increase in fibrosis, is associated with diastolic dysfunction (29, 269). In the current study, significant fibrosis was apparent in the LV of LS and HS fed rats, with the exception of ET trained rats which displayed ~40% less fibrosis. This indicated that ET, but not HIIT, prevented the development of fibrosis in the Dahl/SS rats. The relationship between fibrosis and diastolic dysfunction has long been defined, and many studies have successfully reduced diastolic stiffness by targeting the signaling factors involved in the development of fibrosis, such as transforming growth factor-β, the sodium-hydrogen exchanger, angiotensin II receptor-mediating signaling, and chymase (126, 165, 189). HF manifestation in the Dahl/SS rat has been largely characterized as diastolic dysfunction, and fibrosis is considered to be a mediator of this process. Interventions such as exercise and pharmacological compounds (e.g., resveratrol, angiotensin receptor blockers) reduce the formation of fibrosis and are associated with improved diastolic function in Dahl/SS rats (38, 50) and other animal models (197, 213, 269). Therefore, the current data suggests that ET promoted a
phenotype that would improve LV relaxation, decrease end diastolic pressure and improve overall filing, which are characteristics of improved diastolic function. The signals that have elicted the divergent results in fibrosis between ET and HIIT remain unclear.

While alterations in blood pressure could represent a potential explanation, in the current model, neither ET nor HIIT altered resting blood pressure (Table 4.2). However, it is unclear how blood pressure was altered in these animals during or immediately after exercise. Regardless, our results indicate that only ET prevented fibrosis, data that is supported by previous findings that ET protects against, and reverses, the development of fibrosis in Dahl/SS rats in the later stages of disease progression (40). Therefore, with respect to fibrosis, ET represented the optimal exercise intervention in presence of hypertension.

4.5.2 Cardiac hypertrophy and cardiac fibre cross-sectional area

Pressure-induced cardiac remodeling is initially associated with concentric cardiac hypertrophy that is beneficial. While HS did not cause hypertrophy, HIIT elicited both LV hypertrophy and a reduction in cardiac fibre CSA, responses not observed in ET animals. Cardiac hypertrophy occurs in response to various stimuli such as chronic exercise training (volume overload) and hypertension (pressure overload), and the hypertrophy that develops is specific to the stimuli that caused the increase in LV mass. Physiological hypertrophy is characterized by normal organization of cardiac structure and normal or enhanced cardiac function. In contrast, pathological hypertrophy is commonly associated with the upregulation of fetal genes (e.g., β-MHC) (167, 259), fibrosis and cardiac dysfunction and is recognized as an independent risk factor for morbidity and mortality.
(36, 130, 183, 202). Early progression to HF involves the development of hypertrophy and a reorganization of cardiomyocytes. The etiology of the reduction of CSA in the HIIT animals remains unclear, and was an unexpected finding. However, given the various stages of remodeling as a result of hypertension, this may be due the timing of our study. The animals were hypertensive for 4 weeks, and to our knowledge there are no studies that have investigated the adaptations occurring in the cardiomyocytes at this early time point.

In the setting of disease, initially myocardial hypertrophy is a compensatory mechanism, by which the LV adapts to an increased systolic load (e.g., hypertension, aortic stenosis), with the aim of normalizing/restoring LV wall stress and the maintenance of Q (127). While in the early stages of disease progression, HS did not cause hypertrophy in the SED or ET animals, while HIIT caused significant hypertrophy to develop. This strongly supports the notion that in the presence of hypertension, HIIT promotes pathological remodeling of the LV.

4.5.3 Brain and atrial natriuretic peptides and β-myosin heavy chain

Neurohormonal activation is a physiological response and important prognostic marker in patients with chronic HF. Specifically, in HF an elevated BNP level is associated with impaired left ventricular ejection fraction and early mortality (169). HIIT animals demonstrated significantly higher LV BNP levels when compared to ET or HS alone. In response to increased filling pressure BNP is released primarily from the LV promoting vasodilatation and natriuresis (216). On the other-hand, ANP is released in response to exercise and remains elevated in the presence of HF, but returns to baseline post-exercise in healthy individuals (360). Therefore, the fact that we did not see an
elevation in ANP is not surprising, as the time point studied represented the early stages of HF progression. Furthermore, we did not detect an increase in β-MHC typically seen in advanced models of HF (167, 259) confirming the absence of overt HF in our animals after 4 weeks of HS. The elevation of BNP in the HIIT group, along with hypertrophy and alterations in CSA, again suggests that HIIT promoted a phenotype that may transition into end-stage HF at an accelerated rate.

4.5.4 Capillarization, eNOS, and HIF1α content

The balance between cardiac growth and coronary angiogenesis is a key determinant of cardiac function, and disruption of this balance is implicated in the transition from physiological to pathological hypertrophy and HF (303). In addition to protecting against fibrosis, ET promotes angiogenesis in this model, and an increase in LV capillary/fibre ratio (65). eNOS is found primarily in the vascular endothelium and a concomitant loss of endothelium and eNOS content is associated with various disease states, such as hypertension, diabetes and HF (78, 141, 184). Endurance exercise is effective at restoring vascular reactivity and eNOS levels in these disease states (85, 212, 359). Although we did not see a significant reduction in eNOS due to HS at this early time point in the disease progression, ET increased eNOS content, while HIIT did not. This may also have implications for the development of pathological hypertrophy seen in our HIIT animals, as previous studies have demonstrated that nitric oxide (NO) production through eNOS plays an important role in the regulation of cardiac hypertrophy (359). The studies in which eNOS signaling was augmented by the administration of a calcium antagonist or angiotensin-I converting enzyme inhibitors demonstrated improvements in myocardial remodeling and HF (187, 282).
The elevation of eNOS protein content and increase in capillary/fibre ratio in the ET animals occurred together with a decrease in HIF1α protein content. HIF1α is considered to be the mediator of physiological and patho-physiological responses to hypoxia and exercise training. HIF1α protein content decreases in response to chronic exercise training in both heart and skeletal muscle (190, 250, 296). The physiological mechanisms by which cells respond to hypoxic stimuli are only beginning to be elucidated at the molecular level. However, the role of HIF1α in the activation of VEGF gene transcription in hypoxic cells is well established (94, 296). Hypoxia, and the molecular responses to hypoxia, play an important role in the pathology of major causes of mortality, such as myocardial ischemia, and chronic heart and lung diseases (296).

The development of myocardial hypertrophy transiently causes hypoxia and HIF1α accumulation, leading to the initiation of angiogenesis (284). Angiogenesis supports the growth and survival of the newly thickened myocardium. However, as hypertrophy continues to develop, prolonged hypoxia leads to p53 accumulation, which mediates the inactivation of HIF1α and results in eventual cardiac failure due to further inhibition of angiogenesis (284). The fact that our animals demonstrated hypertrophy in response to HIIT, together with an increase in HIF1α protein, and without a concomitant increase in VEGF protein, suggests that the adaptive molecular signals are in the early stages and prior to compensatory angiogenesis typically seen in myocardial hypertrophy. The lack of response of VEGF protein content was not expected, although it is known that the effect of training on VEGF protein is temporal (222). Angiogenesis as a result of chronic exercise training is dependent on the complex coordination of the metabolic signals responsible for both positive and negative angiogenic factors. While studies in
healthy rodents have shown that interval and moderate exercise training had similar effects on LV capillary density (238), to our knowledge the present data are the first to demonstrate that HIIT does not induce angiogenesis in the hypertrophied LV in the presence of hypertension prior to the development of end stage HF.

The current data support the supposition that, classical ET attenuates cardiac fibrosis, ameliorates pathological hypertrophy, and stimulates angiogenesis in the LV in the presence of hypertension, supporting the longstanding belief in the effectiveness of ET in disease. In contrast, HIIT did not reduce fibrosis or promote angiogenesis. Critically, myocardial weight increased along with an elevated BNP expression as a result of HIIT, all of which are consistent with pathological remodeling and deleterious cardiac function. The negative impact of HIIT on the LV in the presence of hypertension highlights the need for further research on the effects of HIIT in the presence of disease. Altogether, our data demonstrates that ET and HIIT induce divergent molecular signatures in the hearts of hypertensive rats.
CHAPTER FIVE

A CALL FOR ADULT CONGENITAL HEART DISEASE PATIENT PARTICIPATION IN CARDIAC REHABILITATION
5.1 INTRODUCTION

Given advancements in the diagnosis and management of infants born with congenital heart disease, 90% are now expected to reach adulthood: the adult population with congenital heart disease is continuously growing. It has been demonstrated that exercise capacity diminishes with advancing age in congenital heart disease (74), therefore, new therapies targeting improvements in exercise capacity should be considered. Historically, adult congenital heart disease (ACHD) patients have not participated in traditional cardiac rehabilitation (CR) programs. Exercise training in CR is effective at improving exercise capacity in chronic heart failure, a group that appears to share many factors related to low exercise capacity with ACHD patients (106).

Impaired exercise capacity is a common feature in ACHD, and is reduced by up to 50%, even in patients with ‘simple’ lesions (74). Diminished exercise capacity [e.g., metabolic equivalents (METs) achieved on a graded exercise test] has been correlated with mortality and hospitalization in this population (74), further suggesting that appropriate interventions should be focussed on improving exercise capacity. The cause of reduced exercise capacity in ACHD is multi-factorial, including both central (e.g., inadequate Q) and peripheral (e.g., low skeletal muscle metabolic capacity) factors, of which some or all may respond to exercise training. It is also important to recognize that education regarding exercise and its benefits and limitations is suboptimal, even in specialist clinics (314) where more than 70% of ACHD patients report a moderate to extreme level of concern regarding exercise (135). Previous advice in childhood, inadequate advice in adulthood and a high level of concern may lead to a sedentary
lifestyle, thus exacerbating any physiological limitations present in ACHD. Although the majority of ACHD patients do not meet recommended national guidelines for participation in exercise for health (314), as a group ACHD patients have expressed interest in exercise and a desire for more formal exercise advice (135).

American and European ACHD guidelines recommend that patients participate in some form of exercise [5, 6], guidance that is largely based on theoretical knowledge rather than a scientific foundation supporting both efficacy and safety. Therefore, we evaluated the impact of a traditionally structured CR program on ACHD patients of moderate to great complexity. Traditional evidence-based CR programs involve medically-supervised exercise prescription, in addition to nutritional, vocational, psychological and pharmacological counselling provided by an interdisciplinary team tailored to a patient’s risk profile and disease state. However, no reports have assessed the safety and effectiveness of including ACHD patients in a traditionally structured CR program.
5.2 METHODS

5.2.1 Experimental Design

A retrospective analysis of ACHD patients who were referred to the Cardiovascular Rehabilitation and Prevention Program of the Peter Munk Cardiac Centre, University Health Network in Toronto, Canada between 2005 and 2010 was performed. With regard to safety, we investigated the occurrence of any adverse events related to exercise (e.g., arrhythmia or exacerbation of symptoms, such as shortness of breath, chest discomfort, etc.). To evaluate effectiveness, we compared graded exercise test results (Bruce treadmill protocol) and treadmill-walking times and speeds (miles per hour) pre and post-CR. Data were analyzed using paired sample t-tests. This study received institutional ethics approval.

5.2.2 Subject Characteristics and Exercise Intervention

The study population included 11 ACHD patients (4 female and 7 male, age 32.6±12.2 years). Seven patients had defects of moderate complexity and 4 had defects of great complexity (344). Table 5.1 presents the specific diagnoses and treatment history of all 11 patients. As with all CR participants, ACHD patients received exercise prescription appropriate for their medical diagnoses (e.g., self-limiting, progressive cardiovascular exercise). There were no alterations or deviations from the traditional CR program (e.g., length of program, frequency of interaction with health professionals, education regarding exercise and diet, scheduling of exercise sessions were all consistent with current CR guidelines).
<table>
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<th>Primary Diagnosis</th>
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<th>Arrhythmia history</th>
<th>Transplant assessment</th>
<th>Documented anxiety/depression</th>
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<td>AF</td>
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<td>complete AV block</td>
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<td>AF/VT/VF</td>
<td>H/dL</td>
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<td>AF</td>
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<td>TGA repaired (Mustard procedure)</td>
<td>1</td>
<td>intra-atrial reentrant tachycardia</td>
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<td>yes</td>
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</table>

| Incidence | 26 | 64% | 27% | 64% |

ToF, tetralogy of Fallot; TR, tricuspid regurgitation; PA, pulmonary artery; VT, ventricular tachycardia; CoA, coarctation of the aorta; AS, aortic stenosis; BAV, bicuspid aortic valve; AVSD, atrioventricular septal defect; sAS, subaortic stenosis; AV, atrioventricular valve; PH, pulmonary hypertension; ASD, atrial septal defect; VSD, ventricular septal defect; AF, atrial fibrillation; VF, ventricular fibrillation; H/dL, heart lung transplantation; ccTGA, congenitally corrected transposition of the great arteries; RV, right ventricle; TGA, complete transposition of the great arteries.
5.3. RESULTS

5.3.1 Outcomes and Improvements in Exercise Capacity

Seven patients completed the full CR program (minimum 32 exercise sessions over 4-6 months). Reasons for non-completion were; 1 patient returned to university studies, 1 returned to work; 1 was investigated for right heart failure symptoms (gastrointestinal upset) that preceded CR initiation, and 1 was non-adherent with prescribed diuretics and therefore ineligible for CR participation. Among all participants across a total of 369 sessions, there were no occurrences of adverse events during CR, despite the fact that 64% of patients had a prior history of atrial and/or ventricular arrhythmias (Table 1). Exercise capacity was significantly higher after full completion of CR (p<0.05, n=7, Figure 5.1 A). Treadmill walking time and speed were significantly higher when comparing the first to the last CR session for all 11 patients, even if they did not complete the full program and undergo a post-CR graded exercise test (p<0.05, Figure 5.1 B, p<0.05, Figure 5.1 C, n=11).
Figure 5.1. The effects of cardiac rehabilitation on ACHD patients. A: Metabolic equivalents (METs) achieved on the Bruce Treadmill Protocol at the start of cardiac rehabilitation (pre-CR) and upon completion of the full program (post-CR), n=7. B: Treadmill walking speed in kilometers per hour at start of cardiac rehabilitation (pre-CR) and at the last session of cardiac rehabilitation (post-CR), n=11. C: Treadmill walking time in minutes at start of cardiac rehabilitation (pre-CR) and at the last session of cardiac rehabilitation (post-CR), n=11. Data are expressed as mean ± S.E.M., *p<0.05 pre-CR vs. post-CR.

5.4 DISCUSSION

In summary, a traditionally structured CR program can provide a safe and effective intervention for ACHD patients with varying degrees of disease severity. The main focus of exercise training in a CR setting is moderate level dynamic exercise, which is inherently safe. While this study provides support for the recently published guidelines endorsing participation in exercise as part of the management of ACHD (13, 344) further research investigating the physiological mechanisms responsible for the improvement in exercise capacity is required. It is unknown whether improvements in this group of ACHD patients were due to adaptations in central or peripheral physiological
mechanisms, or a combination of both (e.g., improvements in Q and/or increases in skeletal muscle mitochondrial function). Regardless, CR programs are encouraged to include ACHD patients, and clinicians are encouraged to consider CR as part of the ongoing management of ACHD patients, as it is a safe and effective intervention that results in improved exercise capacity.

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology (54).
CHAPTER SIX

INTEGRATIVE DISCUSSION
6.1 INTRODUCTION

Although the portion of our genome determining basic anatomy and physiology has remained relatively unchanged over the past 40,000 years, our diet and physical activity patterns have become increasingly divergent from our ancestral hunter-gatherers. It is estimated that Stone Age men and women walked, or ran, 5-10 miles per day in search of water, food and protection from the elements (61), while current estimates of physical activity indicate the majority of adults in Western societies fail to accumulate 30 min of moderate daily exertion (57). The growing number of people living with, and the healthcare costs associated with, cardiovascular diseases, as a result of increasingly sedentary lifestyles further demonstrates this point.

There is little doubt that high levels of physical fitness, or an improvement in fitness through chronic exercise training has profound effects on various physiological systems, including the respiratory, cardiovascular, and metabolic (136, 179, 182, 230). While the effects of exercise capacity (25) and the impact of exercise training on mortality (24) are well recognized, there is much to be learned about the optimal intensity of exercise in the setting of disease. As demonstrated in this thesis, this is particularly evident in pathologies that include compromised cardiovascular function. This thesis provides evidence that ET had conclusive overall positive effects in settings of disease (hypertension and ACHD), however, the adaptations as a result of HIIT were diverse. While these results remain to be demonstrated in humans, they do encourage a cautionary approach when prescribing HIIT in clinical settings, and illustrate the critical need for further research in this area.
The current thesis demonstrated that in a rodent model of hypertension, HIIT promoted pathological adaptations. In the skeletal muscle, there was an absence of a coordinated oxidative response to HIIT, and in cardiac muscle, HIIT promoted fibrosis, hypertrophy in the absence of angiogenesis, and increased molecular markers of HF. This suggests that in a model of hypertension, HIIT promotes a pathological phenotype that may accelerate the progression towards overt HF. In contrast to HIIT, ET promoted a phenotype in skeletal and cardiac muscle associated with reduced risk of disease and improved outcomes. Specifically, ET improved the oxidative capacity in both tissues, and prevented pathological cardiac hypertrophy and fibrosis. Due to the lack of convincing data around the safety and effectiveness of HIIT in the presence of compromised cardiac function, as well as the pathological adaptations outlined in the Dah/SS rat in the current thesis, only ET was utilized in a patient population with extremely complex and compromised heart function. Importantly, ET was effective at improving exercise capacity and tolerance in ACHD patients. Therefore, the current thesis supports the longstanding belief that ET is beneficial in clinical populations (87, 206) with compromised cardiac performance, while HIIT may stimulate at least some negative adaptations.

It remains to be determined why ET and HIIT have divergent effects in the presence of hypertension. However, since historically “intermittent” exercise is known to be effective and safe within a clinical setting (63), the divergent effects may manifest as a result of the “high intensity” nature of the exercise. The following discussion will focus on the potential for several mechanisms to account for the detrimental effects of HIIT, including: i) increased reactive oxygen species; ii) alterations in the local regulation of
blood flow; iii) attenuated baroreceptor sensitivity, and finally; iv) regulation of mean arterial pressure. Combined, these responses may explain the apparent negative adaptations following HIIT in a model of hypertension.

6.1.1 Mechanisms within skeletal muscle and the importance of ROS

Mitochondrial biogenesis is activated by various mechanisms, including Ca\(^{2+}\), energy turnover (AMPK), ROS, epinephrine and general stress-activated kinases (e.g., P38 MAPK, ERK1/2) (148, 219). Previous work in healthy individuals has shown similar activation of these signals following ET and HIIT, and therefore these signals may be saturated above a moderate intensity of exercise (38, 39, 110, 111). In addition, these processes are likely not influenced by compromised blood flow or hypoxic conditions, as these conditions would be expected to increase several of these signaling pathways, however exercise-induced desaturation with COPD (306), and athletes training in hypoxic situations maintain mitochondrial content and/or induce normal responses (199). This likely explains why ET and HIIT both increased mitochondrial content to the same degree in the current thesis in the presence of hypertension.

Mitochondrial biogenesis and fibre type transition are both linked to PGC-1\(\alpha\) regulation (18), and therefore it could have been expected that the fibre type transition would also have been similar in response to ET and HIIT. However, in the current study, ET promoted an increased expression of hybrid type IIAX fibres, while HIIT promoted an increased expression of type IIB fibres. While this may seem paradoxical, a recent report has divorced the relationship between PGC-1\(\alpha\) and fibre type, suggesting additional regulation on fibre type beyond the activation of PGC-1\(\alpha\) (123). Since fibre type adaptations are similar in healthy individuals in response to ET and HIIT, the divergent
effects reported in this thesis appear to be specific to hypertension. In addition, a reduction in skeletal muscle hybrid fibres and a transition towards a faster MHC isoform has been linked to cardiovascular disease. While the underlying mechanisms remain unknown, a logical inference would be that an intensity-dependent metabolite within skeletal muscle contributes to this response.

For instance, exercise causes an increase in various metabolic intermediates, including ADP\textsubscript{f}, AMP\textsubscript{f}, and IMP\textsubscript{f}. The degree to which each is elevated is dependent on intensity, or the rate of ATP hydrolysis (Table 6.1). Compared to after 10 min of ET, ADP\textsubscript{f} is 1.5 then 3 fold higher and AMP\textsubscript{f} is 3 then 10 fold higher following HIIT and sprint interval training (SIT), respectively (150, 228, 240). Therefore, after either HIIT or SIT, a significant increase in AMP\textsubscript{f} could lead to higher levels of IMP\textsubscript{f}, (139) and therefore flux through xanthine oxidase, which is a near-equilibrium enzyme (Figure 6.1).

<table>
<thead>
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<th>Parameter</th>
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<th>HIIT</th>
<th>SIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCr mmol/kg/dry wt</td>
<td>47</td>
<td>13</td>
<td>7.6</td>
</tr>
<tr>
<td>ADP free umol/kg/dry wt</td>
<td>230</td>
<td>366</td>
<td>650</td>
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<tr>
<td>AMP free umol/kg/dry wt</td>
<td>2</td>
<td>7</td>
<td>20</td>
</tr>
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</table>

*Phosphocreatine, PCr; adenosine diphosphate, ADP; adenosine monophosphate, AMP; sprint interval training, SIT. Adapted from (150, 228, 240).*
Figure 6.1. *AMP stimulated formation of reactive oxygen species (ROS).* Adenosine monophosphate, AMP; inositol monophosphate, IMP; purine nucleotide phosphate, PNP; xanthine oxidase, XO. XO acts a mini electron transport chain where O$_2$ is the final electron acceptor during the XO reaction, which leads to the formation of H$_2$O$_2$.

This will increase ROS production, as xanthine oxidase acts a mini electron transport chain, where O$_2$ is the final electron acceptor leading to the formation of H$_2$O$_2$. H$_2$O$_2$, is of particular interest due to its’ higher stability versus oxygen radicals and its ability to cross biological membranes (35). In addition, NADPH oxidase flux is increased during exercise, and therefore so is the production of superoxide. Superoxide reacts with NO to make peroxynitrite, which reduces NO bioavailability. While the presence of ROS is required for many normal physiological signaling processes, excessive oxidative stress has been linked to cardiovascular diseases, including hypertension (315) and HF (44,
In addition, exercise training in conditions with chronically elevated levels of ROS (e.g., aging) attenuates the beneficial effects of exercise, suggesting extremely high levels of ROS are detrimental in situations with oxidative stress/diminished antioxidant defenses (327, 328). This may explain why HIIT exerted some negative adoptions in the current thesis, as the higher metabolite profiles following HIIT likely leads to increased ROS production. While ROS was not directly measured in the current study to support this supposition, HIF1α content was increased following HIIT, and ROS activates the expression of HIF1α (218). Further support for an increased production of ROS after intense exercise is provided from the reduction in NO-bioavailability, as measured by impaired flow-mediated dilation (FMD), after intense (19, 71, 122), but not moderate levels of exertion (161).

Vascular adaptations to exercise training may be dependent on repetitive increases in shear stress, which may be offset by higher power outputs due to oxidative stress. The mechanisms by which an elevation in HIF1α did not result in angiogenesis may be explained by a local redox imbalance, as ROS stabilizes HIF1α by preventing hydroxylation by propyl hydroxylases, eventual and ubiquination and breakdown via the von Hippel-Lindau complex (215). Therefore, the elevation of HIF1α in response to HIIT in our animals supports the likelihood that disproportionate levels of ROS exist in both cardiac and skeletal muscle. Further evidence for this argument comes from the regulation of VEGFR2, where low levels of ROS activate the receptor (35), however disproportionately high levels of ROS inactivate the receptor (164). In this manner, ROS may activate HIF1α without a functional effect, as HIF-mediated increase in VEGF cannot activate the VEGFR. This may explain why skeletal muscle capillary to fibre ratio
was reduced in response to HIIT in the current thesis. In addition, ROS (specifically H$_2$O$_2$) has been shown to cause capillary rarefaction in both the Dahl/SS (322) and spontaneously hypertensive rodent models of hypertension (6). The cardiac isoform of NADPH oxidase (NOX4), is upregulated in the hearts of spontaneously hypertensive rodents, and the use of antioxidants prevents impaired angiogenesis in this model (362).

A reduction in skeletal muscle capillary to fibre ratio in skeletal muscle may increase afterload, thereby increasing the work of the heart. HF has also been linked to oxidative stress within the cardiac muscle. In addition, since HR has a linear relationship with power, HIIT training will elicit a higher HR, increasing the potential for ROS production within the heart in a similar manner to skeletal muscle. Presumably, since this occurred in the presence of hypertension-induced oxidative stress, HIIT induced ROS may account for the observed loss of capillarization. The increase in ATP turnover as a result of a higher afterload would be negatively influenced by a reduction in capillary density in the myocardium due to increase in O$_2$ diffusion distances from the capillary to the mitochondria, exacerbating the production of ROS during exercise. Future research should focus on the potential for ROS to mediate the negative adaptations observed following HIIT, as this could have implications for other pathologies that are associated with oxidative stress (e.g., aging, insulin resistance and diabetes) (4, 181).

6.1.2 Local regulation of skeletal muscle blood flow

In addition to ROS-mediated process within cardiac and skeletal muscles, HIIT may compromise venous return during passive recovery, due the intensified elevation of factors responsible for local blood flow distribution. This would be expected to exacerbate the production of ROS, thereby contributing the negative adaptations observed in the
presence of hypertension. This situation could also create transient hypoxic signaling in both skeletal and heart muscles, that in normal individuals have no effect, but in the presence of hypertension results in negative responses. Skeletal muscle blood flow is regulated through multiple redundant systems that converge on the vascular smooth muscle (VSM). The most potent signal for VSM relaxation is a reduction in intracellular Ca\(^{2+}\) due to uptake via the sarcoplasmic reticulum, or extrusion via Ca\(^{2+}\) pumps on the cell membrane (285). However, important Ca\(^{2+}\)-independent mechanisms also play a role in the regulation of muscle blood flow. Local metabolic control is critical for the maintenance of MAP, as muscle blood flow can increase up to 100-fold over resting values (280). Areas in which ET and HIIT may differ in their impact on muscle blood flow include the three main signals responsible for increased blood flow: metabolic, endothelium-dependent, and myogenic vascular control. Enhanced muscle activity results in the generation of mediators, or ‘metabolic signals’, including increases in pCO\(_2\), lactate, K\(^+\), and adenosine (see Figure 6.2).

![Diagram](image)

**Figure 6.2.** “Classical” metabolic factors leading to dilation in vascular smooth muscle. Adapted from (285).
Doubling of $P_{CO2}$ and increases in lactate of 2mmol/L within 30 sec of intense exercise have been documented (33). Muscle action potentials lead to the release of $K^+$ ions from skeletal muscle cells, and under conditions of high rate action potentials (e.g. HIIT), the re-uptake of $K^+$ cannot be compensated for by the $Na^{2+}-K^+$ATPase leading to a further rise in $K^+$. In fact, interstitial $K^+$ can reach 10 mmol/L early on in exercise and remain elevated throughout (285). The hydrolysis of ATP leads to a rise in adenosine concentration, which acts as a potent vasodilator, that is proportional to exercise intensity. All of these substances can easily diffuse to the VSM of adjacent arterioles thereby increasing muscle blood flow. In response to HIIT, whereby there is widespread motor unit recruitment, and high rates of ATP turnover, the local metabolic environment increasing blood flow during and post-exercise would be more potent, potentially impairing venous return, and therefore SV as a result of HIIT.

The endothelium also plays a vital role in the control of vascular tone and blood flow. Specifically the endothelial cells (EC) can detect blood-borne, as well as physical forces such as shear stress and vascular stretch (176). Elevation in MAP and flow stimulate the release of endothelial-derived factors such as nitric oxide (NO), prostacyclin (PGI$_2$) and endothelial derived hyperpolarizing factor (EDHF), all of which lead to vasodilation of VSM (180, 252, 345). These factors work through stretch activated receptors on EC. During the exercise, HIIT may increase MAP and flow to a greater degree than ET, thereby increasing endothelium-dependent vasodilation during or post exercise, increasing the demand on the heart, which may not be able to sustain MAP, although this remains speculative. It should be noted, however, that the HIIT animals showed significant signs of fatigue (e.g., dropping of hind limbs) throughout the 4th week.
of training, and struggled to complete the exercise sessions, further suggesting a potentially acute maladaptive response to HIIT.

The myogenic mechanisms proposed to increase muscle blood flow work primarily through changes in transmural pressure as a result of muscle contraction, or the “muscle pump”. Potential mechanisms of action for the muscle pump include venous emptying produced by muscle contraction promoting flow through a widening of the pressure gradient (175). Again, assuming widespread recruitment of skeletal muscle as a result of HIIT, the stretch activated receptors on both the VSM and EC would be activated to a greater degree to increase blood flow, through a reduction in total peripheral resistance (TPR). Drastic reductions in TPR could potentially lead to the inability to maintain Q and MAP. This could further compromise Q during the next exercise bout, thereby creating a vicious cycle in which the system is constantly working to maintain Q homeostasis, promoting a stress which is much greater than in response to ET. This stress may lead to chronic activation of systems such as the baroreceptor reflex in response to HIIT.

6.1.3 Baroreceptor sensitivity and sympathetic outflow

Sympathetic nervous system (SNS) activation, via the modulation of autonomic nerve activity, is governed by the baroreceptor reflex. The role of the baroreceptor reflex in controlling MAP is well defined (64) and reduced baroreceptor sensitivity (BRS), as a result of increased chronic sympathetic activation, has been implicated in the pathogenesis of both hypertension and HF (77). The absence of inhibitory influences (e.g., beta-blockade), due to impaired BRS, allows excessive SNS output to be
unrestrained (293) which promotes remodeling and increases risk of sudden death (292). It is hypothesized that reduced BRS elevates the risk of ventricular arrhythmias due to the absence of modulation from the parasympathetic nervous system (PNS). Baroreceptors in the carotid sinus are sensitive to stretch, rather than pressure, therefore if MAP falls as a result of unadorned reductions in TPR as proposed in sections 6.1 and 6.2, the reduced BRS may play a role in the divergent responses demonstrated as a result of ET and HIIT. In addition, if NE is elevated to a greater degree as a result of HIIT, in a state of excitatory sympathetic activation, this could have further promoted pathological remodeling since the link between excessive NE and hypertrophy is well defined (321). Reducing SNS, through the use of β-blockers in these animals during HIIT could determine if this mechanism is responsible for our divergent results, however, although blockage of the receptors occurs, the circulating levels of NE remain high, thereby potentially confounding any results.

In addition to pathological remodeling, reductions in cardiac function, and transient HF have been reported as a result of elevated plasma catecholamines, such as NE (232, 302, 353), even in the absence of significant coronary disease. Although the data are limited, metabolic and hormonal responses differ even between ET and HIIT matched for work, demonstrating higher levels of metabolites as a result of HIIT (233), and a significant elevation in central femoral flow that is maintained for 60 min post exercise (156). This further indicates that HIIT may simply represent a stimulus that has a U-type function, which in healthy people does not promote pathological adaptations. However, in our model of hypertension, HIIT may have elicited a chronically higher level of NE, thereby partially explaining why HIIT and ET differ their responses. Studies have
demonstrated a reduction in sympathetic outflow (direct measure of muscle SNS activity, or urinary NE levels) as a result of ET (60 min 3 times per week) (95, 274), coinciding with improvements in BRS (101, 260). However, no studies have investigated the effects of HIIT on BRS, although strength training does not affect BRS in healthy males (60) and it has been suggested that the MAP response as a result of HIIT and strength training are similar (46). If this is the case, again, using β-blockers to reduce the effects of NE may prevent the pathological phenotype due to HIIT.

The mechanism of altered control of MAP, or the inability to maintain MAP during exercise and/or recovery could be determined through telemetry monitoring in the animals under the same exercise conditions. However, it must be stated that even if telemetry concluded that the regulation of MAP is altered as a result of the model or exercise intervention, this may be a limitation to the work, as humans may have a more well defined and responsive system to control MAP.

6.1.4 Regulation of mean arterial pressure

The control of one of the most tightly regulated physiological variables, MAP, is altered in cardiovascular disease, therefore a review of the potential effects of ET and HIIT on MAP to explain the divergent results is necessary. As eluded to in previous sections, the maintenance of MAP is achieved through an elegant balance between central and peripheral factors. The required Q is dictated by the need for O₂ and the production of ATP. When this requirement increases, as with acute exercise, alterations are made to the following variables: HR, SV and TPR. All three are influenced by the level of SNS output, such that increases in output increase HR and contractility, increase vaso and veno-constriction, and promote cross-bridge cycling in skeletal muscle to promote venous
return, and therefore increase SV. Importantly, TPR is under local metabolic control to allow for blood flow to be directed to the most metabolically active tissues, while vasoconstriction “diverts” flow from areas with low metabolic rates to maintain MAP. It has been noted that skeletal muscle has a greater capacity for Q than can be supported by the central circulation, therefore tight control is required. When whole body exercise is ceased, metabolic byproducts promote widespread vasodilation. The vasodilation is proportional to the intensity of exercise performed, such that higher intensities will promote more blood flow to the previously active skeletal muscle. When “central command” is off following exercise, signals for vasoconstriction are absent, and without mechanical forces (the muscle pump) venous return, central venous pressure (CVP) and therefore end diastolic volume (EDV) and SV could all be transiently reduced (see Figure 6.1). The baroreceptors sense this and increase SNS output to increase HR and vasoconstriction in an attempt to restore SV, Q and MAP.

Figure 6.3 Regulation of mean arterial pressure.
There are many levels of MAP control where HIIT and ET may differ. Metabolic rate, and therefore the cytosolic concentration of metabolic byproducts (CO$_2$, H+, lactate, K$^+$) are higher as a result of more intense exercise. If this is the case, a larger blood volume may be diverted to the hind limbs during the ‘recovery’ periods of HIIT. Given 70% of a rodent’s blood volume normally sits above the heart, which is not the case in the human, they may not have mechanisms in place to protect against drastic changes in venous return. Perhaps in the rodent model used, a drop in MAP post high levels of exercise present a larger stress than would be in the human, as they do not have a hydrostatic column to the degree in which a human does, therefore, SNS output or other mechanisms to maintain MAP may not be as sensitive or able to respond to such a stress. While the volume of blood dispersed to the periphery in rodents will be dramatically lower than in humans, since the resting HR of rodents is ~ 6 fold higher, small reductions in venous return would still be expected to decrease SV, however the effect on Q and MAP remains speculative at this time. If Q and MAP are not maintained in HIIT, as a result of ineffective SNS output and impaired BRS, as well as large decreases in TPR due to higher levels of dilating factors/metabolic byproducts in active muscles, this may have contributed to the pathological phenotypic response demonstrated as a result of HIIT.

6.1.5 Adult congenital heart disease

ACHD patients represent an extreme level of de-conditioning, complex physiology, compromised Q, risk of arrhythmia and HF, therefore as a first pass, and to maintain safety, it was appropriate to utilize ET as a modality to improve the fitness of these complex patients. There were no previous reports of ACHD patients participating in a comprehensive, structured cardiac rehabilitation (CR) program aimed at acquired HD
patients. CR programs in tertiary centres are experienced with heart transplant patients, left ventricular assist device patients and those with severe HF. Given these patients are at a high risk for life threatening arrhythmias, staff are trained in Advanced Cardiac Life Support (ACLS) and have access to the equipment required to treat and monitor high risk patients. CR programs utilize an evidence-based approach for exercise prescription, and as there are likely many physiological parallels between acquired HF and ACHD related HF, the decision was made to utilize current guidelines as a template to exercise train ACHD patients.

There were no adverse events due to the exercise training and all patients experienced an increase in exercise capacity (measured by metabolic equivalents on the Bruce treadmill protocol) and perhaps more importantly, exercise tolerance. The minor change in METs was not surprising, as it was hypothesized that many patients would be unable to centrally adapt to exercise training in the same way individuals with ‘normal’ cardiac structure and physiology would (see Figure 6.4 for documented and hypothesized factors influencing O₂ uptake in ACHD).
For example, it is unlikely patients would experience a measurable increases in blood volume, given the strict regulation on fluid balance in an attempt to reduce pulmonary and hepatic congestion. Whether or not physiological cardiac hypertrophy could develop was also questionable, as many patients had either, right systemic, single or severely altered ventricles. Ultimately, given the multiple structural abnormalities and surgical interventions, the heart itself may not produce appreciable adaptations in response to exercise. However, one must be reminded of the striking plasticity of skeletal muscle and the changes that occur as a result of exercise training.

ACHD patients represent arguably the most sedentary model of humankind, as the restrictions placed on them as children by parents and physicians were often extreme and
unnecessary (209). This could make the skeletal muscle highly responsive to even minor increases in daily exertion in these patients. The significant increase in exercise tolerance is undoubtedly due to alterations at the level of the skeletal muscle, including; increased mitochondrial content, activities of oxidative enzymes, and capillary networks. Further studies are required to elucidate the mechanisms behind the improvement in exercise capacity and tolerance as a result of ET in ACHD patients. In the meantime, appropriately stable ACHD patients are encouraged to participate in formal exercise rehabilitation programs, guided by the defect-specific flow charts found in Appendix A.

6.2 CONCLUSIONS

In conclusion, this thesis presented data indicating that in a rodent model of hypertension, ET and HIIT have divergent results. ET promoted an overall beneficial effect on both skeletal and cardiac tissues, and in high-risk ACHD patients, while HIIT provoked capillary loss, pathological cardiac hypertrophy and fibrosis. While ET has been shown time and time again to not only be safe, but effective, in high-risk patients, where evidence is sparse or lacking, HIIT may be detrimental. While these results remain to be demonstrated in humans, they encourage a cautionary approach when prescribing HIIT in clinical settings. Therefore the current thesis illustrates the critical need for additional research on the effects of HIIT in clinical populations.
6.3 LIMITATIONS

The major limitation of the current thesis was the use of rodents as a preclinical model, as opposed to hypertensive humans. While the translational nature of studies done in rodents to humans can be somewhat limited, the nature of the current work dictated the use of rodents as a first step. The fibre type profile in rats differs from humans in that rodent muscle has a larger spectrum between type I and IIB fibres. However, the rat is more similar to humans than a mouse, and the rat RG was chosen for analysis because it represents a phenotype closest to human muscle. Nevertheless, some of the observations made in the current thesis may not extend to humans, especially in the WG (primarily IIX and B), which is vastly different from any human muscle (see Figure 6.5 below) (288).

![Image of a chart showing species differences in shortening velocity among muscle fibre types.](chart.png)

**Figure 6.5. Species differences in shortening velocity among muscle fibre types.**
Correlation comparing the velocity of in vitro myosin shortening (Vf) and maximal single fibre unloaded shortening (Vo) across humans, rats, mice and rabbits. Redrawn from (288).
Clearly, to demonstrate the effects of HIIT in a clinical population, the use of human participants is ideal, however, to study the effects of HIIT on the heart specifically, would require heavy reliance on indirect methods (e.g., echocardiography) and provide limited insight into the molecular regulation on adaptations as provided in this thesis. Where tissue is available (e.g., from the right atria (RA) of pacemaker recipients) the assumption that the RA possesses similar morphologic properties as the LV would have to be made, and this is unlikely.

Another limitation of the current thesis is the use of HIIT on 5 days per week versus 3 days per week, which is typically used in the literature. However, the aim of the current thesis was to use an extreme exercise insult in order to determine whether any negative consequences would ensue. Furthermore, the hypotensive effects of exercise are limited to a 24-hour period (131), therefore daily exercise is essential in the treatment of hypertension. Human studies demonstrating the chronic elevation and desensitization of NE as a result of overtraining may help to explain the findings in this thesis. In addition, overtraining in rodents is associated with lower CS levels and higher oxidative stress in muscle (90). In the current study, the WG skeletal muscle had lower CS activity following HIIT, suggesting overtraining may have occurred. Whether or not 3 days per week would ameliorate or prevent the pathological adaptations observed following HIIT, remains to be seen, however, it is my belief that exercising every day is optimal in the clinical management of hypertension. Therefore, given the clear ‘intensity threshold’, beyond which exercise promotes pathological adaptations in both cardiac and skeletal muscle, ET appears to be the safer intensity of exercise in the presence of hypertension.
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APPENDIX A

ADULT CONGENITAL HEART DISEASE EXERCISE PRESCRIPTION

FLOW CHARTS
Atrial Septal Defect

ASD

Ass\textsuperscript{4} lesions
(30% of cases)

- PVS
- MVS or MVP
- VSD
- PDA
- Coarctation of the aorta

Absence of other lesions or complications

Most adults with repaired ASD’s are in NYHA class I or II and require NO restrictions

Complications

- AV valve regurgitation
- Sinus node dysfunction
- Atrial flutter/fibrillation
- LV dysfn\textsuperscript{n}
- Right HF
- Systemic Hypertension
- Paradoxical TE
- PH
- Endocarditis

Use current guidelines

- No isometric
- NO Exercise

\textbf{ACHD Flow Chart 1}: *when present in isolation (e.g., no associated complications as outline in the flow chart)
PVS pulmonary value stenosis, VSD ventricular septal defect, MVS mitral valve stenosis, MVP mitral valve prolapse, PDA patent ductus arteriosus, HD heart disease, AV atrioventricular, LV left ventricular, HF heart failure, PH pulmonary hypertension, TE thromboembolism
Atrioventricular Septal Defect
*Moderately complex ACHD

AVSD

Complete AVSD

Intermediate AVSD

Partial AVSD

Complications

Avoid strenuous exercise

Atrial arrhythmias

No activity restrictions for patients with small VSD’s, no associated lesions and normal LV function

Complications

Eisenmenger Physiology

See Flow Chart 7

Monitor for desaturation

Avoid Isometric

Use current guidelines

PH

Use current guidelines

Subaortic or subpulmonary stenosis

Low-mod intensity

See Flow Chart 5

TOF

LVOT

ACHD Flow Chart 2:*when present in isolation

TGA transposition of the great arteries, TOF tetralogy of fallot, LVOT left ventricular outflow tract obstruction, LV left ventricular, PH pulmonary hypertension
**Ventricular Septal Defect**

*Moderately complex ACHD

**VSD**

- **Non-restrictive**
  - Complications
  - Eisenmenger Physiology
    - See Flow Chart 7
    - Monitor for desaturation
    - Monitor for dehydration

- **Moderate to Lg restrictive**
  - Complications
  - Avoid strenuous exercise
  - PH
  - Atrial arrhythmias
    - No Isometric
    - Use current guidelines

- **Small restrictive VSD**
  - Complications
  - No activity restrictions for patients with small VSD’s, no associated lesions and normal LV function
  - Subaortic or subpulmonary stenosis
    - Use current guidelines

See Flow Chart 6
See Flow Chart 8
See Flow Chart #

**ACHD Flow Chart 3:** *when present in isolation
TGA transposition of the great arteries, TOF tetralogy of fallot, LVOT left ventricular outflow tract obstruction, LV left ventricular, PH pulmonary hypertension
Patent Ductus Arteriosus
*Moderately complex ACHD

PDA

3 Classes

Ass
g with

CoA
See Flow
Chart 5

ASD
See Flow
Chart 1

VSD
See Flow
Chart 3

Large PDA

With
Complications

Eisenmenger
Physiology

PH

See Flow
Chart 7

No
isometric

Monitor for desaturation and dehydration

Moderate PDA

With
Complications

Atrial
arrhythmias

Use current
guidelines

Small or repaired PDA

No exercise restrictions, normal life expectancy

ACHD Flow Chart 4:*when present in isolation (not closed)
CoA coarctation of the aorta, ASD atrial septal defect, VSD ventricular septal defect, PH pulmonary hypertension
Coarctation of the Aorta*
**Moderately complex ACHD

CoA

Ass'd lesions

LVOT  VSD  PDA

Avoid Isometric  See Flow Chart 3  See Flow Chart 4

Complications

Exercise should be encouraged b/c of early atherosclerosis, however extreme isometric and high intensity (e.g., start stop) exercise should be avoided

Atrial flutter/fibrillation  LV dysfn^  Systemic Hypertension  Thrombotic Events  Endocarditis

Use current guidelines^  Monitor BP closely

ACHD Flow Chart 5:*blood pressure to be measured in the right arm, **when present in isolation
LVOT left ventricular outflow tract obstruction, VSD ventricular septal defect, PDA patent ductus arteriosus, LV left ventricular
current guidelines refer to those from cardiac rehabilitation regulatory bodies (ACSM, CACR, AACVPR etc.)
Complete Transposition of the Great Arteries

ACHD of great complexity

TGA

VSD

CoA

LVOT

See Flow Chart 2

See Flow Chart 5

Avoid Isometric

Rastelli Procedure

Avoid strenuous and isometric exercise

Atrial Switch/Baffle (Mustard)

With Complications

LV tunnel obstruction

RV to PA conduit obstruction can cause RV angina

Atrial arrhythmias

Left AV valve regurgitation

CHF

RV dysf

AV valve regurgitation

PS

CAD

With Complications

With Complications

Use current guidelines

Restricted to class 1A type activities

Telemetry may be required

ACHD Flow Chart 6: VSD ventricular septal defect, CoA coarctation of the aorta, LVOT left ventricular outflow tract obstruction LV left ventricular, RV right ventricular, PA pulmonary artery, AV atrioventricular, PS pulmonary stenosis, CAD coronary artery disease, CHF congestive heart failure; current guidelines refer to those from cardiac rehabilitation regulatory bodies (ACSM, CACR, AACVPR etc.)
Eisenmenger Physiology
Greatly complex ACHD

EP

Ass^3 lesions
(30% of cases)

ASD  VSD  VSD

Use current guidelines

PDA  Coartation of the aorta

See Flow Chart 4  See Flow Chart 5

Complications

Monitor for dehydration
Monitor for desaturation

Telemetry recommended

AV valve regurgitation  Sinus node dysfunction  Atrial flutter/fib  LV dysfn^a  Right HF  Systemic Hypertension  Paroxysmal TE  PH  Endocarditis

Use current guidelines

Avoid isometric  NO Exercise

ACHD Flow Chart 7: *when present in isolation (e.g., no associated complications as outline in the flow chart)
PVS pulmonary valve stenosis, VSD ventricular septal defect, MVS mitral valve stenosis, MVP mitral valve prolapse, PDA patent ductus arteriosus, HD heart disease, AV atrioventricular, LV left ventricular, HF heart failure, PH pulmonary hypertension, TE thromboembolism
**The Single Ventricle and Fontan Circulations**

ACHD of great complexity

- **1V/Fontan**
  - **Ass° with**
    - TGA
    - CoA
    - PS/PA

- **RA enlargement**
- **CHF**
- **AV valve regurgitation**
- **Sinus or AV node dysfn requiring PPM**
- **Atrial arrhythmias**
- **Thromboembolic events**
- **Persistent right sided PE**

**Use current guidelines**

- **Telemetry may be required**
  - Monitor for dehydration
  - Monitor for desaturation

**Avoid contact sports**

- **Avoid strenuous and isometric exercise**

- **Hepatic congestion/dysfunction**
- **Pulmonary vein obstruction**
- **Obstruction in Fontan circuit**
- **Protein-losing enteropathy**

**See Flow Chart 6**

**See Flow Chart 5**

**With anticoagulation**

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**ACHD Flow Chart 9:** TGA transposition of the great arteries, CoA coarctation of the aorta, PS/PA pulmonary stenosis/pulmonary atresia, RA right atrial, CHF congestive heart failure AV atrioventricular PPM permanent pacemaker, PE pulmonary effusion; "current guidelines refer to those from cardiac rehabilitation regulatory bodies (ACSM, CACR, AACVPR etc.)"
**Tetralogy of Fallot**
*(&Right Ventricular Outflow Tract Obstructions)*

*Moderately complex ACHD*

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**ToF**

- Absence of other lesions or complications

**Complications**

- Require NO exercise restrictions provided QRS duration < 180ms

- Pulmonary regurgitation
- RV dilatation
- LV dysfunction
- SV arrhythmia
- AR
- VT
- Residual VSD
- Residual RVOT
- Aneursymal dilatation of the RVOT
- Endocarditis

**Use current guidelines**

**NO Exercise in absence of ICD**

**See Flow Chart 2**

**NO Exercise**

**Telemetry may be required**

**Restrict to class I A type activities**

---

ACHD Flow Chart 7: *when present in isolation*

RV right ventricular, LV left ventricular, SV supraventricular, AR aortic regurgitation, VT ventricular tachycardia, VSD ventricular septal defect, RVOT right ventricular outflow tract obstruction
Ebstein’s Anomaly of the Tricuspid Valve

Ebstein

Ass’ lesions (50% of cases)

ASD, PFO

Absence of other lesions or complications

Most adults with repaired Ebstein have good medium-term prognosis

Restrict to class 1A type activities

Complications

See Flow Chart 1

TV regurgitation, TV stenosis, Atrial arrhythmia, Complete heart block, Right HF, Enlargement of Right Atrium

Use current guidelines

Cardiomegaly (cardiothoracic ratio >65%)

NO Exercise

ACHD Flow Chart 10: ASD atrial septal defect; PFO patent foramen ovale; TV tricuspid valve; HF heart failure

*current guidelines refer to those from cardiac rehabilitation regulatory bodies (ACSM, CACR, AACVPR etc.)
**Congenitally Corrected Transposition of the Great Arteries**

ACHD of great complexity

ccTGA

Ass° with

- VSD
  - See Flow Chart 3
- Ebstein-like valve(s)
  - See Flow Chart 10

2 Procedures

- Classic Repair
  - With Complications
    - Restricted to class I A type activities
      - Atrial arrhythmias
        - Telemetry may be required
      - TV regurgitation
      - LV dysfn
      - RV dysfn°
      - PH
        - Use current guidelines°

°current guidelines refer to those from cardiac rehabilitation regulatory bodies (ACSM, CACR, AACVPR etc.)
APPENDIX B

METHODS
**Muscle Homogenate Tissue Preparation**

**Reagents:**

1. **Buffer 1**

   43.5 g KCL (FW 74.56)---1.167 M
   13.0 g Tetra-sodium pyrophosphate (FW 446.05, Na₄P₂O₇•10H₂O)---58.3 mM
   Dissolve up to 500 mL with ddH₂O

2. **Buffer 2**

   121.1 mg Tris-Base (FW 121.1)---10.0 mM
   37.22 mg EDTA (FW 372.24)---1.0 mM
   Dilute to 100 mL with ddH₂O, adjust pH to 7.4

3. **Buffer A**

   71.89 g Sucrose (FW 342.3)---210 mM
   0.7608 g EGTA (FW 380.4)---2 mM
   2.338 g NaCl (FW 58.44)---40 mM
   7.15 g Hepes (FW 238.3)---30 mM
   Dissolve up to 1000 mL with ddH₂O, adjust pH to 7.4

4. **20 mM EDTA in Buffer A**

   1.8612 g EDTA (FW 372.24) dissolved in 250 mL Buffer A

5. **Homogenizing Buffer (enough for 12 samples)**

   39 mL Buffer A
   13 mL 20 mM EDTA in Buffer A
   312 µL PMSF solution (0.0215 g PMSF in 350 µL DMSO)
   Stir for 5-15 minutes, rest on ice while homogenizing
**MUSCLE PREPARATION**

***Do all homogenizing on ice, and keep samples on ice***

1. Make sure **70.1 Ti** Rotor is stored in fridge

2. Turn on ultracentrifuge and activate vacuum to lower temperature to 4°C while homogenizing

3. Get liquid N$_2$, flask of distilled H$_2$O for rinsing, tweezers/scoopula etc to handle and clean homogenizer and round styrofoam container filled with ice for homogenizing samples

4. Rinse homogenizer head with EtOH or H$_2$O$_2$, followed by ddH$_2$O and wipe dry.

5. Weigh out approximately **50-80 mg** of a frozen muscle sample and put into an ultracentrifuge tube.

6. Add **2 mL** homogenizing buffer and homogenize on ice for **15sec x 2**

7. Rinse with ddH$_2$O and clean homogenizer between samples.

8. Add another **2ml** homogenizing buffer.

9. Add **3 mL** of Buffer 1, vortex, and set on ice for 15 minutes. (or put all samples in rotor and into refrigerated centrifuge and let sit there as vacuum will take 10-15 minutes to drop to the level required for centrifuge to start).

10. Centrifuge at **50,000 rpm** for **75 min** at **4°C**.

11. Discard supernatant and wipe walls with cotton swab. Optional** add **0.5-1 mL** Buffer 2 to wash the wall and discard, then dry with cotton tip**

12. Add **600 µL** Buffer 2, and homogenize for **10 sec x 2** at Polytron setting 7.

13. Add **200 µL** 16% SDS and keep off of ice. Vortex.

14. Centrifuge at **3000 rpm** for **15 minutes** at RT using rotor SH:24 (tabletop Beckman).

15. Remove the supernatant and store in labeled 1.5 mL eppendorf tubes and store at –80°C.
**Western Blotting – General Procedure**

**SDS-PAGE:**

- Prepare running gel and stacking gel (do not add APS & Temed to stacking gel until ready to use)
- Assemble clear stand with green gel holder and grey pad
- Pour running gel up to 1 cm below comb
- Add dH₂O to cover gel and tap on bench to ensure no bubbles have formed

<table>
<thead>
<tr>
<th></th>
<th>Stacking</th>
<th>5%</th>
<th>6%</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>6.8 ml</td>
<td>11.4</td>
<td>10.6</td>
<td>9.4</td>
<td>8</td>
<td>6.7</td>
</tr>
<tr>
<td>1.5M Tris-HCl, pH 8.8</td>
<td>***</td>
<td>5 ml</td>
<td>5 ml</td>
<td>5 ml</td>
<td>5 ml</td>
<td>5 ml</td>
</tr>
<tr>
<td>1M Tris-HCl, pH 6.8</td>
<td>1.25 ml</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>1.70 ml</td>
<td>3.4 ml</td>
<td>4 ml</td>
<td>5.3 ml</td>
<td>6.7 ml</td>
<td>8 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µl</td>
<td>200 µl</td>
<td>200 µl</td>
<td>200 µl</td>
<td>200 µl</td>
<td>200 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>100 µl</td>
<td>200 µl</td>
<td>200 µl</td>
<td>200 µl</td>
<td>200 µl</td>
<td>200 µl</td>
</tr>
<tr>
<td>Temed</td>
<td>20 µl</td>
<td>20 µl</td>
<td>20 µl</td>
<td>20 µl</td>
<td>20 µl</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

- Turn on heat block to 95°C
- Thaw samples on ice
- Wait 30 minutes for gel to set
- Soak up water layer using blotting paper
- Using a pipette, add stacking gel up to top of glass plate
- Immediately insert the comb diagonally
- Wait ~ 20-30 minutes until gel has set
**Sample Preparation:**

- Prepare samples in Laemmeli’s buffer (see below)
- Mix quantities determined from protein assay in centrifuge tubes, vortex
- Boil for 5 min @ 95°C using heat block
- Centrifuge

**4X Laemmeli’s Buffer:**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Quantities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>40 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>8.2 g</td>
</tr>
<tr>
<td>0.5 M Tris-HCl (pH 6.8)</td>
<td>50 ml</td>
</tr>
<tr>
<td>1 % Bromophenol Blue</td>
<td>500 µl</td>
</tr>
<tr>
<td>Complete to… pH to 6.8</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**NOTE:** Before use, add 31 mg DTT to 500 µl of 4X Laemmeli’s buffer

**Electrophoresis:**

- After polymerisation, place gel plates in white holder and slide holder into white reservoir (use thick clear plastic plate if not running 2 gels)
- Carefully remove comb to expose the sample wells, then fill reservoir with 1X running buffer and rinse wells out with running buffer using 5 ml syringe
- Pour cold running buffer to fill tank ~1/2
- Add 5 µl of MW marker to end well and load samples
- Load blank wells with water; load wells R to L
- Run the gels @ 160 V for 1hr or until samples have run off bottom of the gel

**Setup of Transfer:**

- Carefully separate glass plates; using scalpel blade, carefully cut down sides of gel and cut top right hand corner for gel orientation
- Label PVDF transfer membrane with name of gel top right corner
- Pre-soak PVDF membrane 1 min in methanol, 1 min in H₂O, 15-20 min in transfer buffer to equilibrate
- Pre-soak Whatman paper & cotton blot pad in transfer buffer
• Assemble immuno-blots sandwich under transfer buffer at all times:
  o 1 x cotton blot pad
  o 2 x Whatman
  o Gel (cut at left corner)
  o PVDF membrane (cut corner on cut gel corner)
  o 2 x Whatman
  o 1 x cotton blot pad

There should be no air bubbles between gel and membrane; Use rolling tube to smooth membrane and layers but be careful not to stretch gel

• Put stirrer in bucket (between black and red)
• Put bucket in low plastic container
• Insert sandwich (white (red) side top, black facing black)
• Insert ice block
• Run transfer @ 100 V for 1 h

After transfer, carefull remove membrane (with forceps) and place protein side up in a clean plastic container; you should be able to visualize the major bands.
DETECTION USING CHEMILUMINESCENCE

- ECL reagent
- Pipette with 500 µl tips
- Forceps
- Blotting paper
- Plastic petri dish for ECL reagent
- Spare plastic petri dish for your membranes
- Membranes in wash buffer

Detection and Photos:

- Combine 3-3 ml of each ECL solution
- Blot membrane and place in petri dish containing ECL mixture for 1 min
- Blot membrane, wrap in saran wrap and mark the MW with fluorescent pen
- Adjust magnification, focus and position, open aperture all the way .95
- Put wrapped membrane in imager
- Go to movie mode; chose # images and click on stack images
- Preview time; set exposure time based on this
- Image comes up as black; reverse image, autocontrast to get the best image
- Ensure lanes are not saturated
- Save best image and print

Quantification:

- Use analysis tools tab in software
### Western Blotting – Preparation of Buffers

**10 X Running Buffer:**

<table>
<thead>
<tr>
<th></th>
<th>500 ml</th>
<th>1 L</th>
<th>2 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>15 g</td>
<td>30 g</td>
<td>60 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>72 g</td>
<td>144 g</td>
<td>288 g</td>
</tr>
<tr>
<td>SDS</td>
<td>5 g</td>
<td>10 g</td>
<td>20 g</td>
</tr>
</tbody>
</table>

**1 X Running Buffer:**

10 X Running buffer 100 ml
dH2O 900 ml

**10 X Western Transfer Buffer:**

<table>
<thead>
<tr>
<th></th>
<th>500 ml</th>
<th>1 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>52.5 g</td>
<td>105 g</td>
</tr>
<tr>
<td>Tris Base</td>
<td>22.5 g</td>
<td>45 g</td>
</tr>
</tbody>
</table>

**1 X Western Transfer Buffer:**

10 X Transfer buffer 80 ml
Methanol 160 ml
dH2O 560 ml

**10 X TBS (Tris Buffered Saline):**

<table>
<thead>
<tr>
<th></th>
<th>500 ml</th>
<th>1 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>12.1 g</td>
<td>24.2 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>40 g</td>
<td>80 g</td>
</tr>
</tbody>
</table>

Adjust to pH 7.6 with HCl
1 X TBST:

<table>
<thead>
<tr>
<th></th>
<th>1 L</th>
<th>2 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X TBS</td>
<td>100 ml</td>
<td>200 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>900 ml</td>
<td>1800 ml</td>
</tr>
<tr>
<td>Tween 20</td>
<td>500 µl</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Note: Ensure solution is stirring when adding Tween

10 X PBS (Phosphate Buffered Saline):

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>80 g</td>
</tr>
<tr>
<td>KCl</td>
<td>2 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>14.4 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.4 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>850 ml</td>
</tr>
</tbody>
</table>

pH with HCl; Bring up to 1 L

1 X PBS: Follow same instructions as 1 X TBST
WESTERN ANTIBODIES

NOTE: Unless indicated otherwise*, runs are 1 hour 150V, transfers are 1 hour 100V, washes are 3 x 15 min @ RT

α-tubulin:
MW: 50 kDa
Blocking 5% NFM
  • 1g NFM in 20 ml PBST

1° antibody: 1: 500 1% NFM overnight
  • 100 mg NFM in 20 ml PBST

2° antibody: 1:1000 goat anti-rabbit
  • 10 µl antibody to 10 ml PBST

ANP: Abcam, ab110413
MW: 16 kDa
Blocking 5% NFM
  • 1g NFM in 20 ml PBST

1° antibody: 1:500 1% NFM overnight
  • 100 mg NFM in 20 ml PBST

2° antibody: 1:1000 goat anti-rabbit
  • 10 µl antibody to 10 ml PBST
**BNP:** Abcam, ab19645

**MW:** 18 kDa

**Blocking 5% NFM**
- 1g NFM in 20 ml PBST

**1° antibody:** 1:500 1% NFM overnight
- 100 mg NFM in 20 ml PBST

**2° antibody:** 1:1000 goat anti-rabbit
- 10 µl antibody to 10 ml PBST

---

**eNOS:** Abcam, ab5589

**MW:** 133 kDa

* run for 1.25 hours at 150V

**Blocking 5% NFM**
- 1g NFM in 20 ml PBST

**1° antibody:** 1:1000 1% NFM overnight
- 100 mg NFM in 20 ml PBST

**2° antibody:** 1:1000 goat anti-rabbit
- 10 µl antibody to 10 ml PBST
**β-MHC**: Abcam, ab172967

MW: 223 kDa

* transfer buffer w/out methanol; transfer in cold room for 4 hours, changing ice pack and buffer every hour

Blocking 5% NFM

- 1g NFM in 20 ml PBST

1° antibody: 1:500 1% NFM overnight

- 100 mg NFM in 20 ml PBST

2° antibody: 1:1000 goat anti-rabbit

- 10 µl antibody to 10 ml PBST

---

**VEGF**: Abcam, ab46154

MW: 43 kDa

Blocking 5% NFM

- 1g NFM in 20 ml PBST

1° antibody: 1:500 1% NFM overnight

- 100 mg NFM in 20 ml PBST

2° antibody: 1:1000 goat anti-rabbit

- 10 µl antibody to 10 ml PBST
**MAFbx** Santa Cruz, sc33782  
MW: 40 kDa  
Blocking 5% NFM  
  • 1g NFM in 20 ml PBST  

1° antibody: 1:2000 1% NFM overnight  
  • 100 mg NFM in 20 ml PBST  

2° antibody: 1:1000 goat anti-rabbit  
  • 10 µl antibody to 10 ml PBST  

---

**MuRF1** sc-32920  
MW: 40 kDa  
Blocking 5% NFM  
  • 1g NFM in 20 ml PBST  

1° antibody: 1:500 1% NFM overnight  
  • 100 mg NFM in 20 ml PBST  

2° antibody: 1:1000 goat anti-rabbit  
  • 10 µl antibody to 10 ml PBST
**HIF1α** Abcam, ab463

MW: 97 kDa

Blocking 5% NFM overnight

- 1g NFM in 20 ml TBST

1° antibody: 1:1000 1% NFM overnight

- 100 mg NFM in 20 ml TBST

2° antibody: 1:1000 goat anti-mouse

- 10 µl antibody to 10 ml TBST

---

**FOXO3a Ser253**: Abcam, ab47285

MW: 105 kDa

Blocking 5% NFM

- 1g NFM in 20 ml PBST

1° antibody: 1:500 1% NFM overnight

- 100 mg NFM in 20 ml PBST

2° antibody: 1:1000 goat anti-rabbit

- 10 µl antibody to 10 ml PBST
VEGFR2:
MW: 220 kDa
* transfer buffer w/out methanol; transfer in cold room for 4 hours, changing ice pack and buffer every hour

Blocking 5% NFM
• 1g NFM in 20 ml PBST overnight

1° antibody: 1: 500 1% NFM overnight
• 100 mg NFM in 20 ml PBST

2° antibody: 1:1000 goat anti-rabbit
• 10 µl antibody to 10 ml PBST

OXPHOS:
MW: Complex I 20 kDa, II 30 kDa, III 40 kDa, IV 48 kDa, V 55 kDa

Blocking 5% NFM
• 1g NFM in 20 ml PBST

1° antibody: 1: 500 1% NFM 4 hours
• 100 mg NFM in 20 ml PBST

2° antibody: 1:1000 goat anti-rabbit
• 10 µl antibody to 10 ml PBST
**Citrate Synthase**

This reaction involves the measurement of citrate synthase activity by linking the release of CoASH to the colorimetric agent DTNB 5,5-dithiobis-2-nitrobenzoate. Changes in absorbance are followed at 412 nm.

\[
\text{Oxaloacetate + acetyl CoA} \rightarrow \text{CoASH + Citrate} \\
\text{CoA-SH + DTNB}_{\text{pale yellow}} \rightarrow \text{CoA + DTNB}_{\text{dark yellow}}
\]

**Preparation of Buffers**

**100mM TRIS buffer (pH 8.3)**
Dissolve 3.0275g in 250 ml of distilled water. Use HCl to adjust pH to 8.3. Store in refrigerator.

**1mM DTNB (Sigma D-8130) Make fresh every 2 weeks**
Dissolve 3.96 mg of DTNB in 10 ml of 100mM TRIS buffer (pH 8.3). Store in opaque bottle in refrigerator.

**10mM Oxaloacetate (Sigma O-4126) Prepare fresh daily**
Dissolve 13.2mg in 10ml of 100mM TRIS buffer, pH 8.3.

**3mM Acetyl CoA (Sigma A-2897) Make fresh daily.**
Dissolve 3.1mg Acetyl CoA in 1ml of distilled water. Make stock and store at –20°C.

**Homogenizing Solution**
Add 1.36g of 0.1M KH$_2$PO$_4$ and 50 mg of BSA to ~ 80 ml H$_2$O. Adjust pH to 7.3 with KOH and top to 100 ml with H$_2$O. Store in fridge for 4-6 months.

**Procedure**
1. Chip a muscle 6-10 mg wet mass piece and weigh in cryovial.
2. Add 100 µl of homogenizing solution/mg wet mass and homogenize until muscle is fully broken up; immediately freeze sample in liquid N$_2$.
3. Ts and Em are diluted 20 X (5 µl mitos + 95 µl S+M)
4. MH and Ts go through 2 sets or freeze/thaw cycles
5. Prepare DU70 set up as follows:

<table>
<thead>
<tr>
<th>ON/IDLE</th>
<th>TEMP controller at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VISIBLE light source</td>
</tr>
<tr>
<td></td>
<td>TIME DRIVE</td>
</tr>
</tbody>
</table>
MENU SETUP: Kinetics; 412 nm, 20 sec intervals, 5min run; 0 and 1.5
abs limits, multi cell sampling device; 6 cells

6. Prepare the following in quartz cuvettes in duplicates:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Total Muscle (MH)</th>
<th>Total Suspension (ts)</th>
<th>Extra mitochondrial (em)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer (µl)</td>
<td>150</td>
<td>150</td>
<td>160</td>
</tr>
<tr>
<td>DTNB (µl)</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Acetyl-CoA (µl)</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>M. homogenate (µl)</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Triton (10%) (µl)</td>
<td>10</td>
<td>10</td>
<td>--</td>
</tr>
</tbody>
</table>

7. Use water as blank; press **START** to calibrate

8. Insert 6 cuvettes, add 15 µl of **OAA** and stir thoroughly, close lid and push **RUN**

9. After reaction is complete, select Tabulation press COPY to print

**CALCULATIONS:**

\[
\text{CS Activity} = \frac{\text{Abs/min} \times \text{Total Volume} \times \text{dilution factor (101)}}{13.6 \times \text{Sample Volume}} = \frac{\text{µmol/min/g}}{}
\]

\[
\text{Change in absorbance per minute} = 0.172 \\
\text{Total volume (in cuvette)} = 250 \mu l \\
\text{Sample volume (muscle homogenate)} = 10 \mu l \\
\text{Dilution factor} = 100
\]

\[
\text{CS Activity} = \frac{0.172 \times 250 \mu l \times 100}{13.6 \times 10 \mu l} = 31.93 \text{ µmol/min/g}
\]

**EXPECTED VALUES:**

**Rat tissue:**
- Plantaris ~ 10 – 30 µmol/min/g
- Soleus ~ 20 – 35 µmol/min/g
- White vastus ~ 10 – 15 µmol/min/g

**Human muscle:**
- Untrained ~ 10 – 20 µmol/min/g
- Trained ~ 30 – 60 µmol/min/g
IMMUNOFLOUORESCENCE AND HISTOCHEMISTRY

Picro-sirus Red (Fibrosis) Staining

Protocol

1. 2 min Xylene
2. 2 min Xylene
3. 2 min Xylene
4. 2 min 100% ETOH
5. 2 min 100% ETOH
6. 2 min 95% ETOH
7. 2 min 70% ETOH
8. 2 min dH2O
9. 10 min hematoxylin
10. Rinse in running dH2O until Clear
11. 1 hour in Picro-sirus Red

   Sirius red F3B (C.I. 35782) -------------------- 0.5 g
   Saturated aqueous solution of picric acid --------500 ml

12. 2 min in Acid water (5ml of Glacial Acetic Acid in 1L of dH2O) with agitation
13. 2 min in Acid water (5ml of Glacial Acetic Acid in 1L of dH2O) with agitation
14. 2 min 95% ETOH
15. 2 min 100% ETOH
16. 2 min 100% ETOH
17. 2 min Xylene
18. 2 min Xylene
**SKELETAL MUSCLE MYOSIN HEAVY CHAIN**

1. Cut O.C.T.-embedded muscle into 10 µm cross-sections and store at −80°C

2. Air-dry sections for 10 min.

3. Block with 10% goat serum in PBS for 60 min

4. Incubate with 1° antibody cocktail for 60 min

5. Wash 3 x 5 min PBS

6. Apply 2° antibody cocktail for 60 min (Invitrogen)

7. Wash 3 x 5 min PBS

8. Mount coverslips with Prolong® Gold antifade reagent

Immunofluorescence analysis of MHC expression primary antibodies (Developmental Studies Hybridoma Bank):

- **MHC I (BA-F8) 1:50**
  - Alexa Fluor 350 IgG2b 1500 (blue)

- **MHC IIa (SC-71, 2F7) 1:600**
  - Alexa Fluor 488 IgG1 1500 (green)

- **MHC IIx (6H1) 1:50**
  - Alexa Fluor 555 IgM 1500 (red)

- **MHC IIb (BF-F3) 1:100**
  - Alexa Fluor 555 IgM 1500 (red)
CSA of Cardiac Cells

1. Formalin fixation and permeabilization with Triton X-100,

2. Block with 10% goat serum in PBS,

3. Incubate with 1° antibody cocktail overnight with (anti-dystrophin primary antibody; Developmental Studies Hybridoma Bank),

4. Incubate with fluorescent 2° antibody (Life Technologies)

5. Counter stain sections with DAPI (Life Technologies) to visualize nuclei

**Succinate Dehydrogenase (Blanco CE 1988)**

**Reagents:**

Acetone

Deionized water

Gelatin - stored @ RT

Glycerol - Sigma, stored at @ RT

Nitro blue tetrazolium – Sigma, stored desiccated at 0 - 5 °C

Phenol - Fisher, stored @ RT

Sodium dibasic phosphate (Na$_2$HPO$_4$) anhydrous, ACS (FW 141.96)-Sigma or Fisher, stored at @ RT

Sodium dibasic phosphate (Na$_2$HPO$_4$) heptahydrate (FW 268.07) Sigma or Fisher, store at @ RT

Sodium monobasic phosphate (NaH$_2$PO$_4$) monohydrate (FW 137.99), ACS - Sigma or Fisher; stored @ RT

Succinic acid, disodium salt – Sigma, stored @ RT

**Solutions:**

I. 0.2 M phosphate buffer, pH 7.6

0.2 M sodium monobasic phosphate (NaH$_2$PO$_4$) 13 ml (27.8 gm/liter deionized H$_2$O)

0.2 M sodium dibasic phosphate (Na$_2$HPO$_4$) heptahydrate (53.65 gm/liter deionized H$_2$O) or sodium dibasic phosphate (Na$_2$HPO$_4$) anhydrous (28.39g/liter deionized water) 87 ml

II. 0.2 M Succinic Acid (sodium salt) with deionized H2O 5.4 g/100ml.

It is recommended to prepare FRESH.

*A stock solution may be kept refrigerated for two weeks.*
III. Aqueous Mounting Medium (glycerogel)

Gelatin (ICN#960317 - 100 bloom) 4 g  
Glycerol 25 ml  
Phenol 0.5 ml  
Deionized water 21 ml

1. Dissolve gelatin in boiling water.  
2. Cool, but do not allow to solidify.  
3. Add phenol and glycerol.  
4. Mix well.  
5. Allow air bubbles in mixture to dissipate before using!
**Staining Procedure:**

1. Prepare the incubation medium as follows:

   0.2 M phosphate buffer 10 ml
   Dissolve Sodium Succinate 270 mg & NBT 10 mg

2. Incubate coverslips in a staining dish for 60 minutes at 37 °C.

3. Wash with three exchanges of tap or deionized H2O.

4. Prepare approximate solutions of 30, 60 and 90 % acetone using deionized H2O and remove unbound NBT from the sections with three exchanges each of the acetone solutions in increasing then decreasing concentration. Leave the 90 % acetone covering the sections until a faint purplish cloud is seen over the section.

5. Finally, rinse several times with deionized H2O and then mount the coverslips with the aqueous mounting medium.

**Results:**

Purple formazan precipitate is deposited at sites of mitochondria in sarcoplasmic network. Type I fibers are darker than those of type II. Walls of blood vessels also are stained. Best results occur if the sections are stained on the same day that they are cut.