MALADAPTATION OF CARDIAC AND SKELETAL MUSCLE IN CHRONIC DISEASE: EFFECTS OF EXERCISE

A Dissertation

Presented to

The Faculty of the Graduate School

University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Robert Tyler Morris

Frank W. Booth, Ph.D., Dissertation Supervisor

AUGUST 2007

The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled:

MALADAPTATION OF CARDIAC AND SKELETAL MUSCLE IN CHRONIC DISEASE: EFFECTS OF EXERCISE

presented by Robert Tyler Morris

a candidate for the degree of Doctor of Philosophy

and hereby certify that in their opinion it is worthy of acceptance.

____________________________________ Frank Booth, Ph.D.

____________________________________ Marybeth Brown, Ph.D.

____________________________________ M. Harold Laughlin, Ph.D.

____________________________________ Kerry McDonald, Ph.D.

____________________________________ Michael Rovetto, Ph.D.

This dissertation is dedicated to the following:

Ernest A. Hill

(August 4th,1920 - July 4th, 2005)

The memories of my grandfather will last forever. He was kind, compassionate, and hard working. My family has been shaped through his wonderful life.

Patrick N. Colleran, Ph.D.

(October 14th,1968 - Feb 15th, 2006)

Patrick was a great friend, teammate, and colleague. His spirit will continue on through the lives he touched.

ACKNOWLEDGEMENTS

A dissertation involves many people. First, I would like to thank my mentor, Frank Booth, for his continued support and guidance during the last several years. As both a masters and Ph.D. student, he has provided me with exceptional mentorship. His enthusiasm for science and the ability to critically assess challenges within the laboratory is remarkable. I am very grateful for my experience within Dr.Booth's lab and have learned to improve my skills as a scientist during this time. I would also like to thank the members of my dissertation committee, Marybeth Brown, Ph.D.; Harold Laughlin, Ph.D.; Kerry McDonald, Ph.D.; and Michael Rovetto, Ph.D. Your guidance has been extremely helpful. I also appreciate the help from several post-doctoral fellows within the Booth lab including: Espen Spangenburg, Ph.D.; Andrew Shanely, Ph.D.; and Simon Lees, Ph.D. All the members of the Booth lab have been very supportive and a special thanks to Tom Childs for his technical assistance. I also appreciate the participation of Craig Stump, Ph.D., M.D., Deborah Fine, D.V.M., and Suzanne Clark, M.S., for their help in the Ren-2 study. I thank John Thyfault, Ph.D., for providing assistance in the data collection from the OLETF study. My development as a teacher would not have been possible without Tom Hurley, Ph.D. He has helped me on several occasions to improve my abilities as a teaching assistant and allowed me to lecture some of the undergraduate physiology class. I would also like to thank the faculty from both the Department of Medical Pharmacology & Physiology and Department of Biomedical Sciences

for their instruction. I thank my mentor at Drury University, Donald Deeds, Ph.D., for inspiring me about the study of physiology.

I also appreciate the everlasting support provided by my family. To my parents, Bob Morris and April Herrin, for encouraging me to achieve success and always being supportive. You have been wonderful parents. I cannot thank you enough. To my sisters, Holly and Hannah Morris, for looking out for me and keeping me pointed in the right direction. You have given me much love and support. To my grandmother, Georgine “Pocky” Morris, for shaping such a great family and being very supportive in all my endeavors. To my extended family, Cynthia Morris, Jay and Jason Herrin, Frank Bailey, and Claudia Polen, for being a positive influence in my life. Although graduate school can be difficult on relationships, I would like to thank Liz Dunavant and Traci Strauch for their support during my graduate career. Also, to my grandfather, Robert L. Morris (“Grampie”), for inspiring me about academia.

Also, it would not have been possible to pursue my doctoral degree without the blessings and spiritual guidance of my Lord and Savior, Jesus Christ (Romans 5:1-5).

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List of Abbreviations

βARK - β-Adrenergic Receptor Kinase

β-HAD - β-hydroxyacyl-CoA dehydrogenase

$2O^{\bullet -}_2$ - Superoxide

4-HNE – 4-hydroxynonenal

ABI – Applied Biosystems

AC – Aortic Constriction

ACE – Angiotensin Converting Enzyme

AKT – Protein Kinase B

Ang II – Angiotensin II

ANOVA – Analysis of Variance

ANP – Atrial Natriuretic Peptide

AT1 – Angiotensin II Receptor Type 1

ATP – Adenosine Triphosphate

BMI – Body Mass Index

BNP – B-type Natriuretic Peptide

BP – Blood Pressure

CCK-A - Cholecystokinin-A

CO – Cardiac Output

CPT-1 - Carnitine Palmitoyltransferase-1

CR – Caloric Restriction

Cu-Zn SOD – Copper Zinc Superoxide Dismutase

DBP – Diastolic Blood Pressure

DNA - Deoxyribonucleic acid

dPdT – Change in Pressure per Change in Time

EC – Excitation-Contraction

EC-SOD – Extracellular Superoxide Dismutase

ELISA - Enzyme-Linked ImmunoSorbent Assay

ET – Exercise Training

EX-Ren-2 – Exercise Trained TG(mRen2)27

EX-SD – Exercise Trained Sprague-Dawley

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GLUT – Glucose Transporter

GRK – G-protein coupled Receptor Kinase

GST – Glutathione S-Transferase

H_2O_2 – Hydrogen Peroxide

IMTG – Intramuscular Triglyceride

LETO - Long-Evans Tokushima Otsuka

LV – Left Ventricle

LVIDd – Left Ventricular Internal Diameter during Diastole

LVIDs - Left Ventricular Internal Diameter during Systole

MAP – Mean Arterial Pressure

MAPK - Mitogen-Activated Protein Kinase

MDA - Malondialdehyde

MMP – Matrix Metalloproteinase

MNC - Mononuclear cell

Mn-SOD - Manganese Superoxide Dismutase

mtDNA – Mitochondrial DNA

MyHC – Myosin Heavy Chain

NIDDM - Non-Insulin Dependent Diabetes Mellitus

NWR – Normotensive American Wistar Rats

OLETF - OTSUKA LONG-EVANS TOKUSHIMA FATTY

PCR – Polymerase Chain Reaction

PGC-1 - Peroxisome proliferator activated receptor-δ co-activator-1

PI3K- Phosphoinositide-3 kinase

PKA – Protein Kinase A

PLB - Phospholamban

PMN - Polymorphonuclear Leukocytes

PPAR - Peroxisome proliferator activated receptor

RAS – Renin-Angiotensin System

RNA - Ribonucleic acid

ROS – Reactive Oxygen Species

SBP – Systolic Blood Pressure

SD – Sprague-Dawley

SED-Ren2 – Sedentary TG(mRen2)27

SED-SD – Sedentary Sprague-Dawley

SERCA2a – Sarco, Endoplasmic Reticulum Ca^{+2} ATPase

SHHFR - Spontaneously Hypertensive Heart Failure Rat

SHR - Spontaneously Hypertensive Rat

SOD – Superoxide Dismutase

SR – Sarcoplasmic Reticulum

TG - Transgenic

TGF-β - Transforming Growth Factor-β

TPR – Total Peripheral Resistance

MALADAPTATION OF CARDIAC AND SKELETAL MUSCLE IN CHRONIC DISEASE: EFFECTS OF EXERCISE

Robert Tyler Morris

Dr. Frank W. Booth, Dissertation Supervisor

ABSTRACT

The human genome is adapted for daily physical activity (85). Thus, a sedentary lifestyle is capable of promoting detrimental consequences to human health. The linkage between lack of sufficient physical activity and the development of modern chronic disease began to evolve only in the second half of the 20th century. This relationship was initially documented in London where men, who were more sedentary at work or during leisure time, had higher rates of coronary heart disease (242; 243). A multitude of other published reports have since confirmed the health hazards of maintaining an inactive lifestyle (40; 329). In addition, modern chronic diseases attributed to physical inactivity now represent a major burden on direct health care costs in the United States which totaled 83.6 million dollars in 2000 (55; 102). The importance of physical activity has become more apparent and it is now recommended by the US Surgeon General that "every U.S. adult should accumulate 30 minutes or more of moderate-intensity physical activity on most, preferably all, days of the week" (264). Furthermore, physicians are also being recommended to prescribe regular

exercise to sedentary patients as a measure to reduce chronic health conditions (40; 42). However, more experimental investigations are necessary to elucidate how exercise delays or inhibits the development of chronic diseases, including hypertension, obesity, and type II diabetes. Understanding the mechanisms that regulate such conditions may lead to a scientific basis for therapy and cure. The following studies were done to investigate: Part I) the regulation of cardiac structure and function by exercise in the hypertensive (mREN2)27 rat, and Part II) the effects of voluntary exercise on skeletal muscle lipids in an obese OLEFT rat. Lastly, Part III (*supplemental*) provides a brief discussion on the effects of caloric restriction in obesity.

CHAPTER 1: INTRODUCTION

Part I.

PATHOGENESIS OF HYPERTENSION:

Epidemiology and characterization

Despite significant advances in the treatment of cardiovascular diseases since the 1970's, hypertension remains a major health problem in the United States by affecting ~28.7% of Americans (or 58.4 million patients) with many more being undiagnosed (123). Risk factors associated with hypertension include heart disease, stroke, and kidney failure (333). Essential hypertension in humans is characterized by systolic blood pressure (SBP) ≥ 140 mmHg and / or diastolic blood pressure (DBP) ≥ 90 mmHg (124).

Potential regulatory mechanisms

Blood pressure is regulated by a variety of different mechanisms which serves to influence the following: Blood Pressure (BP) = Cardiac Output (CO) x Total Peripheral Resistance (TPR). Figure 1.1 depicts how either CO and / or TPR can result in higher blood pressure through the influence of several factors, such as sodium intake, reduced nephron number, stress, genetics, obesity, and endothelial-derived factors (344).

Although the exact mechanism for the development of hypertension is still unclear, the etiology of hypertension is believed to stem from pathophysiological regulation of blood pressure levels. Long term blood pressure control is believed to be regulated by pressure natriuresis, or "the ability of the kidneys to respond to changes in arterial pressure by altering renal excretion of salt and water" (218).

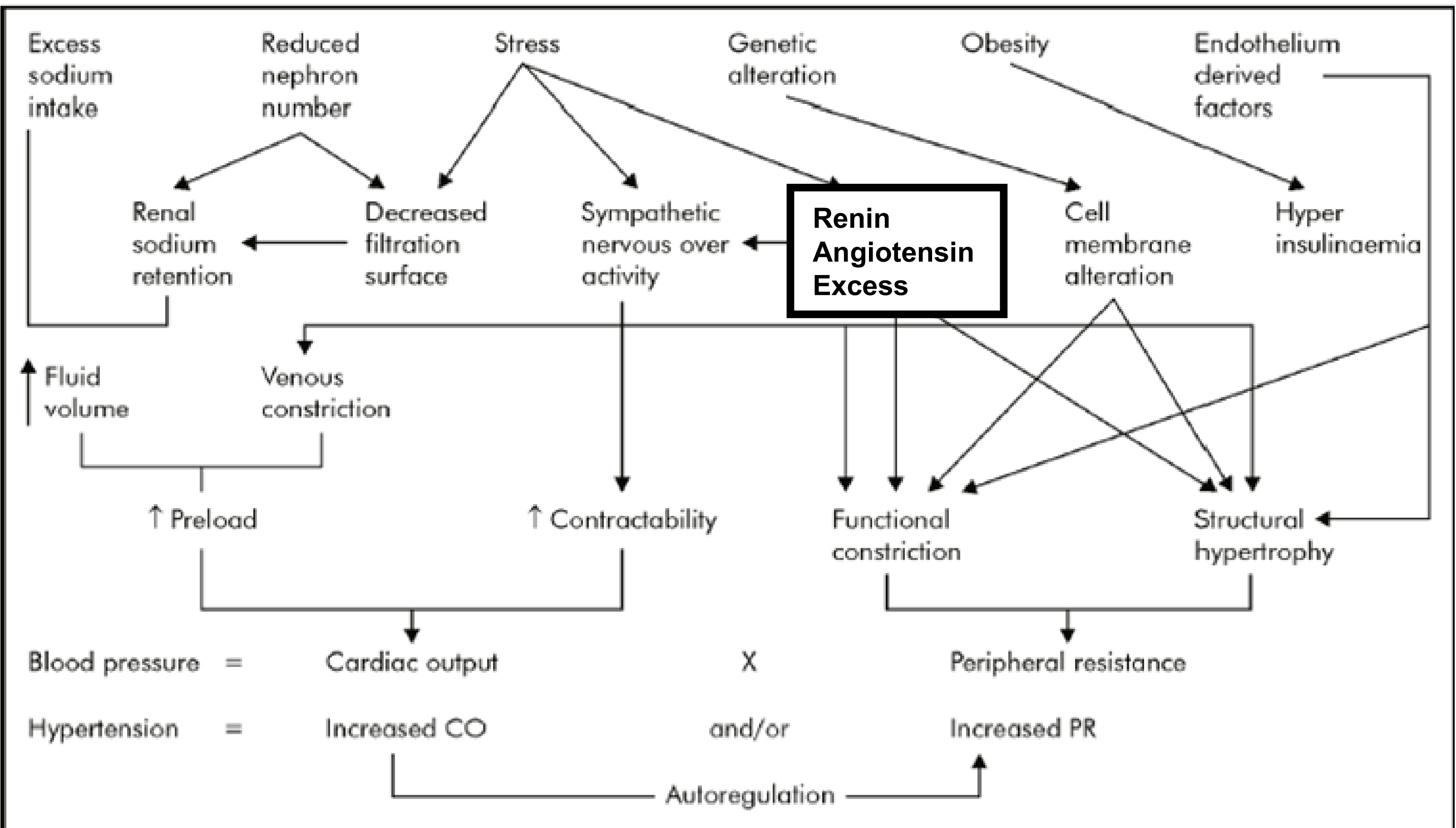

Figure 1.1 - Control of blood pressure

(copyright permission obtained from Dr. BB Rewari Senior Physician,RML Hospital E-197 , Amar Colony,Lajpat Nagar-IV New Delhi-110024 INDIA; Vikrant S et al, *J Indian Acad Clin Med*, 2001)

Hyperactive sympathetic nerve activity is common in subjects with hypertension and can lead to renal vasoconstriction, produce increased sodium reabsorption, abnormal body fluid volume, and altered arterial pressure homeostasis (157; 159; 160). Several factors are known to influence the sensitivity of pressure natriuresis, including the renin-angiotensin system (RAS). When arterial pressure or sodium intake is elevated, the RAS is suppressed in order to promote excretion of salt and water by the kidneys. The relationship between RAS and pressure natriuresis is believed to be very important in the long-term regulation of arterial pressure and the development of hypertension (217). Selective pharmacological agents, such as Angiotensin Converting Enzyme (ACE) inhibitors and Angiotensin II receptor antagonists (AT1 blockers), are widely used therapies to reduce blood pressure in hypertensive patients (349). Interestingly, genetic polymorphisms of the RAS (ACE I/D, AGT M235T, and AT1 A1266C) have been associated with essential hypertension

Lifestyle intervention

A variety of epidemiological studies have supported a role for lifestyle intervention in reducing hypertension (203). For example, Dickinson (2006) used a systematic review of controlled trials to determine the effectiveness of lifestyle factors in lowering high blood pressure (72). Human adult subjects qualified for inclusion with a blood pressure of 145 / 85 mmHg and at least 8 weeks of intervention. Of the 105 trials and 6805 participants, statistically significant

decreases in mean systolic blood pressure were observed for improved diet (5.0 mmHg), regular physical activity (4.6 mmHg), alcohol and salt restriction (3.8 and 3.6 mmHg), and fish oil supplements (2.3 mmHg). Similar effects have been observed in experimental animals with fulminant hypertension (354-356) suggesting that lifestyle choices play an important role in reducing hypertension (71).

PHENOTYPE CHANGES IN THE HEART FOLLOWING PRESSURE OVERLOAD

In response to arterial hypertension, the myocardium adapts by initiating hypertrophic growth, natriuretic peptide expression, myosin heavy chain isoform switching, and interstitial fibrosis. The following sections will characterize these phenotypic changes that occur within the myocardium in response to the mechanical stress of hypertension.

Myocardial hypertrophy

Cardiac hypertrophy, or the phenotypic enlargement of the myocardium, can occur in response to mechanical stress. Classically, two different forms of hypertrophy exist and can be distinguished based upon sarcomere assembly. Eccentric (physiological) hypertrophy results in uniform growth of the myocardium and the addition of sacromeres in series and in parallel. Concentric (pathological) hypertrophy is characterized by thickening of the left ventricle wall and the addition of sarcomeres in parallel. Both forms are considered “compensatory” due to the normalization of systolic wall stress. However,

concentric hypertrophy due to prolonged hypertension can further develop eccentric hypertrophy and chamber dilation; a process characterized by depressed myocardial contractility and known as *decompensation*. Also, eccentric cardiac hypertrophy can be produced by endurance training and is known to increase contractility and maximize cardiac output.

Cardiac hypertrophy has been characterized based upon the type of stimulus introduced. Adaptive (or developmental) hypertrophy is the physiological growth which commonly occurs in athletes and results in proportional increases in the chamber dimension. In contrast, maladaptive hypertrophy occurs in response to pathophysiological stimuli, such as hypertension, and can also lead to chamber dilation resulting in cardiac dysfunction.

(Figure 1.2)(138).

Changes in workload are regularly introduced in the heart and thus, the transduction of mechanical forces into gene expression in the cardiac myocyte serves an important function. Cardiac myocytes possess mechanical sensors that can activate a variety of signaling pathways known to regulate the gene expression of the cell. For example, the integrin protein family, specifically β1-intergrin, is stimulated by interactions between the extracellular basement membrane and the sarcolemma. During pressure overload in the heart, mechanical forces cause adhesion complexes of integrin proteins to activate both MAPK and PI3K / AKT signaling pathways (322). This form of

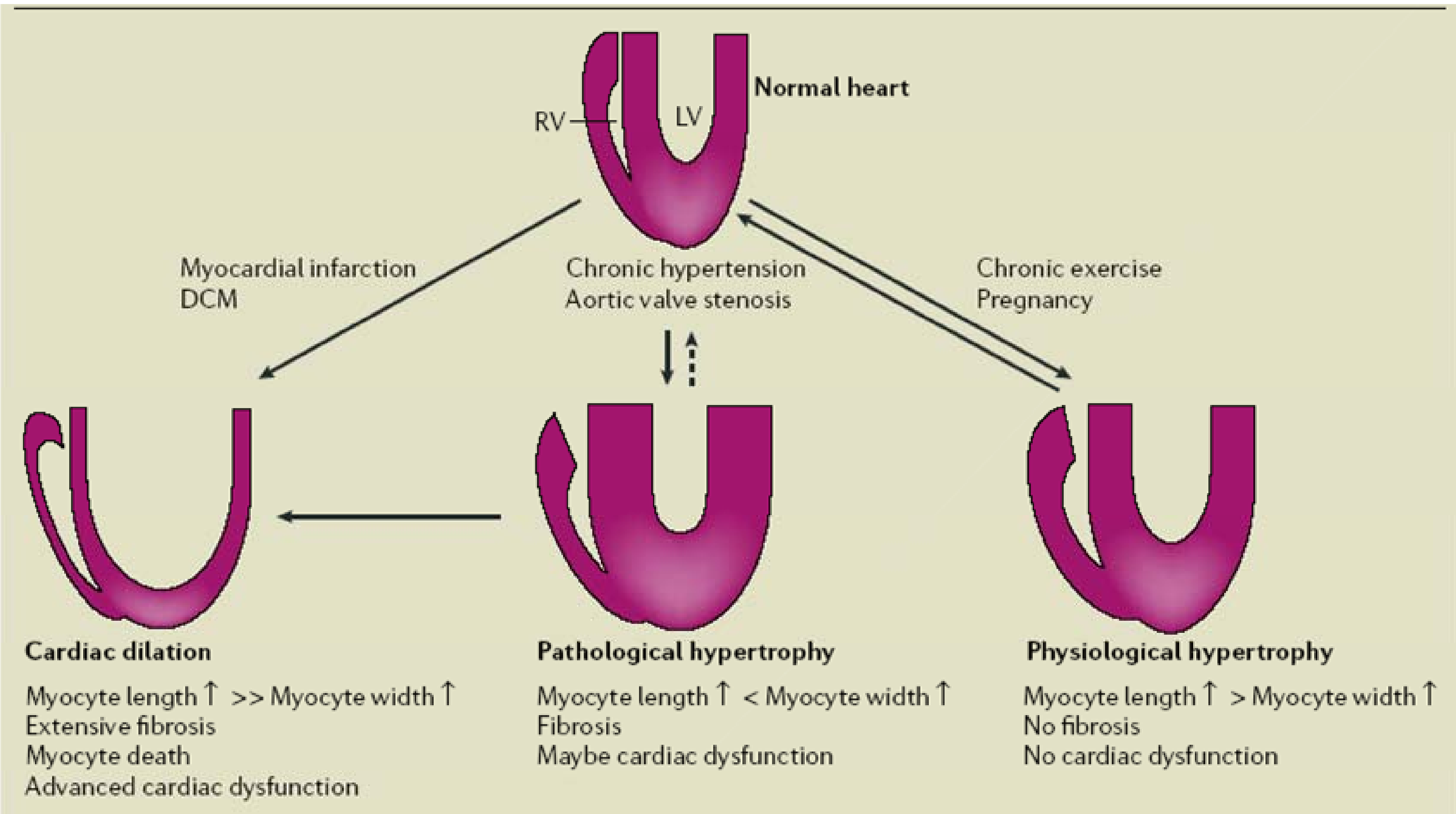

Figure 1.2 – Types of Cardiac Hypertrophy

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mechanotransduction links mechanical stress at the sarcolemma with the nucleus of the myocyte.

Cardiomyocyte hypertrophy occurs through enhanced cell size, increased rates of protein synthesis, decreased protein degradation, and reorganization of the sarcomere (98). Because adult cardiac myocytes are post-mitotic nondividing cells, organ growth is promoted via cellular hypertrophy (361). For example, Sadoshima (1992) evaluated the effects of mechanical stretch (20%) on neonatal rat ventricular cardiomyocytes after 48 hours and observed a significant increase in ^{3}H-phenylalanine incorporation into protein and relative protein content normalized to DNA (292). Stimulation of cell growth in cardiac myocytes occurs via mechanotransduction and thereby can respond to stimuli such as mechanical stress.

Protein synthesis in the myocardium also increases in response to mechanical stress. Adult Sprague-Dawley rats hearts subjected to 120 mmHg of aortic perfusion pressure in a Langendorff heart preparation had higher rates of protein synthesis within 1-2 hours when compared to hearts perfused at 60 mmHg (185). This observation suggests that elevated aortic pressure and subsequent ventricular wall stress has a stimulatory effect on cardiac protein synthesis rates.

Left ventricular hypertrophy has been shown to reflect increases in mean arterial pressure (MAP) in spontaneously hypertensive rats (SHR). For example, 12-month-old SHR had significantly higher left ventricular (LV) weights normalized to body weight (mg/g) compared to age-matched normotensive

American Wistar rats (NWR). This observation was paralleled by higher MAP in the SHR (268). Within the same study, 24-month-old SHR were treated for 10 months with captopril, an angiotensin converting enzyme inhibitor, which resulted in both reduced LV weight and MAP (269). These important observations provide evidence for pressure-overload induced cardiac hypertrophy *in vivo*.

Natriuretic peptide expression

The atrial natriuretic peptide (ANP) was discovered in the cardiac atrium during the early 1980's and shown to have potent effects on regulating body fluid homeostasis (97; 163). In normal adult hearts, ANP is expressed exclusively by the atria. However, during both volume and pressure overload, the ANP gene is overexpressed in the left ventricle and in the plasma (6; 32; 52). This response is considered a hallmark symbol of cardiac hypertrophy and ventricular remodeling. Brain Natriuretic Peptide (BNP) is also a member of the natriuretic peptide family found in the left ventricle and serves a similar function as ANP (254; 282). However, plasma BNP levels are substantially lower than ANP in healthy men, but can be synthesized more rapidly during ventricular overload (244; 245; 360). Thus, BNP is an index of hypertrophy and may have a pathophysiological role during the development of maladaptive cardiac hypertrophy.

Myosin heavy chain isoform switching

Myosin heavy chain (MyHC), a major component of force production in the heart, serves to control the level of mechanical performance (241). The β MyHC isoform is expressed during fetal development and α MHC is induced after birth

via thyroid hormone (240). In a variety of pathophysiological models of pressure overload, isoform switching in rodents occurs from the faster, more powerful α-MyHC to the slower, more efficient β-MyHC. Lowering of α-MyHC in this model is thought to occur by dilution of mRNA content by the β-MyHC and not through inhibition of α-MyHC synthesis (96). In humans, α-MyHC represents a much smaller proportion of the total MyHC in the left ventricle, but heart failure patients appear to maintain a similar pattern of isoform switching compared to the rodent heart (220). Interestingly, reversal of pressure overload results in regression of cardiac hypertrophy and subsequently returns both α- and β-MyHC mRNAs to normal levels (121). The recovery of α-MyHC is much quicker and suggests that mechanical load-related signals regulate each of the separate genes via different mechanisms.

Interstitial Fibrosis

Collagen constitutes ~85% of the extracellular matrix proteins and provides high tensile strength to the myocardium (136; 350). Interstitial fibrosis, or increased collagen deposition, is unique to maladaptive hypertrophy and not commonly associated with physiological stimuli such as exercise training (30). Two different types of cardiac fibrosis have been observed, namely, reparative and reactive (318). Reparative fibrosis results from loss of myocardial cells due to apoptosis or necrosis; typically following myocardial infarction or senescence. Reactive fibrosis is commonly observed in response to perivascular inflammation which can lead to interstitial fibrosis. Reactive fibrosis occurs in the early stages

of hypertension and subsequently develops into reparative fibrosis following myocyte loss due to apoptosis.

Aortic constriction (AC) is a rapid model of elevated blood pressure and cardiac hypertrophy accompanied with isolated diastolic dysfunction. This model of pressure overload stimulates cardiac fibroblast proliferation and myocardial fibrosis (Figure 1.3) (161). The activity of angiotensin converting enzyme, or ACE, is upregulated during the 1st day of AC followed by fibroblast proliferation by day 3 and myocardial fibrosis by day 28 (162). Cardiac fibroblasts are a major source of collagen production and it has previously been shown that exogenous administration of angiotensin II *in vitro* stimulates cardiac fibroblast proliferation as well as collagen synthesis via an angiotensin II type 1 receptor (AT1) (291; 362). In addition, matrix metalloproteinases (MMP) are capable of degrading collagens and ultimately reducing collagen production. Angiotensin II has been shown to reduce MMP-1 activity in adult human cardiac fibroblasts and enhance the production of tissue inhibitors of metalloproteinase-1 (TIMP-1) in endothelial cells (50; 101). Angiotensin II also upregulates transforming growth factor-beta (TGF-beta) in neonatal and adult cardiac

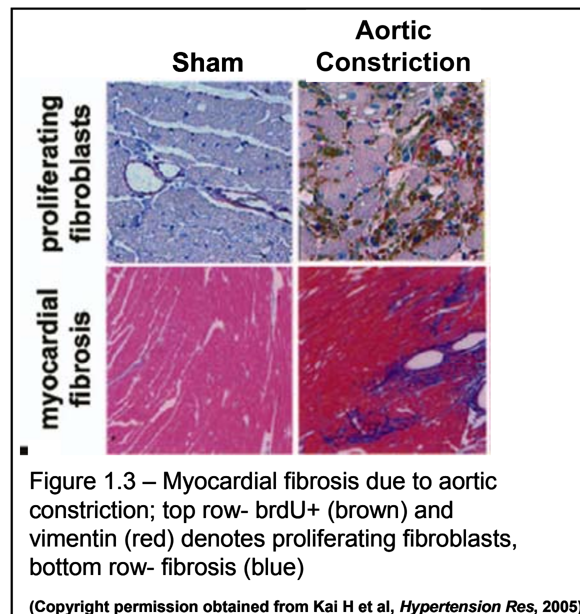

Figure 1.3 – Myocardial fibrosis due to aortic constriction; top row- brdU+ (brown) and vimentin (red) denotes proliferating fibroblasts, bottom row- fibrosis (blue)

(Copyright permission obtained from Kai H et al, *Hypertension Res*, 2005)

fibroblasts (294). Thus, Ang II has been previously shown to stimulate collagen synthesis and promote myocardial fibrosis.

Taken together, the changes in myocardial phenotype following pressure overload, such as concentric hypertrophy, natriuretic peptide synthesis, MyHC isoform switching, and fibrosis, are likely involved in the maladaptive process of the heart.

CARDIAC FUNCTION FOLLOWING PRESSURE OVERLOAD AND MALADAPTIVE PHENOTYPE CHANGES

Although the direct mechanism is still unclear, it is well established that hypertension increases the risk of heart failure (195). Several studies indicate that pressure overload compromises myocardial performance via expression of the maladaptive phenotype. Cardiac fibrosis and myosin heavy chain (MyHC) switching are believed to impair left ventricular function, particularly in pathophysiological states such as hypertension. Mounting evidence indicates these adaptations are disadvantageous to cardiac functionality.

Fibrosis and collagen deposition diminish cardiac function

The detrimental effects of increased myocardial fibrosis are documented in the following reports. A study conducted with Dahl salt sensitive rats placed on 8% NaCl diet at 7 weeks of age demonstrated hypertension, LV hypertrophy, and isolated diastolic dysfunction at age of 13 weeks (79). LV end diastolic pressure was significantly elevated compared to age-matched controls and suggested impaired myocardial relaxation. By 19 weeks of age, overt diastolic

congestive heart failure was established whereby the majority of the rats died by 21 weeks of age. Also, at 19 weeks of age, markers of collagen deposition, such as % area of fibrosis (Azan Mallory stain) and hydroxyproline concentrations, were increased compared to controls (78). These observations suggest that abnormal passive stiffness of the LV wall facilitates diastolic failure and is associated with a phenotype for collagen deposition.

In a separate study, rats subjected to abdominal aortic constriction for 10 weeks had significantly higher dPdT / MAP following stimulation with isoproterenol when compared to a sham group (31). Hydroxyproline analysis of the LV revealed increased collagen deposition compared to sham-operated animals. Also, the ratio of collagen type III:I was reduced in the experimental hypertension group. Since collagen type I is less compliant than type III, a lower ratio of type III to I represents a stiffer, less compliant ventricular wall in this model and may promote impaired dPdT / MAP.

Influence of myosin heavy chain on cardiac function

Circumstantial evidence suggests that the shift from α- to β-MyHC is disadvantageous and promotes the progression of myocardial disease (158; 238). While β-MyHC is more economically efficient with the usage of ATP than α-MyHC, sole expression of this isoform produces lower power output and displays accelerated dysfunction in response to cardiac stress (146; 247).

The α-MyHC is primarily expressed in the adult rodent heart while β-MHC comprises a much lower percentage. However, during pressure overload α-MHC is downregulated while β-MyHC expression is concomitantly stimulated (150).

Several studies have investigated the impact of this response on cardiac function. For example, Korte (2005) utilized hypothyroid and euthyroid rats to express 0% to 100% of cardiac β-MyHC. Isolated working heart preparations demonstrated a linear relationship between increasing β-MyHC and decreased power output (192). The same relationship was observed in skinned cardiac myocytes which suggests a functional consistency between single myocytes and working heart preparations. Although α-MHC is expressed at a much smaller percentage than β-MyHC in human hearts, small increases in α-MyHC expression (~12%) also been translated into large improvements in myocyte power output (~52%) (141). Taken together, these studies demonstrate that changes in the relative isoform expression of MyHC have important functional consequences.

Expression of β-MyHC alone does not induce disease states. Studies suggest that the switch to β-MyHC coupled with mechanical stress can promote impaired LV function. Transgenic mice (TG) with near complete expression of β-MyHC were subjected to various forms of mechanical stress, including myocardial infarction, isoproterenol administration via osmotic pump, and involuntary exercise, to assess if this adaptation has beneficial or detrimental effects to myocardial performance (198). Under normal conditions, the TG mice with high β-MyHC demonstrated moderate reductions in cardiac contractile function, but appeared morphologically unaffected at baseline. Post-myocardial infarction revealed that TG mice incur accelerated LV dilation and an overall more rapid decline in fractional shortening. In addition, chronic isoproterenal

challenge produced augmented LV hypertrophy and decompensated ventricular function compared to the non-TG group. Following 4 weeks of swim training, both TG- and non-TG mice developed ~20% increase in LV / body weight ratios, but TG mice displayed greater end diastolic and systolic LV diameters compared to the trained, non-TG. This study provides evidence that β-MyHC expression in mice may be a disadvantageous adaptation for cardiac function following mechanical stress (197). Furthermore, normal human hearts, as compared to rodents, express a higher percentage of β-MyHC, but still maintain physiological cardiac function. This adaptation may exist due to the slower heart rate within humans.

CARDIAC FUNCTION FOLLOWING EXERCISE TRAINING IN HEALTH AND DISEASE

Exercise training and cardiac function in health

Cardiac output is believed to have a pivotal role in regulating maximal oxygen consumption (VO2 max) and maximal aerobic capacity. A.V. Hill, a Nobel Laureate (1922) and pioneer of exercise physiology, originally proposed that ability of the heart to deliver oxygen can have a major role in limiting aerobic exercise capacity (143). More recently, other studies have supported Hill's contention by the selective breeding of low- and high-capacity running rats (189). Following only 3 generations of breeding, wild-type rats artificially selected for high- capacity running had augmented cardiac performance, such as cardiac output and stroke volume, in isolated working hearts compared to the low

capacity group (149). Further characterization of these animals revealed upregulated cardiac α-MyHC mRNA and protein expression in the high-capacity running group (17). These observations suggest that physical fitness may be an important factor regulating cardiac function.

Contrary to pressure overload, exercise training induces beneficial effects on cardiac function. When compared to both age-matched or body weight-matched controls, treadmill training for 13 weeks (5 days / week) of wild-type rats produced significant improvements in cardiac function and gene expression different from pathological hypertrophy (155). Hemodynamic measurements determined via echocardiography demonstrated increases in resting cardiac index (or cardiac output / body weight) and stroke volume. Also, exercise training upregulated α-MyHC mRNA levels with no effect on β-MHC, ANP, or Collagen I mRNAs (156).

An improved contractile response at the cellular and molecular level may in part contribute to improved myocardial performance following exercise training. For example, Diffee (2003) provided evidence that exercise training in the rat evoked an increase in peak power output of single permeabilized myocyte preparations (73). This effect was irrespective of changes in MyHC isoform expression between sedentary and exercise groups. However, other studies have suggested that Ca^{+2} sensitivity of the contractile apparatus are enhanced with exercise. Steady-state tension is augmented in single myocytes following exercise training as determined by Ca^{+2} concentrations producing 50% of

maximal tension (76). Thus, exercise improves myocardial contractility and benefits cardiac function.

Exercise training and cardiac function in disease

The number of studies that have evaluated the effects of exercise training on cardiac function in disease models, such as hypertension and diabetes, are limited. However, many key observations suggest that exercise training can delay or reduce the onset of chronic heart failure in pathophysiological states. Emter (2005) demonstrated that low-intensity exercise slows the onset of decompensated heart failure in spontaneously hypertensive, heart failure rats (90). This particular strain of rat is genetically predisposed to accelerated development of hypertension and subsequent mortality as a result of heart failure. Six months of low-intensity treadmill exercise delayed overt heart failure and improved survival independent of changes in blood pressure. Exercise training delayed the shift in MyHC isoforms from α to β compared to the sedentary group (89). Also, Veras-Silva et al (341) have shown that low intensity, not high intensity, treadmill exercise for 18 weeks reduces the rise in heart rate and subsequently lowers cardiac output in spontaneously hypertensive rats. Systolic, diastolic, and mean arterial blood pressure were all reduced only in the low intensity exercised group. Furthermore, exercise for 10-12 weeks via swim training in rats with renal hypertension demonstrated that cardiac function was improved (295). Ejection fraction and percent fractional shortening in isolated working hearts were either partially or completely restored to that of control rats with no hypertension. Together, these observations suggest that

superimposition of exercise training in models of accelerated or moderate forms of hypertension can be beneficial to overall performance in the heart.

Other studies have investigated cardiac function in diabetic heart disease, or diabetic cardiomyopathy. Streptozotocin-induced diabetes in Sprague-Dawley rats results in impaired stroke volume and cardiac output in working heart preparations in response to increasing left atrial filling pressures. Eight weeks of progressive treadmill exercise in diabetic rats produced significantly higher cardiac output per dry weight of the heart at 10, 15, 20 cm H_2O of atrial pressure (69). Additionally, similar experiments using streptozotocin-induced diabetes have shown that the myocardial ultrastructure, such as collagen cross-sectional area, is maintained at control levels following 9 weeks of daily treadmill exercise (297). In agreement, exercise in similar models has been shown to attenuate myocardial stiffness with no effect on cardiac hydroxyproline, a marker of collagen concentration (352). Alternative models of animal disease, such as diabetic dyslipidemic pigs, have demonstrated improvements in fractional shortening (%), peak LV systolic pressure (mmHg), and the development of LV dP/dt (mmHg/sec) following 20 weeks of moderate-intensity exercise (194).

BACKGROUND TO THE (mREN2)27 MODEL

The hypertensive process involves the interaction between environmental factors and a polygenetic background. Several animal disease models, such as the spontaneously hypertensive rat (SHR) and the spontaneously hypertensive heart failure rat (SHHFR), have been widely used to study this phenotype.

However, the regulatory genes involved in this complex disease remain largely unknown.

Unlike naturally occurring disease, transgenic animal models promote a phenotypic effect through a single genetic change. Due to the complex nature of the hypertensive phenotype, causal effects are often difficult to differentiate from secondary effects. Importantly, the renin-angiotensin system (RAS), through its regulation of multiple genes, plays a major role in the control of fluid homeostasis and blood pressure. Thus, the transgenic TGR (mRen2)27 rat (mREN2)27) offers a unique approach to study the pathophysiology of hypertension and its interaction with environmental factors through the selective enhancement of RAS.

The (mREN2)27 rat overexpresses the mouse renin gene which enhances Angiotensin II (Ang II) levels in multiple extrarenal tissues and fails to increase plasma Ang II (34). In heterozygous animals, systolic blood pressure is significantly elevated by 4-5 weeks of age compared to control Sprague Dawley rats and reaches maximum levels of 240 mmHg in males (207). Additionally, in response to a hyperactive RAS, these animals undergoe several maladaptive cardiac responses including the following: cardiac hypertrophy, MyHC isoform switching $\alpha \longrightarrow \beta$, increased collagen deposition, ventricular ANP synthesis, and impaired left ventricular function (230; 260; 274).

Importantly, voluntary exercise has been previously demonstrated in the (mREN2)27 rat. A study by Kinnick et al (180) determined that the average running distance in the (mREN2)27 rat can peak at up to 6-7 km / day and serves

to significantly reduce systolic blood pressure ~16%. Thus, the (mREN2)27 rat is a model which develops essential hypertension and will effectively engage in daily exercise.

Part II.

OBESITY AND THE PATHOGENESIS OF TYPE II DIABETES:

Epidemiology of Type II Diabetes

During the past two decades, the prevalence of type II diabetes has escalated into a global epidemic. In 2000, the estimated number of individuals worldwide with this disease was ~151 million (Figure 1.4) (367). The number affected by type II diabetes is predicted to increase to 220 million by the year 2010 and 300 million thereafter in 2025 (5; 178; 366). These statistics suggest that the diabetes epidemic will continue to worsen rather than improve in the future unless proper measures are taken within the public health community.

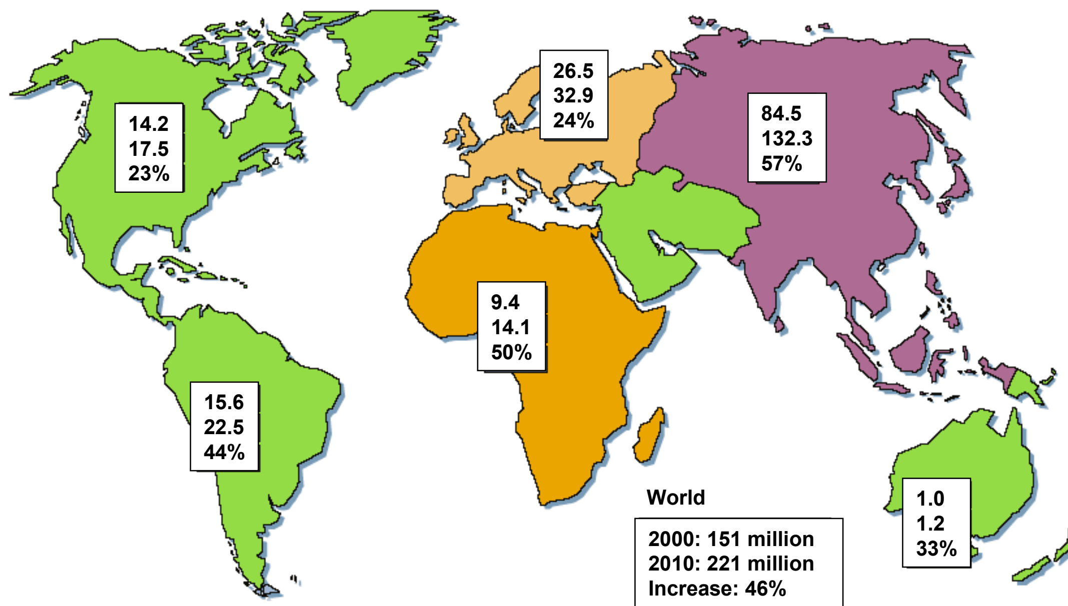

Figure 1.4 – Estimated world wide population with diabetes for 2000 and 2010; Top row- estimation of diabetic individuals (millions) in 2000, middle row- estimation of diabetic individuals (millions) in 2010, bottom row- % increase from 2000 to 2010

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Obesity as a Risk Factor for Type II Diabetes

The defining pathophysiological factors or processes responsible for the development of type II diabetes are not completely understood. However, there is strong evidence to indicate a role for obesity in the pathogenesis of type II diabetes. For example, body mass index (BMI kg/m^2), a statistical measure of body weight according to height, has been shown to be an important risk factor for developing type II diabetes. This measurement was invented by a Belgian mathematician, Adolphe Quetelet, between 1830 and 1850. Today, it has become a crucial health index used by diabetes physicians and within clinical research. Clinical studies demonstrate that the relative risk of type II diabetes in women is 4-fold higher if they have a BMI 23-25 kg/m^2 compared to those with a BMI <20 kg/m^2 (56). Additionally, a BMI > 35 kg/m^2 in women raises the relative risk of type II diabetes to 93-fold compared to lean counterparts (128). Similar correlations also exist in men. U.S males aged 40-75 years were found to have a strong positive correlation between obesity determined by BMI and the incidence of type II diabetes. Those with a BMI of 35 or higher had a significantly greater relative risk of developing diabetes when compared to cohorts with a BMI of < 23 kg/m^2 (43). Due to such a strong relationship between obesity and type II diabetes within a variety of observational studies, the term “diabesity” has even been proposed to redefine obesity as a primary cause of type II diabetes (7).

Lifestyle Intervention

The susceptibility for obesity and type II diabetes can result from a genetic predisposition as well as environmental risk factors. Although the genotype responsible for the onset of type II diabetes has not been elucidated, alterations in lifestyle including increased levels of physical activity and proper diet are believed to significantly influence disease progression. The Finnish Diabetes Prevention Study Group demonstrated that overweight subjects (mean BMI = 31 kg/m^2) with impaired glucose tolerance who participated in a lifestyle intervention for ~3.2 years reduced the incidence of type II diabetes by 58% (326). Annual glucose tolerance tests were performed to diagnose and confirm the development of diabetes according to the 1985 criteria of the World Health Organization. These subjects were instructed to reduce body weight by decreasing fat intake and by engaging in daily physical activity. Mean body weight was significantly reduced following the first (4.2±5.1 kg) and second (3.5±5.5 kg) year of intervention when compared to a non-intervention, control group. The study demonstrated that type II diabetes can be largely prevented by lifestyle changes in high-risk, overweight subjects (327).

A similar study by the Diabetes Prevention Program Research Group has compared the impact of lifestyle changes vs. metformin, a common anti-diabetic drug, in nondiabetic persons with elevated fasting and post-load glucose levels (187). Both treatments significantly reduced the incidence of diabetes when compared to a placebo group, but the lifestyle intervention group was significantly more effective than the metformin group. Interestingly, only 74% of

the participants in the intervention group during the first year adhered to the 150 minutes of prescribed physical activity per week. In the second year, only 58% of participants performed the minimum physical activity requirements suggesting that the potential benefit of the lifestyle intervention could be underestimated. (186).

PHENOTYPE CHANGES IN SKELETAL MUSCLE WITH OBESITY

Lipid accumulation, lipotoxicity, and insulin resistance

The cellular and molecular mechanisms responsible for insulin resistance in skeletal muscle are unknown to date. This metabolic defect is characterized by an inadequate response to basal levels of insulin, decreased glucose uptake via a membrane-bound GLUT4 transporter, and subsequent hyperinsulinemia and hyperglycemia. Many studies suggest that decreased skeletal muscle insulin sensitivity occurs due to lower oxidative capacity and reduced rates of fatty acid oxidation (168; 305; 306). This observation is supported by the association of obesity with a higher percentage of fast, glycolytic muscle fibers (type IIx) (320); but not in all studies (321; 365). Type IIx muscle fibers primarily rely upon glycolytic enzyme activity and have a reduced oxidative capacity compared to type I or type IIa fibers. For example, rat skeletal muscle enriched with oxidative, type I muscle fibers demonstrated a higher capacity for lipid oxidation in comparison with muscle highly expressing type II fibers (84). Taken together, this phenotype could potentially contribute to the development of insulin resistance and type II diabetes.

Intramuscular lipid accumulation is also a common phenotype observed in obesity and type II diabetes compared with lean subjects. Kelley et al (134) investigated lipid content of the vastus lateralis muscle of lean, obese, and type II diabetic humans in relation to muscle fiber type. The amount of oil red O staining for lipid content was significantly higher for all 3 muscle fiber types (I, IIa, IIb) in both obese and type II diabetic subjects compared to the lean group. Additionally, there was not a significant difference between the percentages of muscle fiber types (I, IIa, or IIb) between groups, but the ratio of glycolytic-to-oxidative enzyme activity for each of the three fiber types was higher in both obesity and type II diabetes (135).

Furthermore, Goodpaster et al (105) hypothesized that skeletal muscle lipid content is associated with insulin sensitivity. To determine this, human subjects characterized as lean, obese, type II diabetic, and exercise trained were recruited for measurements of muscle lipid accumulation and insulin sensitivity via hyperinsulinemic / euglycemic clamping. The results demonstrate that intramuscular lipid was elevated in both type II diabetic and exercise trained groups compared to lean, sedentary subjects. Interestingly, the exercise trained group had a significantly higher index of insulin sensitivity compared to the type II diabetic group (107). This observation suggests a paradox between the skeletal muscle lipid content and insulin resistance, as both diabetic subjects and exercise trained athletes have high intramyocellular lipid content while possessing different insulin sensitivities (low in diabetic, high in exercise trained). However, Keizer et al (332) has provided evidence that skeletal muscle lipid

content may not be related to insulin sensitivity. Intramuscular lipid accumulation in mixed fiber types (average of type I and II) of the vastus lateralis was substantially elevated in endurance trained athletes in comparison with obese and type II diabetic subjects suggesting that elevated skeletal muscle lipid content is unlikely to be directly related to insulin resistance.

The effects of weight loss in obese and type II diabetic patients on intramuscular lipid accumulation have been evaluated. Lipid content in the vastus lateralis muscle was assessed by oil red O staining before and after a 16 week medically supervised weight loss program. The mean BMI for both the obese and type II diabetic group was > 33 and for the lean subjects it was 23. Weight loss via caloric restriction successfully decreased body weights by 14-15 kg and concomitantly reduced intramuscular lipid content (111). An additional study which determined the effects of weight loss + physical activity did not demonstrate a decrease in total lipid content. However, the average size of the lipid droplets was significantly decreased which suggests a differential response to exercise-induced weight loss (132). Weight loss + physical activity was also associated with a 46% increase in insulin sensitivity.

Confirming and extending the above, Russell et al (287) demonstrated that skeletal muscle lipid content was similar between insulin sensitive, endurance-trained athletes and insulin-resistant, obese humans. However, the degree of lipid peroxidation and subsequent production of 4-hydroxynonenal (4-HNE) (Figure 1.5) was elevated in skeletal muscle from obese humans compared with lean and endurance trained athletes. This finding indicated that

exercise training was associated with lower lipid peroxidation per unit of intramyocellular lipid. Furthermore, exercise training is a powerful stimulus for increased antioxidant capacity and may serve to reduce the peroxidation of lipids in skeletal muscle via improvements of reactive oxygen species scavenging (60; 300; 337).

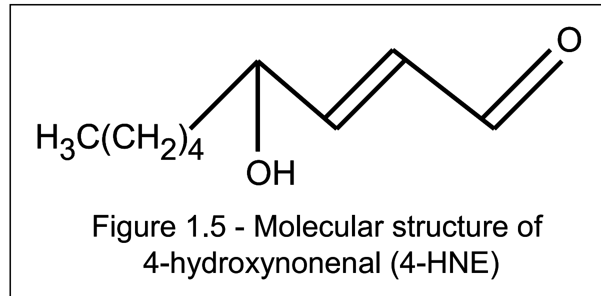


Figure 1.5 - Molecular structure of 4-hydroxynonenal (4-HNE)

This important adaptation will be discussed in a later section. Although both endurance trained and obese subjects had comparable levels of accumulated intramuscular triglycerides (IMTG), the ratio of 4-HNE / Intramuscular lipid accumulation was significantly higher in the obese subjects. This study suggests skeletal muscle lipid peroxidation, not lipid content, may influence insulin sensitivity. However, further studies are needed to evaluate the potential role of lipid peroxidation in development of insulin resistance.

In summary, these observations suggest a possible link between obesity-induced insulin resistance and phenotype alterations in skeletal muscle, including the ratio of lipid peroxidation to intramyocellular lipid content.

PROTECTION AGAINST REACTIVE OXYGEN SPECIES VIA ANTIOXIDANTS

Diabetes is often accompanied by evidence of increased oxidative stress and impaired antioxidant capacity. Oxidative stress induced by reactive oxygen species is believed to have a role in glycemic dysregulation and the development of insulin resistance (139). Supplementation of antioxidants in models of chronic disease has been shown to attenuate oxidative stress and improve glucose

tolerance. For example, Zucker diabetic fatty rats were treated with antioxidants (N-acetyl-L-cysteine or aminoguanidine) from 6-12 weeks of age. This intervention was shown to prevent a rise in blood levels of lipid peroxides (malondialdehyde and 4-hydroxy-2-nonenal), partially reduce glucose intolerance, and improve defective insulin secretion (319). Furthermore, (mREN2)27 transgenic rats, a model of angiotensin II-induced insulin resistance, were treated with a reactive oxygen species scavenger (tempol) from 6-9 weeks of age (24). The results demonstrated reduced superoxide production and improved skeletal muscle glucose transport compared to untreated (mREN2)27 rats. Thus, oxidative stress may serve to impair insulin sensitivity and supplementation with antioxidants can attenuate this maladaptation. Endogenous upregulation of antioxidant capacity through superoxide dismutase (SOD) and glutathione S-transferase-α4 (GSTα4) can protect against reactive oxygen species generation.

Superoxide Dismutase 1 and 2

Exercise training is a powerful stimulus for increased antioxidant capacity and may serve to reduce the oxidative stress in skeletal muscle via improvements of reactive oxygen species scavenging (61; 299; 340). Equation 1 depicts the action of SOD by which superoxide ($2O^{\bullet-}_2$) is mutated to hydrogen peroxide (H_2O_2) and molecular O_2.

$$\text{Equation 1:} \quad 2O^{\bullet-}_2 + 2H^+ \rightarrow H_2O_2 + O_2$$

Treadmill exercise training in young, Sprague Dawley rats for 10 weeks (4 days / week) at 60 min / day significantly enhanced both oxidative capacity and total

superoxide dismutase (SOD) activity in soleus muscle (277). However, the exercise training augmented SOD activity in both the soleus and red gastrocnemius muscle, but did not in the white gastrocnemius muscle. This response suggests that the regulation of SOD activity is: 1) mediated via a fiber-type dependent mechanism, or 2) responsive to muscle fibers being actively recruited during exercise.

Also, various isoforms of SOD can be specifically localized within the cellular and extracellular compartments of mammalian skeletal muscle (228). Cu-Zn SOD (SOD-1) is located within the cytosol whereas Mn-SOD (SOD-2) is principally found within the mitochondrial matrix (126). EC-SOD (SOD-3) resides in the extracellular matrix, but primarily functions in the plasma, lymph, and synovial fluids (229). The majority of SOD activity in skeletal muscle occurs within the cytosol (65-85%), and the remaining is in the mitochondria (15-35%). Interestingly, obese mice were shown to have higher levels of EC-SOD protein in several tissues including the liver, gastrocnemius muscle, white adipose tissue, brown adipose tissue, and plasma (249). This response may serve as a compensatory adaptation to oxidative stress resulting from obesity.

Glutathione S-transferase α-4

The GST family of enzymes catalyzes the conjugation of reduced glutathione with electrophilic compounds, such as 4-HNE, via Michael addition (119; 130). The enzymatic reaction produces a glutathione-toxin conjugate which subsequently enters a detoxification pathway and is exported from the cell. Chronic oxidative stress, which can facilitate 4-HNE production, appears to

augment nitrosylation of Akt and other targets leading to the inhibition of PI 3-kinase signaling during diabetic insulin resistance in muscle (210). In order to assess the pathophysiological role of 4-HNE *in vivo*, GSTα-4 knockout mice (GSTα-4 -/-) were generated and exhibited a reduced ability to conjugate 4-HNE (95). These mice had significantly higher steady-state levels of 4-HNE and malondialdehyde (MDA) (end-products of lipid peroxidation by reactive oxygen species) in the liver compared to wild type mice at 4 months of age. Also, the survival times of GSTα-4 -/- mice were significantly reduced compared to wild-type mice following an intraperitoneal injection of paraquot, a generator of superoxide radicals (94). Another study (117) has shown that protein carbonylation by reactive aldehydes, such as 4-HNE, is elevated in adipose tissue by 2-3 fold following diet-induced obesity in C575I/6J mice. The C575I/6J is an inbred strain of mice which are highly susceptible to obesity, hyperglycemia, and hyperinsulinemia following a high fat diet. Consistent with the observation of high protein carbonylation, GSTα4 protein expression was 3-4 fold lower in adipose tissue of these mice. In summary, these studies suggest that GSTα-4 serves to reduce 4-HNE levels *in vivo* and can protect against oxidative stress.

SKELETAL MUSCLE LIPID OXIDATION

Skeletal muscle has a primary role in regulating whole body lipid oxidation. During resting conditions, up to 90% of energy needs in skeletal muscle is obtained from fatty acid oxidation (64). Thus, several studies have investigated the regulation of skeletal muscle lipid metabolism during conditions such as obesity and subsequent weight loss.

Lipid oxidation in obesity

The impaired ability to sufficiently oxidize lipids in skeletal muscle may have a significant role in the development of obesity (148; 169; 172). For example, lipid oxidation rates were determined by measuring $^{14}CO_2$ production from ^{14}C-labeled fatty acids in the vastus lateralis muscle of lean (BMI 23.8 ± 0.9 kg/m^2) and obese humans (BMI 38.3 ± 3.1 kg/m^2) (173). The obese group had significantly lower ($p < 0.05$) oxidation rates for ^{14}C labeled-palmitate, -palmitoyl, and -octanoate (nmole CO_2 /g/hr) and the rates were negatively correlated with BMI. Also, activity of carnitine palmitoyltransferase-1 (CPT-1), an enzyme which transports long-chain fatty acids across the mitochondrial membrane, was impaired in skeletal muscle of the obese group suggesting this enzyme may have a role in reducing fatty acid oxidation rates (174).

Effects of exercise training in obesity

Other studies have evaluated the effects of exercise training on skeletal muscle lipid oxidation rates in obese humans (28; 58; 170). Eight weeks of moderate-intensity endurance training in obese subjects (BMI 36±2 kg/m^2) did not reduce body mass, but improved citrate synthase and β-hydroxyacyl-CoA dehydrogenase (β-HAD) enzyme activities in skeletal muscle (29). This exercise regime also increased basal mitochondrial fatty acid oxidation rates (nmol/hr/mg), stimulated CPT-1 activity (nmol/min/kg), and augmented an index of insulin sensitivity in the vastus lateralis muscle. Furthermore, enhanced fatty acid oxidation in skeletal muscle has been associated with improved insulin sensitivity following exercise-induced weight loss in obese subjects (109). Thus, physical

activity and weight loss in obesity may serve to lessen the risk of type II diabetes and insulin resistance through the regulation of fatty acid metabolism.

MOLECULAR SIGNALS FOR PROTECTION AGAINIST OBESITY AND REGULATION OF LIPID OXIDATION GENES

Peroxisome proliferator activated receptor-δ

Since its discovery in 1990, investigations on peroxisome proliferator activated receptors (PPAR) have become a fascinating field of research. Through genetic manipulations of PPARs in mice, it was discovered that this family of orphan nuclear receptors can protect against the development of obesity and regulate basic metabolic pathways (15; 219). For example, overexpression of the PPARδ (+/+) isoform effectively protected against diet-induced obesity by significantly reducing body weight when compared to wild-type mice (348). This response was associated with activation of genes required for increased oxidative capacity and thermogenesis in brown adipose tissue. Also, differentiated C_2C_{12} myotubes infected with an adenovirus expressing PPARδ augmented fatty acid β-oxidation and increased mRNA levels for acetyl CoA carboxylase-2 and fatty acid transporter protein 4. Further, overexpression of PPARδ (+/+) within skeletal muscle has been shown to induce expression of type I, oxidative muscle fibers while also increasing the endurance running of the animals (347). PPARδ expression and activation appear to have important physiological roles in enhancing fatty acid oxidation and muscle fiber type formation.

Peroxisome proliferator activated receptor-δ coactivator-1

A specific transcriptional coactivator of PPARs is peroxisome proliferator activated receptor-δ co-activator-1, or PGC-1. Studies have demonstrated that PGC-1 has a powerful role in regulating energy metabolism through mitochondrial biogenesis, glucose/fat metabolism, and muscle fiber type switching (213). Upon activation via a putative p38 signaling pathway, PGC-1 can serve to regulate the stimulation of PPAR nuclear receptors (334). Transfection of CV-1 cells with a target reporter plasmid containing a PPARα responsive element gene promoter was activated six-fold by an expression construct of RXRα / PPARα. Upon co-transfection with RXRα / PPARα and PGC-1, the effect was further enhanced (335). Vega et al (336) also demonstrated that co-infection with PPARδ and PGC-1 retroviruses in preadipocytes (3T3-L1) effectively increased gene expression for lipid oxidation and subsequently augmented rates of palmitate oxidation. Thus, PPAR activation can occur through the transcriptional coactivator PGC-1 and serve to mediate changes in energy metabolism.

BACKGROUND TO OTSUKA LONG-EVANS TOKUSHIMA FATTY (OLETF) RAT MODEL

The OLETF rat was discovered in a breeding colony in Japan during the mid-1980's and was selectively bred to produce a model of non-insulin-dependent diabetes mellitus. Characterization of the OLETF rat includes, 1) hyperphagia, 2) late onset hyperglycemia (after 18 weeks of age), 3) mild obesity, 4) a chronic course of disease, 5) clinical onset of diabetes in males, 6)

polyuria and polydipsia, and 7) hyperplasia and subsequent fibrosis of pancreatic islets (166). Further analysis revealed that mRNA expression for the cholecystokinin-A (CCK-A) receptor in the pancreas, small intestine, and brain were undetectable. This finding indicated that the lack of CCK-A receptors likely results in the impaired ability to process satiety signals and, thus, predispositioned for an obese phenotype (18).

The OLETF rat serves as a unique model to study the effects of obesity on metabolic function. Importantly, this line of rats also exhibits an inherent ability to maintain daily physical activity levels using voluntary running wheels, which is absent in most obese animals (19; 315). When introduced at 8 wk of age, voluntary running distance in the OLETF rat increased to a peak of ~9 km/day by 11 weeks of age (20). It was also determined that voluntary exercise of the OLETF rat prevents excessive adiposity and markedly reduced plasma glucose levels (35%) compared to a sedentary group. Meal patterns, food intake, body weight, and leptin levels in exercised OLETF rats were subsequently maintained to levels of non-obese litter mates, or LETOs (21). The OLETF rat represents an exceptional model for this study due to its predisposition for the obese phenotype and its inherent ability to engage in voluntary exercise.

Part III. (*Supplemental*)

EFFECTS OF CALORIC RESTRICTION IN OBESITY

Caloric restriction (CR) has been shown to effectively extend the lifespan of a variety of species and reduce the development of age-related chronic

diseases (137). It was first described in rats in the 1930's by McKay (1935) and has since become an important strategy for managing obesity-induced non-insulin dependent diabetes mellitus (NIDDM) (140; 232). The exact cellular and molecular mechanisms underlying this response have not been elucidated, but several studies provide evidence that CR can improve metabolic function in obesity.

Insulin Sensitivity and Glucose Transport

Insulin resistance is a metabolic abnormality caused by obesity. Weight loss through CR has been associated with enhanced rates of glucose disposal and reduced insulin levels. Overweight, and otherwise healthy, male (25-50 years) and female (25-45 years) volunteers with BMI $\leq$ 25 kg/m^2 underwent 25% calorie restriction for 6 months (205). The intervention effectively reduced body weight, fasting insulin levels (μU/ml), and an index of insulin sensitivity (AIR_g). Although fasting glucose levels (mg/dL) were unchanged, the subjects were not diagnosed as glucose intolerant during the initial screening process. Within the same study, subjects who underwent 12.5% calorie restriction + 12.5% energy expenditure through exercise for 6 months achieved similar benefits as the CR group. These results suggest that improved insulin sensitivity through weight loss in overweight humans can occur via CR alone or with CR + exercise (204).

Additionally, gastric by-pass surgery is an effective measure to reduce caloric intake (250). This intervention has been shown to promote weight loss, improve lifestyle, and ameliorate risk factors for metabolic and cardiovascular disease in morbidly, obese subjects (208; 308). Glucose disposal rates *in vivo*

during a euglycemic clamp, when insulin was clamped at 40 mU/m^2, increased by 78% following 12 months of gastric bypass surgery in obese subjects (p<0.01) (99). Furthermore, maximal insulin-stimulated glucose transport (2-deoxyglucose) in the vastus lateralis muscle was elevated two-fold (p<0.01) after the weight reduction. However, total skeletal muscle GLUT4 protein was unchanged following gastric by-pass surgery suggesting that changes in insulin sensitivity and glucose transport may occur through improvements in enhanced GLUT4 translocation or activation (100).

GLUT4 Expression in Skeletal Muscle and Adipose

Since glucose transport is believed to be the rate-limiting step in glucose metabolism (364), it is critical to understand the effects of CR on GLUT4 cell surface translocation and protein expression. Twenty days of CR via 60% of ad-lib in rats increased glucose transport (3-O-methylglucose) and augmented the cell surface content of GLUT4 in the epitrochlearis muscle when compared to ad-libitum fed group (68). This observation suggests that enhanced levels of glucose transport in skeletal muscle following CR might occur through increased activation of GLUT4 vesicles, rather than increased total protein expression. Also, a study done in the hyperphagic, OLETF rats has shown that improvements in whole body glucose disposal and insulin resistance following CR (70% of ad-lib) was associated with higher levels of GLUT4 in adipose tissue (263). Interestingly, CR had no effect on total GLUT4 protein levels in gastrocnemius muscle and liver samples. Protein levels for other glucose transporter isoforms, such as GLUT1 and GLUT2, were unaltered. Further, CR for 25 months in rats

significantly increased IRS-1 tyrosine phosphorylation upon insulin stimulation in both liver and skeletal muscle which could serve to stimulate higher levels of GLUT4 activation (363). Taken together, improved glucose transport following CR in rats likely occurs through enhanced cell surface translocation of GLUT4 vesicles in skeletal muscle and increased total GLUT4 protein expression in adipose tissue.

Oxidative Stress

A variety of studies have described obesity-related oxidative stress. It is commonly associated with hyperglycemia, tissue lipid accumulation, and chronic inflammation (346). Consequently, oxidative stress in obesity may serve as a unifying mechanism in conditions such as cardiovascular disease and diabetes (142). However, weight loss through CR has known therapeutic benefits in reducing oxidative stress and improving antioxidant defenses.

Levels of oxidative stress generated within the leukocytes appear to be modifiable with CR alone (65). Nondiabetic, obese humans (BMI, 33–64 kg/m^2) were given a diet of 1000 Cal / day for 4 weeks. Mononuclear cells (MNC) and polymorphonuclear leukocytes (PMN) were isolated from the blood and reactive oxygen species (ROS) generation was measured. CR decreased production of ROS in both cell types. Also, further analysis provided evidence that weight loss through CR was associated with a reduction in oxidative-damage to lipids, proteins, and amino acids (66). Similar observations have been made in severely obese children with an average age of 9.2 ± 2 in years. In particular, lipid peroxide levels of malondialdehyde (MDA) were reduced following 6 months

of dietary restriction (67). Few studies have investigated the role of CR-induced weight loss on oxidative stress in other tissues, such as skeletal muscle, from obese subjects.

One explanation for reduced levels of oxidative stress with CR is increased antioxidant activity and expression. Administration of a very low calorie diet for 1 week was shown to significantly increase SOD activity in erythrocytes ($p<0.01$) of obese, type II diabetic patients and was associated with reduced oxidative stress (309). Also, microarray analysis of the gastrocnemius muscle in Sprague-Dawley rats following 36 weeks of CR (60% of control diet) demonstrated that mRNA levels for SOD-1 and SOD-2 were upregulated (311). However, other studies have not observed differences in antioxidant defense with CR (112; 310). In summary, oxidative stress is commonly associated with obesity and CR can serve to ameliorate oxidative damage, possibly through increased antioxidant status (Figure 1.6) (345).

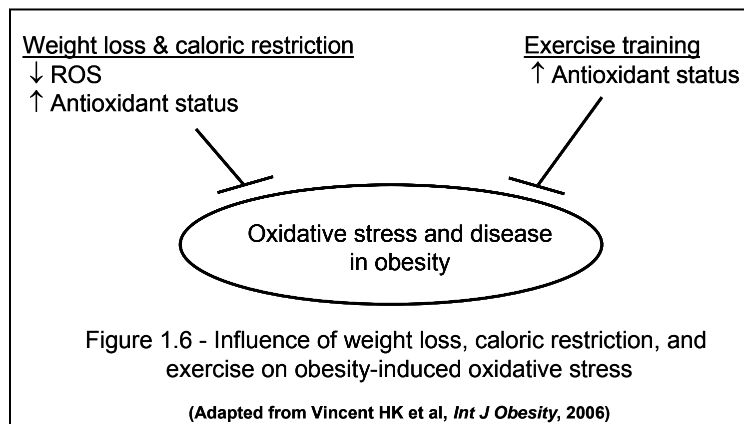


Figure 1.6 - Influence of weight loss, caloric restriction, and exercise on obesity-induced oxidative stress

(Adapted from Vincent HK et al, *Int J Obesity*, 2006)

OVERALL HYPOTHESIS

The overall hypothesis of this dissertation is that exercise protects against maladaptation caused by chronic disease, specifically cardiac dysfunction due to hypertension and skeletal muscle lipid peroxidation due to obesity. It was further

hypothesized that exercise-induced prevention of obesity would not protect against changes in skeletal muscle lipids following short term-physical inactivity for 53 and173 hours.

SPECIFIC RESEARCH AIMS

The specific research aims of this dissertation are:

Chapter 2.

1. To determine if angiotensin II-mediated hypertension in the (mREN2)27 rat will promote cardiac dysfunction and low-intensity exercise training will protect against this response.

2. To determine if angiotensin II-mediated hypertension in the (mREN2)27 rat will promote natriuretic peptide synthesis, myosin heavy chain isoform switching, and collagen deposition and low-intensity exercise training will attenuate these changes.

3. To determine if low-intensity exercise training in the (mREN2)27 rat will reduce systolic blood pressure in angiotensin II-mediated hypertension.

Chapter 3.

1. To determine if exercise-induced prevention of obesity in the OLETF rat will reduce skeletal muscle lipid accumulation.

2. To determine if exercise-induced prevention of obesity in the OLETF rat will protect against skeletal muscle lipid peroxidation.

3. To determine if exercise-induced prevention of obesity in the OLETF rat will augment skeletal muscle fatty acid oxidation.

4. To determine if exercise-induced prevention of obesity in the OLETF rat will not protect against changes in skeletal muscle lipids and maintain fatty acid oxidation rates following short term physical inactivity for 53 and 173 hours.

CHAPTER 2

Exercise training maintains cardiac output and stroke volume in hypertensive TG(mREN-2)27 rats with impaired diastolic function

ABSTRACT.

Excess renin-angiotensin activity can adversely affect cardiac performance; thus we determined the effects of low-intensity exercise training (ET) on *in vivo* cardiac function in hypertensive TG(mREN-2)27 rats (Ren-2). Young Ren-2 rats, overexpressing renin, and age-matched Sprague Dawley (SD) controls (5 weeks) began treadmill exercise every day for 5-6 weeks. Cardiac function was evaluated by echocardiography and blood pressure was measured via tail-cuff method. Cardiac output (ml / min) and stroke volume (ml) per body weight (g) were increased by ET in both 8-wk-old SD and Ren-2. The slope of mitral deceleration time (m/sec^2), a non-invasive measure of diastolic function, was lower in the Ren-2 rats, but not changed by ET in either rat strain compared to SD rats. Systolic, diastolic, and mean arterial blood pressures in Ren-2 rats were higher compared to the SD controls, and ET had no effect. Biochemical measurements were then determined at 10-11 wks of age. LV collagen deposition, assessed by hydroxyproline assay, was not affected by rat strain or ET. Left ventricular B-type natriuretic peptide mRNA levels measured by real-time PCR were higher in the Ren-2 rats (100%), but not affected by ET. Both α (~14.5 fold) and β (~2.5 fold) myosin heavy chain mRNA were higher in the LV of

Ren-2 rats compared to SD ($p < 0.05$), but were not changed by ET. These observations suggest that low-intensity exercise training in Ren-2 rats, a model of angiotensin II-mediated hypertension, maintains cardiac index and stroke volume in the presence of impaired diastolic function at 8 weeks of age.

INTRODUCTION.

For the past 80 years, heart disease has been the leading cause of death within the United States. In 2007, direct and indirect health care costs for this condition are estimated at 151.6 billion US dollars (2). Although the exact mechanisms regulating the pathophysiology of heart disease remain incomplete, primary hypertension is a known cause of cardiac dysfunction and subsequent failure. Interestingly, previous studies indicate that exercise exerts a protective effect toward the development of hypertension in humans. Low and moderate fitness levels have been associated with 3- and 2-fold higher risks, respectively, of developing hypertension than in highly fit humans (37). Also, a US National Committee has concluded that adoption of healthy lifestyles, including increased physical activity, by all individuals is critical for the prevention of high blood pressures and is an indispensable part of the management of those with hypertension (36; 46). In addition to reducing the development of hypertension, exercise may serve a protective role against the events leading toward heart disease.

The renin-angiotensin system (RAS) is an important physiologic mediator of both blood volume and blood pressure. The transgenic TG(mRen2)27 rat

(Ren-2), used in the present study, overexpresses the murine renin gene and exhibits increased angiotensin II (Ang II) levels in multiple extrarenal tissues (33). Unlike other models of hypertension, the pathophysiological phenotype in the Ren-2 rat can be attributed to the selective enhancement of the RAS, including locally within the heart. This model offers a unique approach to study the pathophysiology of heart disease and its interaction with environmental factors such as exercise.

Previous studies suggest that impaired cardiac function and maladaptation occur within 13-17 weeks of age in the Ren-2 rat (257; 272). Both systolic and diastolic rates of pressure change (dP/dt) are reduced in 13-wk old Ren-2 rats when compared to age-matched, wild-type control Sprague-Dawley rats (273). Maladaptation, characterized by re-expression of the fetal gene program and cardiac fibrosis, is commonly associated in left ventricular cardiac dysfunction (152; 153; 283). Interestingly, the Ang II type 1 receptor (AT1) antagonist, TCV-116, blocked the development of cardiac hypertrophy, reduced the ratio of β:α myosin heavy chain (MyHC) mRNA, and altered the expression of the extracellular matrix components in Ren-2 rats by 17 weeks of age (258). Increased levels of physical activity in rats can serve as sufficient treatment for reducing hypertension and hypertensive heart disease (125; 190; 266). For example, in spontaneously hypertensive rats (SHR), 18 weeks of low intensity treadmill exercise (16-20 m/ min) lowered systolic (SBP), diastolic (DBP), and mean arterial (MAP) blood pressures and resting cardiac output and heart rate, while high-intensity training (25-30 m/min for 60 min, 5 times / week) had no

effect (342). Thus, low-intensity exercise influences resting blood pressure and subsequent cardiac function in these animals. Additionally, six weeks of voluntary wheel exercise has been shown to significantly lower ($p<0.05$) resting SBP in the TG(mRen2)27 rat from 198±5 to 167±5 mmHg (sedentary vs. trained, respectively) (181). However, cardiac function following exercise training in a model with selective Ang II-mediated hypertension, such as the TG(mRen2)27 rat, has not been evaluated.

The present study was designed to determine whether treadmill-running at a low intensity would delay cardiac decompensation in the hypertensive TG(mREN2)27 rat. It was hypothesized that exercise training would attenuate increases in blood pressure and preserve normal cardiac output and diastolic function. In addition, left ventricular collagen deposition, levels of α- and β-myosin heavy chain (MyHC) mRNA, and B-type natriuretic peptide (BNP) mRNA were assessed to determine if exercise training would delay the appearance of markers associated with maladaptive cardiac hypertrophy.

MATERIALS AND METHODS.

TG(mRen2)27 rats.

This study utilized a hypertensive rat model to study the effects of exercise training on cardiac function and adaptation. All experimental protocols were approved by the Animal Care and Use Committee at the Harry S. Truman Veterans Memorial Hospital, Columbia, MO.

Male TG(mRen2)27 rats and male Sprague-Dawley (SD) controls were

received from Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC at 3 wk of age. Animals were housed 2-3 per cage with a 12:12-h light-dark cycle in a room maintained at 20-22°C and had free access to rat chow and water. Nails were manicured as needed throughout the experiment in order to prevent potential foot injuries while running on the treadmill. Cardboard backstops were placed at the rear of the running belt, effectively promoting sufficient exercise without the need for electric shock, which could independently affect blood pressure.

Experimental Design.

Rats were selected into running group based upon acclimation to treadmill running and divided into 4 primary experimental groups including: Sedentary SD (SED-SD; n=5), Exercised SD (EX-SD; n=5), Sedentary TG(mRen2)27 (SED-Ren2; n=5), and Exercised TG(mRen2)27 (EX-Ren2; n=5). SD is the background strain from which Ren2 rats were developed (246). At 4 weeks of age, rats were familiarized with the treadmill for 2 weeks (pre-training) followed by 5-6 weeks of exercise training. Rats were run 7 days a week during both the pre-training and training days. Running time was increased during pre-training (2 weeks) until the rats were able to run consecutively for 60 min. During the 5-wk training program rats were given a 2-min daily warm-up and subsequently exercised for 60 min/day. Treadmill speed was progressively increased during this 5-week training period from 14.0 m/min up to 17.5 m/min.

Echocardiography

Echocardiography evaluated left ventricular systolic and diastolic function

following the 3rd week of exercise training. Rats were lightly anesthetized with an intraperitoneal administration of ketamine (50 mg/kg) and diazepam (2.5 mg/kg). This drug cocktail has been previously shown to have minimal cardiorespiratory effects when compared to other suitable anesthetics (317). The hair was removed from the left hemithorax using a depilatory cream to facilitate imaging. The rats were positioned in a right lateral decubitus position. Echocardiograms were performed using a GE Vivid 7 ultrasound system using a 10-mHz transducer and electrocardiographic monitoring. The echosonographer was blinded to the treatment groups. Two-dimensional imaging was used to guide pulsed-wave Doppler evaluation of mitral inflow velocities, and aortic outflow velocities from an apical 4-chamber orientation. M-mode echocardiography of was performed using the parasternal short-axis view of the left ventricle (LV). The guidelines of the American Society of Echocardiography were used for measurement of the LV end-diastolic and end-systolic diameters, and septal and posterior wall thickness. Fractional shortening, an index of contractility was determined from the measurements of LV chamber dimensions and calculated by LV internal diameter in diastole (LVIDd) – LV internal diameter in systole (LVIDs) / LVIDd. Images were captured digitally and a series of 6 consecutive cardiac cycles were measured and averaged for each individual measurement. Stroke volume was calculated using the following formula: aortic outflow velocity time integral X aortic valve cross sectional area. The aortic valve cross sectional area is calculated as follows: aortic diameter^2 X 0.785. Cardiac output was calculated by multiplying stroke volume by heart rate. The cardiac index was

calculated in order to normalize for the increase in cardiac output that occurs with changes in body size. Cardiac index was calculated as: cardiac output / body weight in grams. Rats were allowed to recuperate from the anesthetic for ~1 day before starting daily exercise again.

Blood pressure.

SBP (mmHg) was measured via tail cuff method in conscious rats before the daily exercise on a weekly basis using MC4000 blood pressure analysis system from Hatteras. Prior to the initial (week 1) blood pressure recordings, rats were allowed to acclimate to the blood pressure system over several days.

RNA isolation and cDNA synthesis.

At 10-11 weeks of age, rats were deeply anesthetized and sacrificed. RNA was isolated from a section of the left ventricle using a method adapted from Chomczynski and Sacchi (49). Briefly, the tissue was crushed under liquid N_2 using an autoclaved pestle and mortar. Trizol reagent (1.5 ml) was added to the powdered tissue and homogenized on ice using a Polytron homogenizer (Kinematica) on setting 6 for three times (30 seconds each) with a 1-minute wait between each round. The homogenate was further processed through RNeasy columns and DNase I (Qiagen) to purify the RNA. cDNA synthesis was performed with 2.5μg of purified total RNA using Superscript III first-strand synthesis system (Invitrogen), 50 μM oligo (dT) 20 primer (BNP) or 50ng/ul of random hexamer primers (α- and β MyHC), and a Mastercycler Gradient thermocycler (Eppendorf).

Quantitative Real-time PCR.

The quantitative PCR reactions were performed with an ABI 7000 Sequence Detection System (ABI) using Sybr Green chemistry (ABI). All PCR reactions included 25 ng of cDNA, 90 nM primers for BNP, 900 nM primers for MyHC, and Sybr Green mix. Each sample was assayed in a 25 μl reaction in duplicate. If the duplicate contained a standard deviation of > 0.5, it was re-assayed. Data was normalized to GAPDH. The primer sequence used for rat B-type natriuretic peptide and rat GAPDH were as previously reported (323). (BNP Fw 5' TGGGCAGAAGATAGACCGGA - 3', BNP Rv 5'- ACAACCTCAGCCCGTCACAG - 3'; GAPDH Fw - 5' TGCCAAGTATGATGACATCAAGAAG 3', GAPDH Rv 5' AGCCCAGGATGCCCTTTAGT 3'). Rat α- and β- MyHC primers also were constructed as per previous report (70) (Alpha Fw - 5' TGTGAAAAGATTAACCGGAGTTTAAG 3' , Alpha Rv - 5' TCTGACTTGCGGAGGTATCG 3' ; Beta Fw 5' AAGTCCTCCCTCAAGCTCCTAAGT 3', Beta Rv - 5' TTGCTTTGCCTTTGCCC 3'). All sequences were verified using Primer Express 2.0 (ABI). PCR product formation was checked against a ladder for proper amplicon size using PCR Supermix (Invitrogen) before beginning Sybr Green.

Differences in gene expression were calculated using relative quantification to GAPDH via the comparative C_T method from ABI (User Bulletin no. 2 ABI PRISM 7700 Sequence Detection System). GAPDH was found to be an appropriate normalizer by comparing the differences in raw C_T values which

did not differ between groups. GAPDH expression did not differ with strain (p=0.454), activity (p=0.254), or interaction (p=0.287). Standard curves for each target were run to verify equal efficiency of the PCR reaction.

Hydroxyproline Assay.

The methods for the hydroxyproline assay are previously described (45). Briefly, a section of the LV was weighed, minced on ice, heated in intervals at 60° C until a constant dry weight was obtained; and then incubated overnight at 105-110° C with 6N HCl. A standard curve of hydroxyproline was created and the experimental samples were measured spectrophotometrically at an absorbance of 558 nm. The amount of hydroxyproline was multiplied by the conversion factor 7.46 to calculate total collagen. Lastly, the data were expressed as total collagen normalized to milligrams of left ventricular dry weight (g of LV collagen / mg of LV dry weight).

Statistical Analysis.

Two-way ANOVA was used for all statistical comparisons for echocardiography, blood pressures during the week of echocardiography, real-time PCR, and hydroxyproline assay. There were two possible main effects for these tests: a main effect for exercise training (sedentary and exercise) and a second main effect for strain (SD and Ren2). $P < 0.05$ was considered significant. A three-way ANOVA was used for initial and final blood pressures. A main effect included strain, exercise, or time (initial vs. final) where $p < 0.05$ was considered significant.

RESULTS.

Exercise Reduces Body Weights.

Body weights were significantly greater ($p < 0.05$) in Ren2 compared to SD rats before and after the 5 weeks of treadmill exercise (Table 2.1). EX-SD and EX-Ren2 rats also exhibited lower body weights at the time of the echocardiography compared to SED-SD and SED-Ren2, respectively (Table 2.2).

Exercise Training Improved Cardiac Index and Stroke Volume In TG(mRen2)27 Rats.

Echocardiography was performed after 3 weeks of exercise training (8 weeks of age) and 2-3 weeks prior to sacrifice (Table 2.2). Septal wall thickness during diastole, left ventricular internal diameter during diastole, septal wall thickness during systole, and left ventricular internal diameter during systole were not different between Ren-2 and SD rats when normalized to body weight. Exercise did not have an effect in either rat strain. Also, left ventricular posterior wall thickness (μm) per body weight (g) during diastole and systole were not different. Ren-2 rats exhibited a lower maximum mitral valve inflow velocity (m/sec) (Table 2.2). There was no significant differences between strain or exercise condition for fractional shortening ($p>0.05$).

Since body weights of the EX-Ren-2 and EX-SD were significantly lower at the time of echocardiography compared to their sedentary counterparts (10.4% and 4.0%, respectively), cardiac output (Figure 2.1) and stroke volume (Figure 2.2) were normalized to body weights. During the 3rd week of exercise training, resting cardiac output [stroke volume (ml) x heart rate (beats/min)]

Table 2.1

	Age (weeks)	SED-SD	EX-SD	SED-Ren2	EX-Ren2	$p < 0.05$
n		5	5	5	5	
Initial body weight (g)	3	81.7 ± 4.0	85.8 ± 5.9	136 ± 2.6	127 ± 2.9	*
Final Body weight (g)	10,11	253 ± 13	237 ± 18	298 ± 12.5	273 ± 6.7	*
Total % weight gain		210 ± 4.6	177 ± 15	119 ± 6.2	116 ± 4.1	*
Week 1 run speed (m/min)	5		15.0 ± 0.18		15.0 ± 0.18	
Week 5-6 run speed (m/min)	10,11		17.5		17.5	
Week 1 run distance (km) / day	5		0.90		0.90	
Week 5-6 run distance (km) / day	10,11		1.05		1.05	

Table 2.1 - Body weights, running speeds, and distances. * $p < 0.05$ for main effect of rat strain between Sprague-Dawley (SD) and Ren2 by 2-way ANOVA. Values for each group are mean ± SEM with n = 5 / group.

Table 2.2

	SED-SD	EX-SD	SED-Ren2	EX-Ren2	$p < 0.05$
Body Weight (BW) - (g)	185 ± 3.7	178 ± 10	240 ± 4.2	215 ± 6.3	* †
Heart rate (beats / minute)	519 ± 49	556 ± 6.5	470 ± 21	507 ± 34	
aortic diameter (µm) / BW (g)	12.1 ± 0.42	13.2 ± 0.75	8.97 ± 0.65	10.9 ± 0.32	
Interventr. septum diastole (µm) / BW (g)	11.6 ± 0.58	10.7 ± 0.58	10.1 ± 0.57	11.6 ± 0.63	
LV internal diameter diastole (µm) / BW (g)	28.9 ± 2.1	31.2 ± 1.4	27.5 ± 1.1	30.0 ± 1.1	
LV posterior wall diastole (µm) / BW (g)	13.5 ± 1.1	13.0 ± 0.79	11.3 ± 0.52	12.5 ± 0.75	
Interventr. septum systole (µm) / BW (g)	17.3 ± 1.3	16.8 ± 1.6	15.7 ± 0.58	17.2 ± 0.99	
LV internal diameter systole (µm) / BW (g)	13.2 ± 2.4	16.4 ± 0.74	15.0 ± 1.3	14.9 ± 1.0	
LV posterior wall systole (µm) / BW (g)	19.4 ± 1.6	18.4 ± 2.7	15.6 ± 0.72	17.6 ± 1.2	
% fractional shortening	55.8 ± 4.8	51.0 ± 4.1	45.3 ± 2.6	46.6 ± 2.5	
LV outflow max. (m/sec)	1.07 ± 0.048	1.12 ± 0.088	1.01 ± 0.029	1.15 ± 0.049	
LV outflow mean (m/sec)	0.662 ± 0.038	0.692 ± 0.044	0.618 ± 0.038	0.700 ± 0.044	
Mitral valve inflow max. (m/sec)	1.13 ± 0.078	1.23 ± 0.052	1.11 ± 0.046	0.990 ± 0.053	*
Mitral valve inflow mean (m/sec)	0.700 ± 0.073	0.750 ± 0.058	0.700 ± 0.025	0.596 ± 0.022	

Table 2.2 - Resting echocardiographic measurements determined at 8 weeks of age. * $p < 0.05$ for main effect of rat strain between Sprague-Dawley (SD) and Ren-2 by 2-way ANOVA. † $p < 0.05$ for main effect of exercise within SD and Ren-2 by 2-way ANOVA. Values for each group are mean ± SEM with *n* = 5 / group.

normalized to body weight (g), also known as cardiac index, was significantly greater in both the 8 wk-old EX-SD (21%) and EX-Ren2 (57%) rats compared to their respective sedentary groups ($p < 0.05$; Figure 2.1). Additionally, there was a significant main effect of rat strain on cardiac index which was greater in the SD compared to the Ren-2 ($p < 0.05$). Resting stroke volume (ml) normalized to body weight (g) was also 13% and 43% higher following exercise training in SD and Ren2 rats, respectively, compared to sedentary controls ($p < 0.05$; Figure 2.2). Stroke volume was also larger in the SD strain compared to the Ren-2 ($p < 0.05$). Mitral valve deceleration time (msec) which is the time from the peak velocity of blood flow to the end of flow in diastole was not significantly different (strain $p=0.067$, exercise $p=0.550$; data not shown). However, mitral valve deceleration slope (m/sec^2), which is the change in velocity over the change in time, was shown to be significantly lower in the Ren-2 rats compared to the SD strain ($p < 0.05$; Figure 2.3). In both exercise and sedentary groups, there was no significant difference in mitral valve deceleration with treadmill running in either Ren-2 or SD groups ($p>0.05$).

Figure 2.1

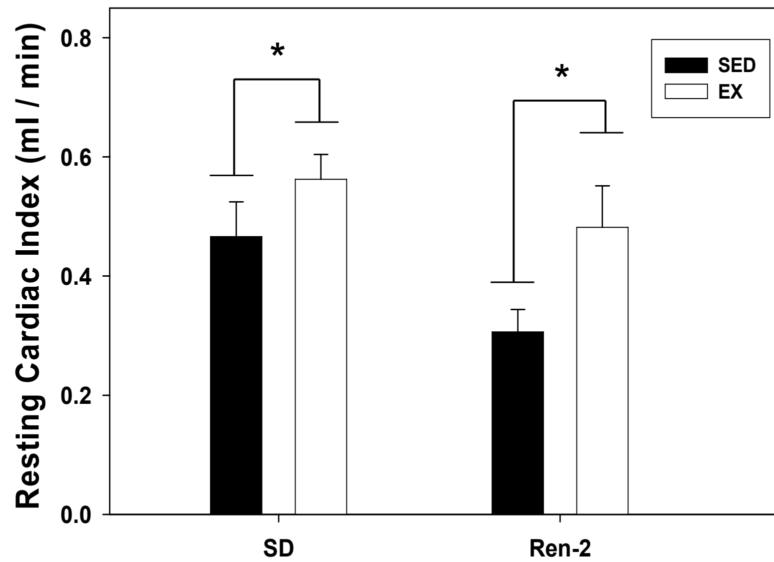


Figure 2.1 - Effect of exercise training on resting cardiac output (ml/min) normalized to body weight (g) [cardiac index] in 8-wk-old Sprague-Dawley (SD) and Ren-2 rats. * indicates for main effect ($p < 0.05$) of exercise within SD and Ren-2 by 2-way ANOVA. Values for each group are mean ± SEM with *n* = 5 / group.

Figure 2.2

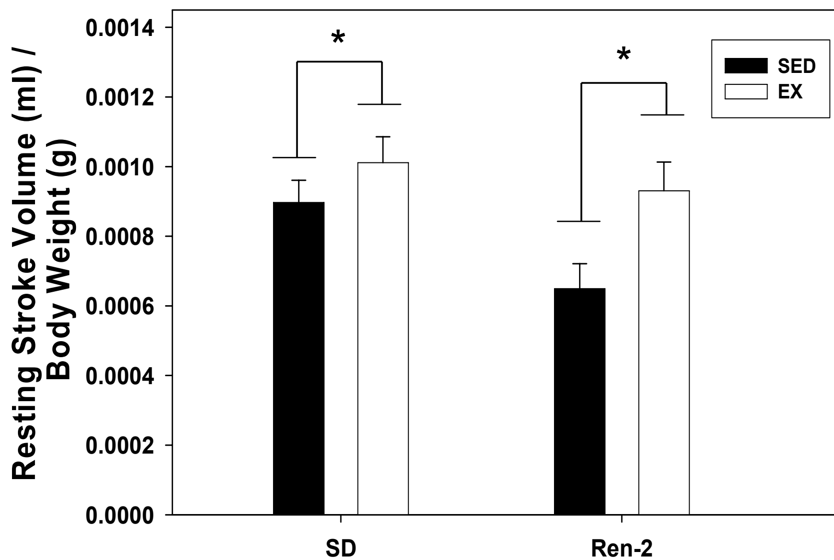


Figure 2.2 - Effect of exercise training on resting stroke volume (ml) normalized to body weight (g) in 8-wk-old SD and Ren-2 rats. * indicates main effect ($p < 0.05$) of exercise within SD and Ren-2 by 2-way ANOVA. Values for each group are mean ± SEM with *n* = 5 / group.

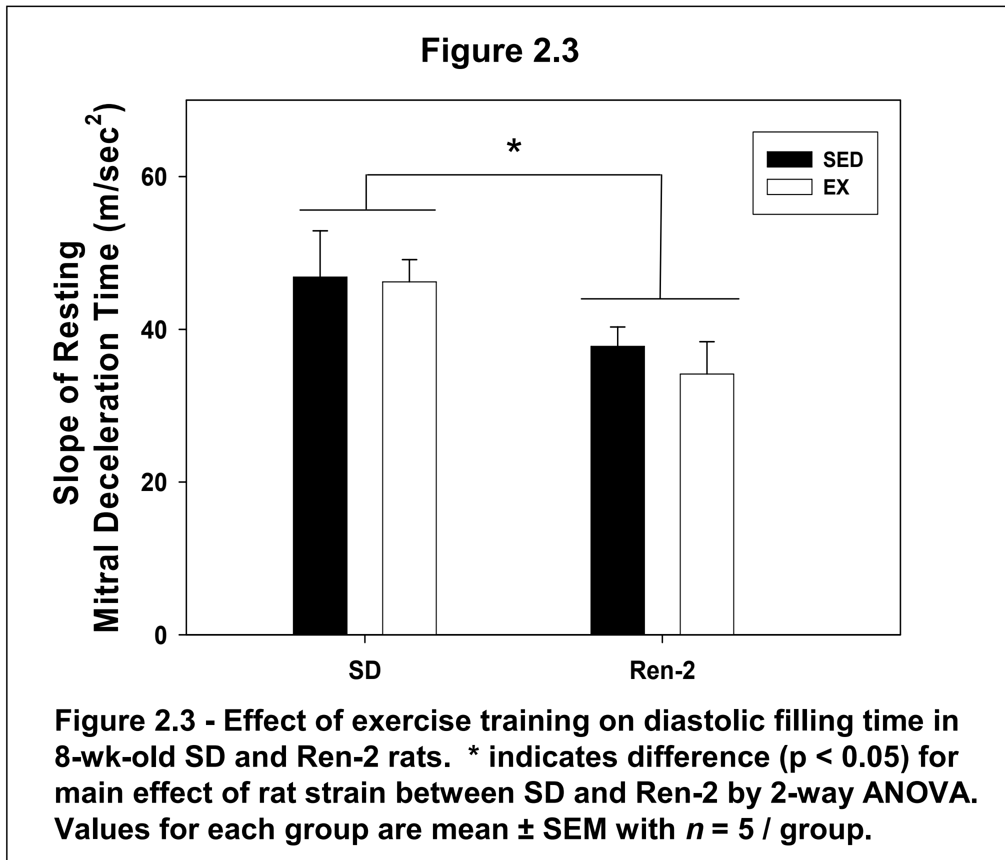


Figure 2.3 - Effect of exercise training on diastolic filling time in 8-wk-old SD and Ren-2 rats. * indicates difference ($p < 0.05$) for main effect of rat strain between SD and Ren-2 by 2-way ANOVA. Values for each group are mean ± SEM with *n* = 5 / group.

Blood Pressures and Heart Rates Were Unaffected by Exercise.

Pre-training, resting systolic blood pressure was not significantly different between sedentary and exercise groups in either rat strain (p=0.184 for SBP; Figure 2.4). Ren-2 rats had a significantly higher pre-training SBP than SD (p<0.05).

During the week of echocardiography (after 3 weeks of exercise training), SBP in Ren2 rats was significantly higher than SD (p<0.05) and SBP of EX-Ren2 rats was greater than SED-Ren2 (p<0.05) (data not shown). After 5 weeks of treadmill running, no significant differences in resting SBP (p=0.478) were observed in EX-SD or EX-Ren-2 rats compared to respective sedentary groups

(Figure 2.4). A significant interaction for strain x time ($p < 0.05$) occurred for SBP. Final resting SBP in EX-Ren2 (67%) and SED-Ren2 (56%) were significantly higher compared to the EX-SD and SED-SD, respectively ($p<0.05$) (Figure 2.4).

Post-training resting heart rates were higher in the SD compared to the Ren2 (p=0.028; data not shown), whereas there was not a significant difference before training. There were no differences in resting heart rates between sedentary and exercise trained groups either pre- or post-training (p=0.821 and p=0.889, respectively; data not shown).

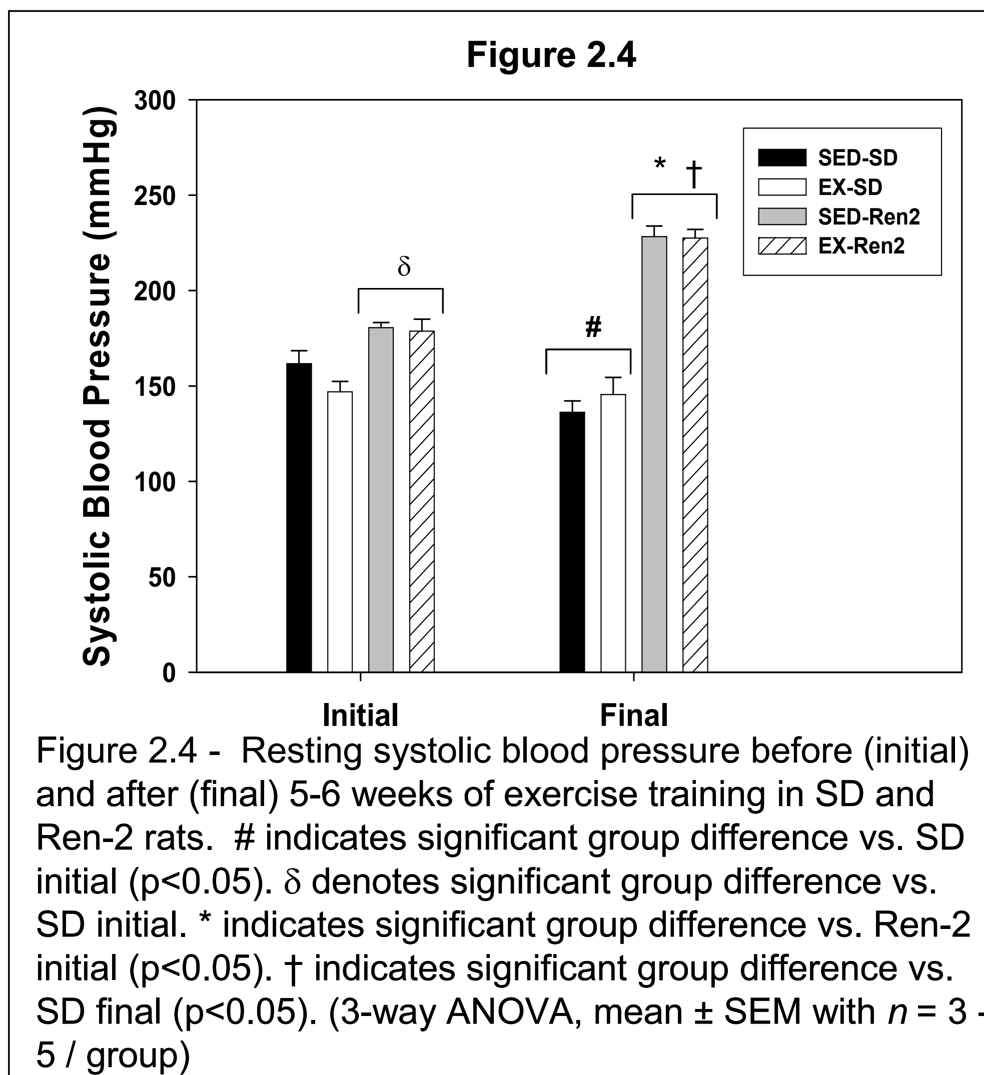


Figure 2.4 - Resting systolic blood pressure before (initial) and after (final) 5-6 weeks of exercise training in SD and Ren-2 rats. # indicates significant group difference vs. SD initial ($p<0.05$). δ denotes significant group difference vs. SD initial. * indicates significant group difference vs. Ren-2 initial ($p<0.05$). † indicates significant group difference vs. SD final ($p<0.05$). (3-way ANOVA, mean ± SEM with *n* = 3 - 5 / group)

B-type Natriuretic Peptide Was Not Changed by Exercise.

Left ventricular B-type natriuretic peptide (BNP) mRNA was significantly higher by 2-fold in 10-11-wk-old Ren-2 rats compared to SD rats ($p<0.05$; Figure 2.5). GAPDH mRNA was used to normalize all mRNA data since it was unchanged by the treatments or strains. Data were then expressed relative to EX-SD. Exercise failed to have an effect on BNP mRNA levels in either the SD or Ren-2 ($p=0.814$).

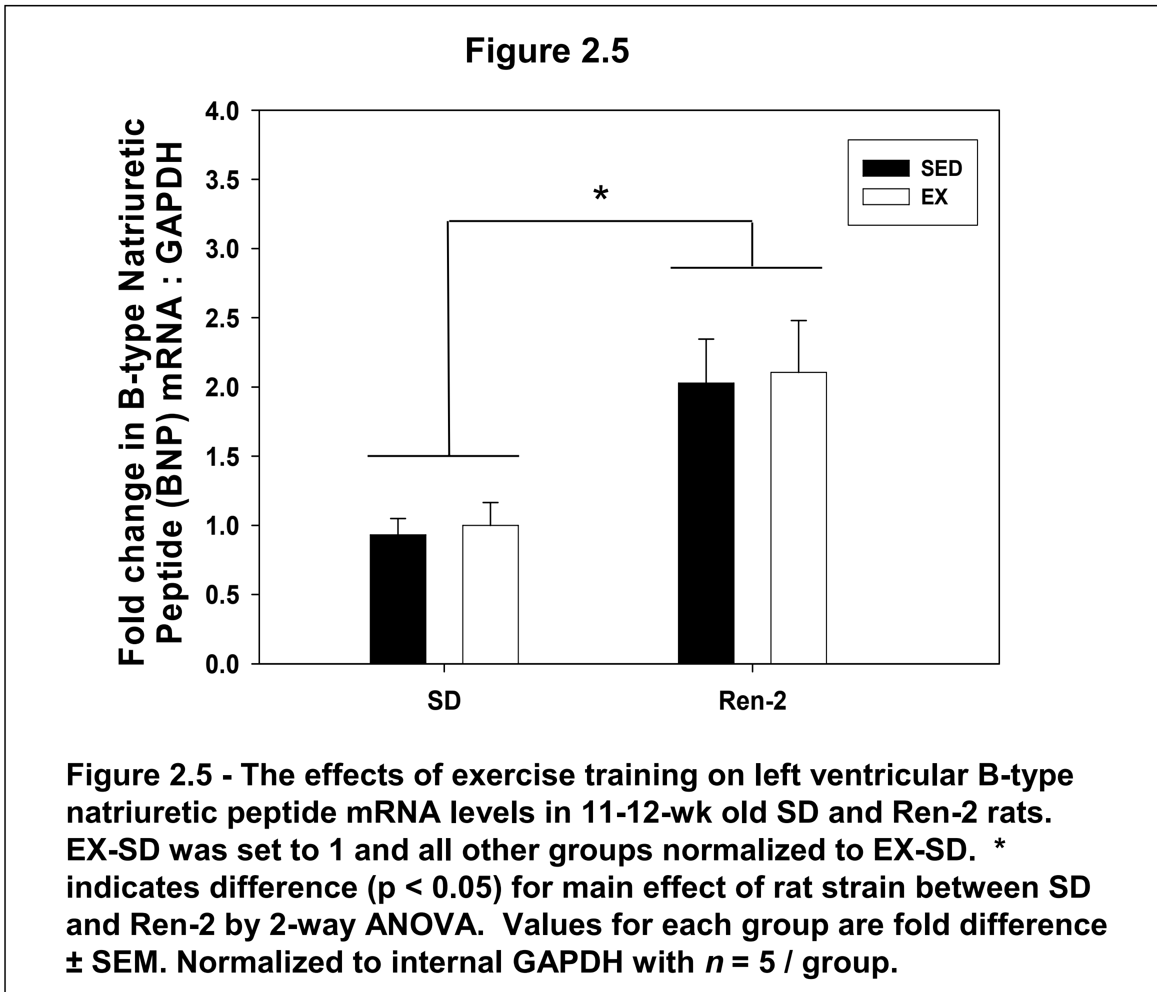


Figure 2.5 - The effects of exercise training on left ventricular B-type natriuretic peptide mRNA levels in 11-12-wk old SD and Ren-2 rats. EX-SD was set to 1 and all other groups normalized to EX-SD. * indicates difference ($p < 0.05$) for main effect of rat strain between SD and Ren-2 by 2-way ANOVA. Values for each group are fold difference ± SEM. Normalized to internal GAPDH with *n* = 5 / group.

Exercise Did Not Alter Myosin Heavy Chain mRNA or Collagen Deposition.

α-myosin heavy chain mRNA levels, normalized to GAPDH, in the left ventricle were significantly higher by ~14.5 fold in 10-11-wk-old Ren-2 rats compared to SD ($p < 0.05$), but were not affected by exercise training in either strain (p=0.693; Figure 2.6). β-myosin heavy chain mRNA levels were significantly higher by ~2.5-fold ($p < 0.05$) in the Ren-2, but not changed by exercise in either rat strain (p=0.388; Figure 2.7).

Left ventricular collagen deposition, as determined by hydroxyproline assay, did not significantly differ between the four groups for either strain of rat (p=0.613) or exercise (p=0.398) (Figure 2.8).

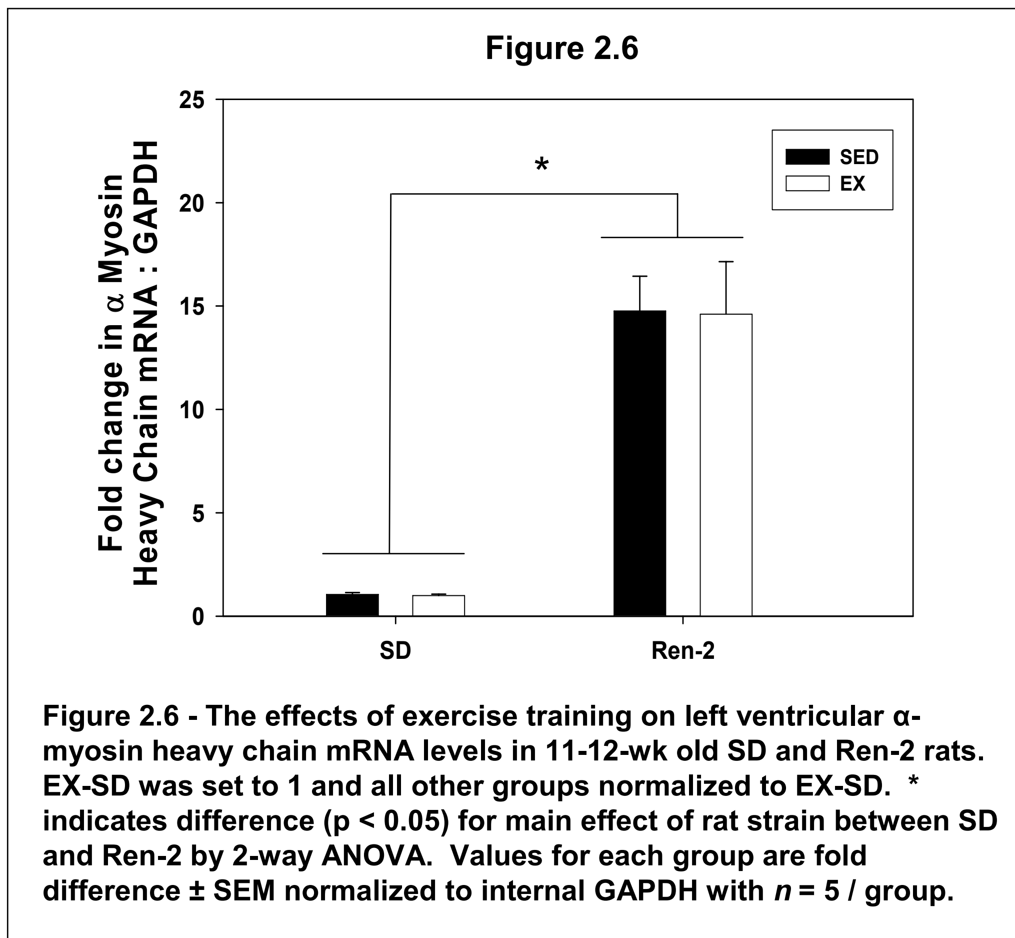


Figure 2.6 - The effects of exercise training on left ventricular α-myosin heavy chain mRNA levels in 11-12-wk old SD and Ren-2 rats. EX-SD was set to 1 and all other groups normalized to EX-SD. * indicates difference ($p < 0.05$) for main effect of rat strain between SD and Ren-2 by 2-way ANOVA. Values for each group are fold difference ± SEM normalized to internal GAPDH with *n* = 5 / group.

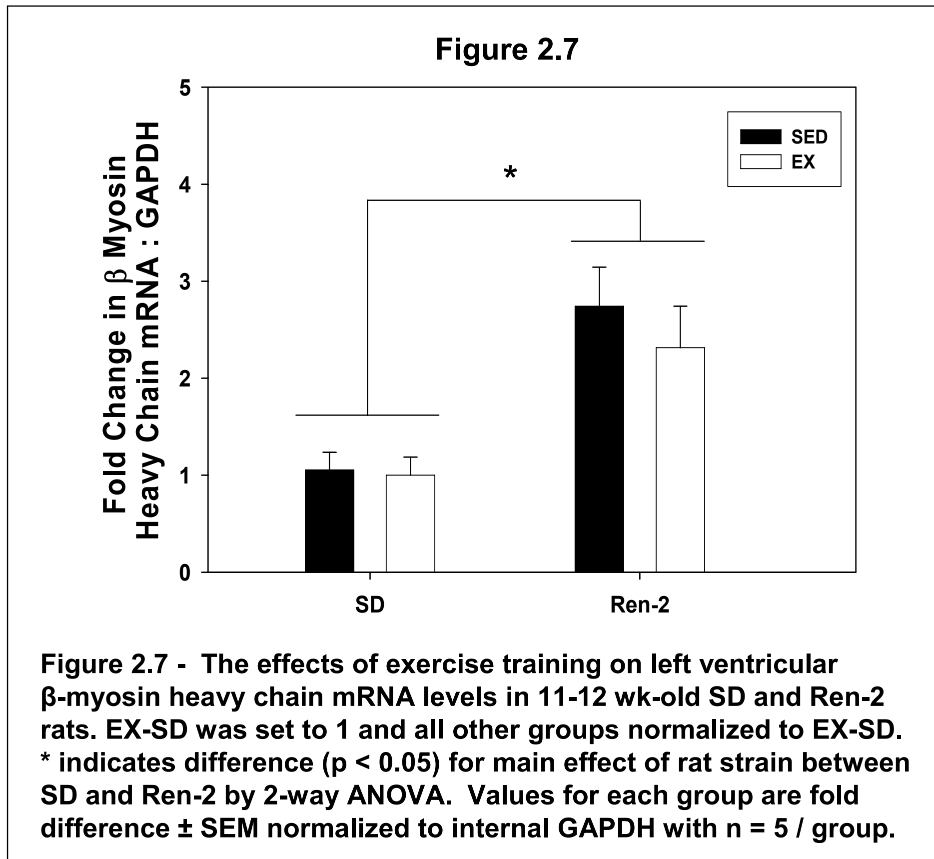


Figure 2.7 - The effects of exercise training on left ventricular β-myosin heavy chain mRNA levels in 11-12 wk-old SD and Ren-2 rats. EX-SD was set to 1 and all other groups normalized to EX-SD. * indicates difference ($p < 0.05$) for main effect of rat strain between SD and Ren-2 by 2-way ANOVA. Values for each group are fold difference ± SEM normalized to internal GAPDH with n = 5 / group.

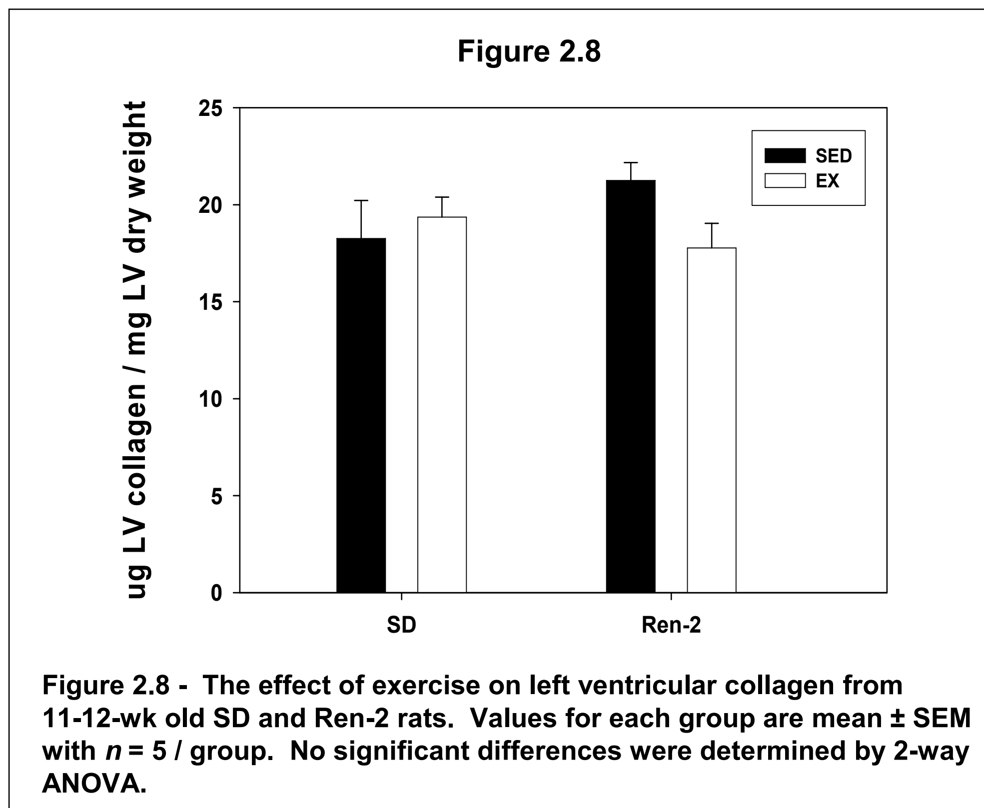


Figure 2.8 - The effect of exercise on left ventricular collagen from 11-12-wk old SD and Ren-2 rats. Values for each group are mean ± SEM with *n* = 5 / group. No significant differences were determined by 2-way ANOVA.

DISCUSSION.

To our knowledge, this is the first study to evaluate the effects of low-intensity, exercise training on cardiac function in an animal model of renin overexpression in cardiac tissue and Ang-II mediated hypertension. According to the literature on Ren-2, we began training the animals at a time prior to cardiac decompensation because it was hypothesized that exercise would attenuate a relative decrease in cardiac output. The novel findings are that introduction of low-intensity treadmill running at a young age (3-4 weeks of age) largely prevented a decrease in relative cardiac function at 8 weeks of age. Echocardiography revealed resting cardiac index (↑ 57%) and stroke volume (↑ 43%) to be significantly higher for the EX-Ren2 compared to their SED-Ren2 counterparts. Interestingly, EX-Ren2 and SED-SD had similar values for resting cardiac index and stroke volume, suggesting that exercise can maintain cardiac systolic function during the early development of hypertension in the Ren-2 model. In addition, mitral deceleration time slope (m/sec^2), a non-invasive estimate of diastolic function, was significantly reduced in the Ren-2 compared to SD rats. However, diastolic dysfunction was not prevented by exercise training in the Ren-2 rats, indicating that the initial preservation of cardiac index and stroke volume occurred in the presence of diastolic dysfunction.

Markers of cardiac remodeling and maladaptation were measured to examine potential factors by which exercise training influenced cardiac function. First, increased myocardial collagen deposition, which has been observed in Ren-2 rats at 17 weeks of age, can be a critical factor in reducing diastolic

relaxation (154). Ang II and RAS have been shown to promote cardiac fibrosis, excess extracellular matrix accumulation, and collagen expression (27; 44; 284; 293; 298). Although our data indicate impaired diastolic function in Ren-2 rat hearts, collagen deposition was not significantly different between Ren-2 and SD hearts suggesting that mechanisms other than collagen deposition are contributing at an earlier stage of diastolic dysfunction. Second, in the present study BNP mRNA levels were significantly higher in Ren-2 rats compared to SD with no protective effect of exercise training observed. It is known that BNP mRNA levels in the heart increase following hypertension and has previously been shown to increase in Ren-2 rats with left ventricular hypertrophy at 12 weeks of age (231; 252).

Myosin heavy chain (MHC) isoform expression plays a significant role in the performance of the LV. α-MHC is more powerful, but less efficient than β-MHC. 10 weeks of treadmill exercise training of normotensive rats has been shown to significantly upregulate both α MyHC mRNA and protein levels (280). This physiological adaptation is believed to improve myocardial contractility and maximize cardiac performance (193). However, following a pathological stimulus, such as hypertension, expression of the β-MHC isoform is often upregulated (122). For example, it was found (259) that Ren-2 hearts have lower α MyHC mRNA at 17-wk of age compared to SD which is consistent with well-defined maladaptive cardiac hypertrophy. The current study provides evidence that both α and β MyHC mRNA levels are significantly increased in the Ren-2 rats and these increases were not altered by exercise training. It remains

unclear whether α MyHC mRNA would have decreased in the SED-Ren2 if the experimental period had been extended, and whether continued exercise would have altered this course in the EX-Ren2 group. Alternatively, differences in the MyHC response might have been more apparent if protein expression was measured in addition to mRNA. However, previous studies have suggested that changes in myosin heavy chain isoenzyme in the heart occurs through pre-translational mechanisms (151).

We demonstrate here that low-intensity exercise training maintained cardiac index and delayed the onset of impaired ventricular function up to 8 weeks of age. Unexpectedly, fractional shortening as measured by echocardiography was not influenced by exercise training in either SD or Ren-2 rats, and this contrasts to the observation of increased cardiac output in the exercised groups. However, it is important to consider that fractional shortening and cardiac output can't be obtained during the same cardiac cycle and are subsequently measured using different imaging modalities. The measurements used to calculate fractional shortening are obtained by slicing the heart into a single imaging plane (M-mode). Fractional shortening is not adequate to assess global cardiac function if regional wall abnormalities or dyssynchronous contractions are present. However, cardiac output via echocardiography can provide a better index of global systolic function. This measure incorporates aortic flow assessed by Doppler (stroke volume), aortic diameter, and heart rate. Furthermore, stroke volume is influenced by preload (Frank-Starling mechanism), afterload, and intrinsic contractility of the heart. Thus, a change in cardiac output

with exercise training likely occurs through mechanisms involving both pre-load and after-load, and is a more sensitive measure of cardiac function as compared to fractional shortening.

Factors commonly associated with cardiac decompensation and remodeling, such as ratios of α- and β- MHC mRNA levels, collagen accumulation and natriuretic peptide synthesis, were unaltered following exercise training in the Ren-2 model. Thus, exercise doesn't appear to maintain cardiac index through such remodeling events. It seems possible that other factors aside from changes in MyHC transcription, collagen deposition, and cardiac morphology are responsible for improved LV function. Another likely candidate involves factors that influence contractility due to significantly improved stroke volume observed following exercise training. It has been shown that Ang II exerts a negative inotropic effect on cardiomyocytes by diminishing calcium transits which, in turn, leads to myocardial dysfunction and remodeling (80; 262). Also, endurance exercise training in rats improved cardiac myocyte contractility through increased sensitivity to Ca^{+2}. In response to the same [Ca^{+2}], single cardiac myocytes from exercise trained rats produced a higher level of 50% maximal tension than the sedentary group (74).

Other studies have shown that low-intensity exercise or voluntary running attenuates blood pressure increases in hypertension prone rats (145; 303). Veras-Silva et al (343), demonstrated that low-intensity treadmill exercise (16-20 m/min, 60 min, 5 times / week) for 18 weeks significantly reduced SBP, DBP, and MAP in the SHR. Interestingly, within the same study a high-intensity exercise

training group (25-30 m/min) failed to show any difference in blood pressure suggesting an exercise intensity-dependent effect. Additionally, another study (182) demonstrated that 6-weeks of voluntary wheel running reduced SBP by 16% in Ren-2 rats, supporting similar results observed in SHR (183). Results from the current study show that SBP blood pressure in the Ren-2 rat did not change in response to 5-6 weeks of low-intensity treadmill exercise. This may be, in part, related to the forced nature of the exercise and a consequent adverse physiological stress. Or, that the total amount (distance ~1 km/d) of running performed at a relatively low intensity (vs. 6-7 km/d voluntary) was insufficient to overcome the Ang II-mediated hypertension. Differences in response between SHR and Ren-2 rats most likely result from multiple factors including differences in the etiology of the hypertension, age (11 vs. 16-18 wks of age), and/or the intensity (low vs. high), duration or distance run, and/or the method of exercise (voluntary vs. forced treadmill).

One limitation to this study is that cardiac hemodynamics measured after 3 weeks may have been different than those following 5 weeks of exercise training. This is important to consider as exercise had no apparent effect on blood pressures after 5-6 weeks in either SD or Ren-2 rats, but after 3 weeks EX-Ren2 rats exhibited higher blood pressures than the SED-Ren2 animals. Also, the low number of animals in the study limits statistical evaluation. Furthermore, echocardiographic measures are typically more conclusive when coupled with invasive measurements such as catheterization of the LV. For example, values for diastolic filling pressures determined via an indwelling catheter would have

provided a better analytical comparison.

Additional studies are needed to further define the effects of RAS-mediated hypertension on cardiac function. Since voluntary exercise on a running wheel in Ren-2 rats has been shown to significantly reduce SBP within 6 weeks (184), evaluation of cardiac function in this type of exercise model may have merit. These studies would help further understand the role of exercise in regulating myocardial function in the hypertensive phenotype.

In summary, these results demonstrate that low-intensity treadmill exercise beginning at an early age in the Ren-2 rats serves to maintain cardiac output and stroke volume without improvements in diastolic function at 8 wks of age. However, typical characteristics of maladaptation, such as collagen deposition and altered MyHC expression ratios, were unaffected via exercise training in RAS-mediated cardiac dysfunction. Thus, it seems likely that improved ventricular performance due to exercise in RAS-mediated hypertension may occur through other existing mechanisms or factors.

CHAPTER 3

EXERCISE-INDUCED PREVENTION OF OBESITY AND HYPERINSULINEMIA IN THE OLETF RAT IS ASSOCIATED WITH REDUCED SKELETAL MUSCLE LIPID PEROXIDATION

ABSTRACT.

Intramuscular lipid peroxidation associated with obesity may influence insulin sensitivity. We hypothesized that exercise-induced prevention of obesity and hyperinsulinemia in the OLETF rat would be associated with the following in the epitrochlearis muscle: 1) lower intramyocellular lipid content, 2) decreased lipid peroxidation, and 3) increased fatty acid oxidation. Hyperphagic, OLETF rats at 4 wks of age were provided with running wheels for 16 wks before they were sacrificed 5 hrs (WL5), 53 h (WL53), or 173 h (WL173) after their wheels were locked. Sedentary (SED) rats that were not given access to running wheels served as age-matched controls. Lipid peroxidation as determined by 4-HNE levels was higher in epitrochlearis muscles of SED compared to WL5, WL53, and WL173. The ratio of 4-HNE levels to lipid content in the SED group was augmented 395% vs. the WL5. Oil Red O staining for intramyocellular lipid accumulation was not statistically different among groups. Glutathione S-transferaseα-4, an enzyme known to specifically metabolize 4-HNE, mRNA levels were higher in WL53 vs. WL173 and SED. In contrast, 4-HNE levels in omental fat were unaltered, indicating tissue specificity for the lipid peroxidation adaptation. Palmitate oxidation ($^{14}CO_2$/g/hr) and relative transcript levels for PPARδ and PGC1α were surprisingly not different between runners

and SED, suggesting that exercise did not improve mitochondrial oxidative capacity in the epitrochlearis muscle of OLEFT runners, despite protection from lipid peroxidation. Future studies are needed to determine the potential influence of lipid peroxidation on skeletal muscle insulin sensitivity.

INTRODUCTION.

Physical inactivity and excessive adiposity independently predict total mortality risk (164), mortality from cardiovascular disease (147; 212), and risk of type 2 diabetes (51; 127; 188; 202; 281; 328). Obesity and diabetes have been categorized as an epidemic by the US Centers for the Disease Control (239); nonetheless, their prevalence continues to increase (179; 255; 359). In addition, approximately half of all U.S. adults >18 yrs old are considered physically inactive and are not achieving the minimal U.S. governmental guidelines for exercise (≥30 minutes per day, ≥5 days per week of household, transportation, and leisure-time activities of moderate-intensity physical activity) (226; 227). One estimate (144) suggests that changes in daily walking distances have decreased energy expenditure by an average of 400-600 kcal/day during the 20th century and the author concluded that reduced physical activity alone could contribute to a widespread epidemic of obesity and type 2 diabetes in the US. Adolescents also experienced an increase in obesity (256) and an unfavorable decline in physical activity patterns from 1999-2004 (265). Therefore, it is imperative to better understand the biology of obesity and inactivity.

The Otsuka Long-Evans Tokushima fatty (OLETF) rat, which is selectively bred for null expression of the cholecystokinin-A receptor, is characterized by hyperphagia, obesity, adult-onset hyperglycemia and hyperinsulinemia, and chronic type 2 diabetes mellitus. Importantly, unlike most obese animal models these unique rats exhibit an inherent ability to maintain daily physical activity levels using voluntary running wheels (22; 316). When introduced at 8 wk of age, voluntary running distance in the OLETF rat increased to a peak of ~9 km/day by 11 weeks of age (23). Meal patterns, food intake, body weight, and leptin were subsequently maintained at levels of non-obese litter mates. Furthermore, long-term voluntary running for 13 weeks was shown to reduce plasma glucose levels and immunoreactive insulin levels in response to a glucose load when compared to a sedentary group (304).

The literature suggests that increased physical activity in normal, healthy rats would facilitate the following adaptations: #1) prevent excessive adiposity (↓ total body fat, ↓ intra-abdominal fat) (201), #2) prevent hyperinsulinemia, #3)alter the status of skeletal muscle lipids (↓ intramyocellular lipid content, ↓ skeletal muscle lipid peroxidation via ↑SOD-1, ↑SOD-2, ↑GST-α4) (106; 110; 278; 288), #4) enhance skeletal muscle lipid oxidation, (↑ mRNAs for PPARδ and ↑PGC-1α) (16; 167; 175; 214), and #5) increase skeletal muscle mitochondrial content (↑ citrate synthase activity) (57). Furthermore, our recent observations (199; 200; 206) would suggest these outcomes would begin to return to pre-activity levels shortly after cessation of physical activity.

Taken together, we hypothesized that exercise-induced prevention of obesity would be associated with decreased intramuscular lipid content, reduced lipid peroxidation, and enhanced fatty acid oxidation in the epitrochlearis muscle of the OLETF rat. Also, it was hypothesized that short-term physical inactivity for 53 or 173 hours would promote a return to the maladaptations observed in the skeletal muscle of SED OLETFs.

MATERIALS AND METHODS.

OLETF rats.

This study utilized a hyperphagic rat model to study the effects of exercise-induced weight loss on skeletal muscle adaptations. All experimental protocols were approved by the Animal Care and Use Committee at the University of Missouri, Columbia, MO.

Forty-seven, male OLETF rats were received from Otsuka Pharmaceutical Company in Japan in the 3rd week of life. Animals were housed 1 per cage in a room with a 12:12-h light-dark cycle maintained at 20-22°C and had free access to rat chow and water. With the exception of the sedentary group, all animals were immediately provided with a running wheel outfitted with a Sigma Sport BC 800 bicycle computer (Cherry Creek Cyclery, Foster Falls, VA, USA).

Experimental Design.

Upon arrival, each rat was randomly designated as either a runner or non-runner. The average initial body weights for runners and non-runners were 79.0±2.5 (g) and 82.2±4.2 (g), respectively. OLETF runners were allowed free

access to voluntary running wheels for 16 weeks until 20 weeks of age. At 20 weeks of age, OLETF runners were divided into 3 additional experimental groups so that the body weight means of each group were comparable: wheel lock for 5 hours (WL5), wheel lock for 53 hours (WL53), and wheel lock for 173 hours (WL173) (Figure 3.1). Also, age-matched, sedentary OLETF (SED) rats did not have access to running wheels throughout the duration of the study. Rat chow (Formulab 5008, Purina Mills, St. Louis, MO) was removed on the day of sacrifice at the time of wheel lock (0600 h). At 20 weeks of age, rats were deeply anesthetized, their tissues removed, and sacrificed.

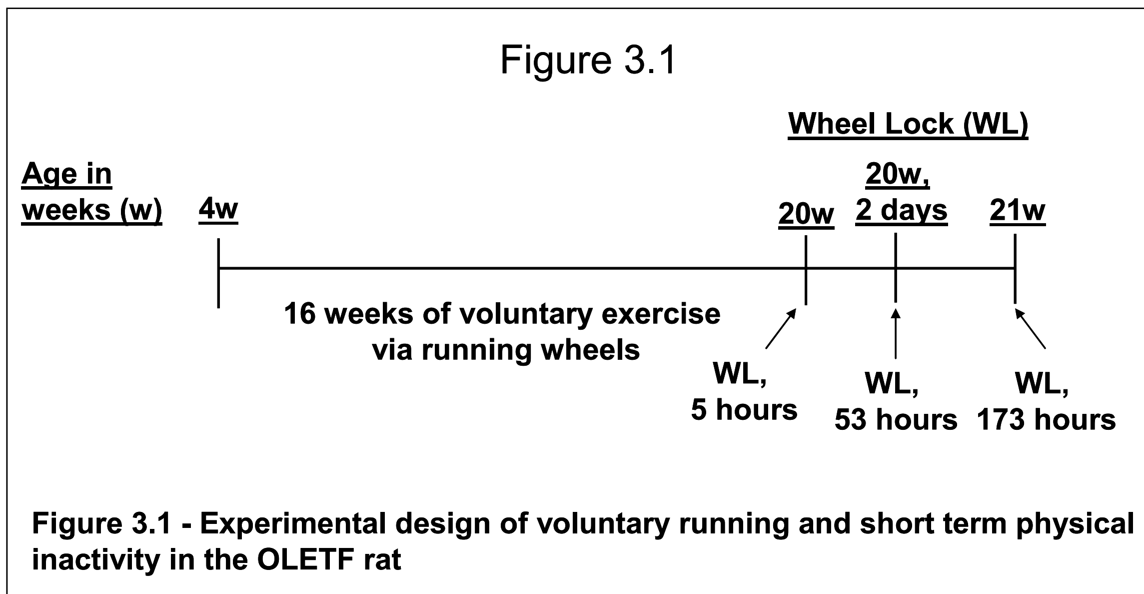


Figure 3.1 - Experimental design of voluntary running and short term physical inactivity in the OLETF rat

Fat Pad Mass and Adipocyte Size.

Epididymal and retroperitoneal fat pads were removed, weighed, and a portion of epididymal adipose was fixed in formalin. For morphometric determinations epididymal sections were stained with hematoxylin and eosin. At least 300 adipocytes/animal were sized under a microscope (Olympus BX40

photomicroscope) at 10X using Image Pro (Silver Springs, MD) imaging software. Average cell diameter was then used to calculate cell volume by using the equation volume = $4/3*\pi *r^3$. Average adipocyte volume was then used to calculate average adipocyte mass by assuming a
0.95 density for adipocytes. Cell number is calculated by using the equation, Cell Number = epididymal fat pad mass / average epididymal adipocyte mass.

Histologic Analysis.

Epitrochlearis muscles were carefully dissected, placed on cork with OTC (Fisher), and submerged in partially frozen isopentane (-160°C) for ~1 min. Frozen muscles sections of 10 μm were sliced at -20°C and stored at -80°C until later use.

Oil Red O staining was adapted from Koopman R et al (191). Stock solution were made by diluting 162.5 mg of Oil Red (Fluka), with 32.5 ml of 60% triethyl phosphate (Fluka), and gently stirred overnight at room temperature. A working solution of Oil Red O was made by adding 30 ml of stock Oil Red to 20 ml of ddH_20 and stirred for 10 minutes at room temperature. The solution was filtered through Whatman Paper number 41 (Whatman, Maidstone, UK) to remove crystallized Oil Red.

Slides were removed from -80°C and thawed 15 minutes at room temperature. Muscle sections were fixed in buffered 10% formalin (Fisher Sci) for 1 hour followed by 3 x 30 second rinses in ddH_2O. Samples were immersed in Oil Red O working solution 30 minutes and rinsed 3 x 30 seconds in ddH_2O. The sections were counterstained with Hemotoxylin (Fisher-Protocol, Gills 3x) 45

seconds and rinsed 3 times in fresh ddH_2O. Finally, slides were rinsed with tap water 10 minutes and mounted with aqueous gel (Biomeda Corp). Oil red O staining was observed via a Zeiss microscope at 20x magnification on the day of staining and done in duplicate with 5 -10 views total / animal (20 x magnification) The relative amounts of intramuscular lipid accumulation were semi-quantified using Image Pro-Plus Software (MediaCybernetics, 2001).

Serial muscle sections were stained for succinate dehydrogenase using a protocol modified by Pette & Tyler (267). Briefly, slides were immersed in an incubation medium (5mM EDTA, 1mM KCN, 0.2 mM phenazine methosulphate, 50 mM sodium succinate, 1.5 nitroblue tetrazolium in 100 mM phosphate buffer pH=7.6) 5 minutes at room temperature, rinsed in ddH_2O, and fixed 15 minutes in formol calcium. This procedure was followed by dehydration and mounting. Serial images were taken to determine the oxidative potential of muscle fibers with Oil Red O staining.

Lipid Peroxidation.

Protein was extracted from muscle and adipose tissues by glass on glass homogenization in Mueller Buffer (50 mM Hepes pH=7.4, 0.1% Triton-X, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7$*H_2O, 100 mM β-glycerophosphate, 25 mM NaF, and ddH_2O), protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail 1 and 2 (Sigma), β-Mercaptoethanol (156 mM), and ddH_2O. Samples were centrifuged at 13,000 g for 10 min and the supernatant was stored at -80°C for later use.

To measure 4-HNE levels, protein concentrations were determined via Bradford (Bio-Rad) and 10 µg of protein were loaded onto a Bio-Dot SF Microfiltration Apparatus (Bio-Rad). Protein loading was measured via Ponceau staining (Sigma) on a nitrocellulose membrane. Membranes were rinsed (TBS-T) and blocked with 5% milk for 1 hour at room temperature. They were then washed 3 x 5 minutes (TBS-T) and incubated with a primary antibody for 4-HNE (A.G. Scientific, Inc.) (1:2500) overnight at 4°C while gently shaking. Membranes were rinsed (TBS-T) for 1 x 10 min, 4 x 5 min and subsequently incubated with an anti-rabbit secondary antibody (Amersham Biosciences) (1:10,000) for 1 hour at room temperature. This was followed by 1 x 10 min, 4 x 5 min washes (TBS-T) and luminescence reagents (Pierce) were placed on the membrane for 5 minutes. Exposures were obtained and quantified by Kodak Molecular Imaging Software.

Western Blot Analysis.

Protein was isolated from muscle via a similar procedure as described above for lipid peroxidation except that BME was not added during the homogenization. Protein concentrations were determined by Bradford (Bio-Rad) and prepared for gel electrophoresis by addition of Lamelli Buffer with BME. Samples were placed on a heating block for 5 minutes at 98°C and stored at -20°C until the next day. Proteins were separated via a 12% gel using a Mini Electrophoresis System (Bio-Rad) and transferred to nitrocellulose membranes for 2.5 hours at 60V using Mini Trans-blot System (Bio-Rad). Membranes were Ponceau stained to verify equal loading and rinsed 2 x 10 min (TBS-T). Primary

antibodies for SOD-1 (Stressgen; 1:5000) and SOD-2 (Stressgen; 1:1250) were incubated overnight with the membrane in 5% milk at 4°C. Membranes were then rinsed (TBS-T) for 4 x 10 min and incubated with an anti-rabbit secondary antibody (1:2500 both) for 1 hour. Finally, membranes were rinsed (TBS-T) for 4 x 10 minutes and exposed via a luminescent reaction (Pierce) and Kodak Molecular Imaging Software.

Fatty Acid Oxidation.

Skeletal muscle homogenate fatty acid oxidation was measured with radiolabeled palmitate (1-^{14}C palmitate from American Radiochemicals) in a fresh muscle homogenate preparation using the methods from Noland et al (253) and as modified by Dohm et al (77). The oxidation rate of ^{14}C palmitate was measured by collecting and counting the $^{14}CO_2$ and acid soluble metabolites produced during incubation in a sealed trapping device. Palmitate (final 200 µM) and U^{14}C Palmitate were bound to 0.5% BSA (final concentration) which was then brought up in reaction buffer to yield the following final concentrations: 100 mM sucrose, 10 mM Tris-HCl, 10 mM KPO_4, 100 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mM L-carnitine, 0.1 mM malate, 2 mM ATP, 0.05 mM CoA, and 1 mM DTT (pH 7.4). 320 µl of the reaction buffer was added to 80 µl of muscle homogenate (normalized to tissue weights) prepared in a sucrose-EDTA buffer in the well of the trapping device. A 0.65 ul centrifuge tube containing 400 ul of NaOH was inserted in the well followed by sealing the well with a rubber gasket. After 60 min of incubation at 37°C on an orbital shaker, 200 µl of 70% perchloric acid was injected through the rubber gasket into the bottom of the well followed

by another 60 minutes of orbital shaking. $^{14}CO_2$ driven out of the reaction by the perchloric acid during the last 60-min incubation was trapped in the NaOH placed in the centrifuge tube in the well which was counted on a scintillation counter. The remaining homogenate was collected and centrifuged at 18,000 rpm for 20 min at room temperature. A sample of the resulting supernatant (acid soluble metabolites) was counted on a scintillation counter. Specific activity was taken by counting a portion of the reaction.

RNA isolation and cDNA synthesis.

RNA was isolated from a section of epitrochlearis muscle using a method adapted from Chomczynski and Sacchi (48). Briefly, Trizol reagent (1.5 ml) was added and the tissue was homogenized on ice using a Polytron homogenizer (Kinematica) on setting 2-3 for three times (30 seconds each) with a 1-minute wait between each round. The homogenate was further processed through RNeasy columns and DNase I (Qiagen) to purify the RNA. cDNA synthesis was performed with 0.586 µg of purified total RNA using Superscript III first-strand synthesis system (Invitrogen), 50ng/ul of random hexamer primers, and a Mastercycler Gradient Thermocycler (Eppendorf).

Quantitative Real-Time PCR.

The quantitative PCR reactions were performed with an ABI 7000 Sequence Detection System (ABI) using Taqman Gene Expression chemistry (ABI). All PCR reactions included 25 ng of cDNA, 90 nM primers (IDT), 250 nM florescent probe, and Taqman Universal PCR master mix (ABI). Each sample was assayed in a 25 µl reaction in duplicate. If the duplicate contained a

standard deviation of > 0.5, it was re-assayed. All experimental samples were analyzed relative to 18S. The primer and probe sequences used for rat peroxisome proliferative activated receptor-δ (PPAR-δ) and rat glutathione S-transferase (GSTA4) were pre-designed by ABI, and rat PPAR-gamma coactivator-1α (PGC-1α) was generously provided by Dr.Tim Koves at Duke University. The PGC-1α sequence was verified using Primer Express 2.0 (ABI). PCR product formation was checked against a ladder for proper amplicon size using PCR Supermix (Invitrogen) before beginning Taqman gene expression.

Differences in gene expression were calculated using relative quantification to 18 S via the comparative C_T method from ABI (User Bulletin no. 2 ABI PRISM 7700 Sequence Detection System). Since no differences in raw C_T values were observed between groups, 18 S was found to be an appropriate normalizer ($p = 0.307$). Standard curves for each target were run to verify similar efficiency of the PCR reaction.

Citrate Synthase Assay.

Fifty micrograms of muscle homogenate from samples were used to determine citrate synthase activity (312). Briefly, samples were diluted to a final volume of 400 µl of 100 mM KH_2PO_4–100 mM K_2HPO_4, pH 7.4–50 µM EGTA–50 µM Na_4EDTA, and kept on ice. Duplicate samples of 100 µl and 200 µl each were mixed to a final volume of 900 µl of 100 mM Tris, pH 8.0–0.167 mM acetyl coenzyme A–0.111 mM 5,5'-dithiobis-(2-nitrobenzoic acid) and placed in a 30°C water bath for 5 min. Oxaloacetic acid (100 µl of 5 mM), was immediately added and the reaction measured in a spectrophotometer at 412 nm for 3 min.

Blood measures.

Blood was collected at the time of sacrifice, allowed to clot for 15 minutes, centrifuged at 3,000 RPM for 10 minutes at 4°C, and the serum was frozen at -80° C. Blood glucose and insulin were measured in the serum via glucose oxidase reagent kit (Sigma) and an ELISA kit (Linco), respectively.

Statistical analysis.

A One-Way ANOVA was used for all statistical comparisons (Sigma Stat 3.5). The WL5 was considered the reference group and $p < 0.05$ was set as a level of significance.

RESULTS.

Alterations in Cardiac Weights, Body Weights, Fat Pad Mass, and Adipocyte Size due to Voluntary Exercise

Wet heart weights (mg) normalized to body weight (g) were 21% lower ($p < 0.05$) in the SED group compared to WL5 (Table 3.1). Left ventricular weights (did not include septum) normalized to body weight were 23% lower ($p < 0.05$) in both SED and WL173 groups as compared to WL5. After 173 hrs without wheel running, LV weight fell 9% from WL5, indicating ventricular atrophy. Final body weights at 20 weeks of age in the SED group were 45% higher ($p < 0.05$) than the WL5, WL53, and WL173 groups as the short-term wheel did not cause changes in body weight when compared to the WL5 group. Also, epididymal and omental fat pad masses in the SED group were 136% and 220%, respectively,

Table 3.1

	WL5	WL53	WL173	SED
n =	5	5	6	6
Heart weight (mg) / BW (g)	3.27± 0.10	3.67 ± 0.37	3.17 ± 0.04	2.59 ± 0.07 *
LV weight (mg) / BW (g)	1.79 ± 0.07	1.73 ± 0.03	1.61 ± 0.05 *	1.38 ± 0.03 *
Final BW (g)	462 ± 16	469.36 ± 13	461 ± 16	671 ± 21 *
Epididymal fat mass (g) / BW (g) (x 1000)	11.0 ± 1.1	10.4 ± 0.89	9.87 ± 0.71	26.0 ± 1.9 *
Omental fat mass (g) / BW (g) (x 1000)	1.29 ± 0.21	2.23 ± 0.37	2.05 ± 0.16	4.13 ± 0.26 *
Epididymal adipocyte volume (pL)	1760 ± 150	1690 ± 100	1630 ± 210	2900 ± 130 *
Epididymal adipocyte diameter (µm)	137 ± 4.2	136 ± 2.9	133 ± 6.0	168 ± 2.9 *
Epididymal adipocyte number x 10^6	35.5 ± 5.4	30.4 ± 3.8	34.8 ± 5.5	66.8 ± 6.8 *

Table 3.1 - Cardiac wet weights, body weights (BW), adipose tissue wet weights, and epididymal adipocyte volumes, diameters, and cell numbers. LV = left ventricle. * denotes $p < 0.05$ vs. WL5. Values are means ± SEM; n= 5-6 / group.

larger ($p < 0.05$). No changes occurred during 173 hrs of WL. In the SED group, epididymal adipocyte volume (pL) and number (10^6) were 65% and 88% greater ($p < 0.05$), respectively, as compared to the WL5 groups.

Voluntary Running Distances for OLETF Rats Between Ages 4 and 20 Weeks.

Initial OLETF running distance at 4 weeks of age averaged between 3-4 km / day. Peak distances of ~9 km / day occurred between 8-11 weeks and thereafter steadily declined to ~5 km / day at 20 wks of age (Figure 3.2).

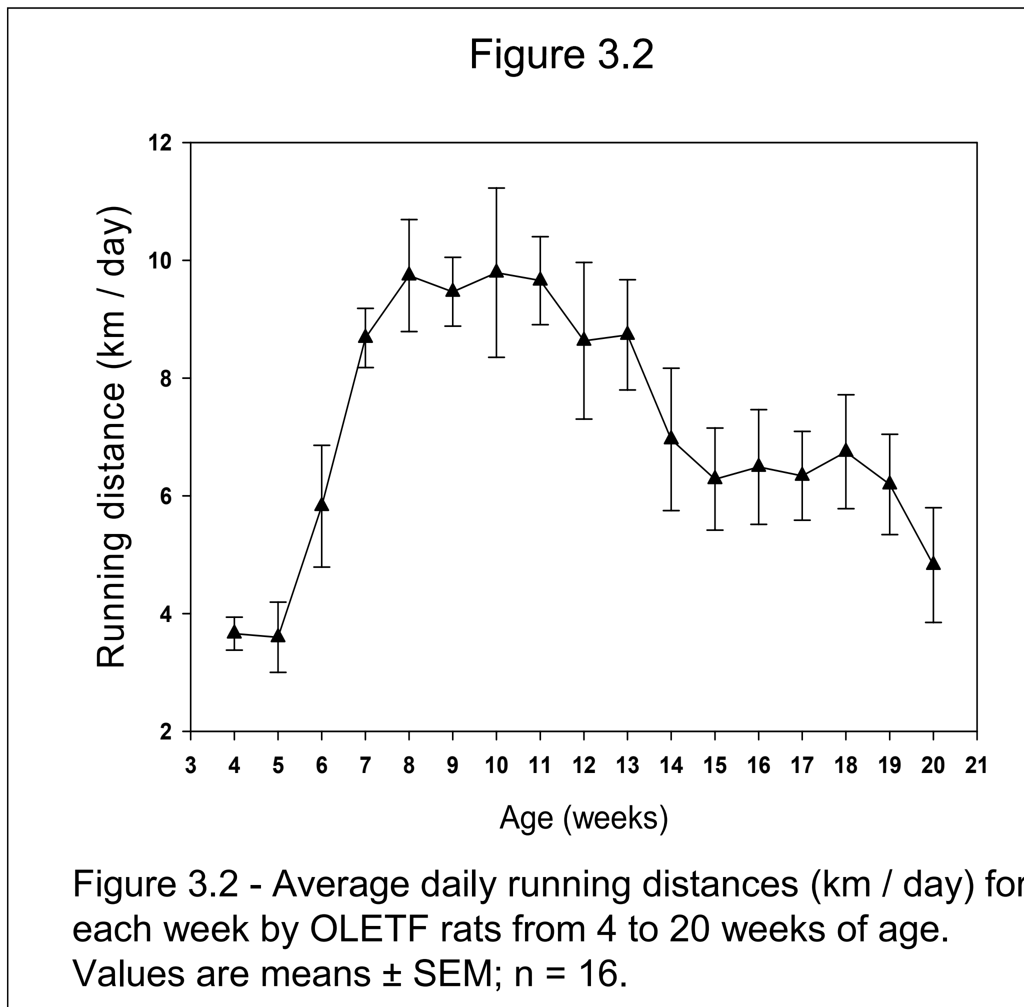


Figure 3.2 - Average daily running distances (km / day) for each week by OLETF rats from 4 to 20 weeks of age. Values are means ± SEM; n = 16.

Lower Food Consumption in OLETF Runners.

Voluntary running significantly reduced food intake ($p < 0.05$) at 6 of the 16 weeks between 4 and 20 weeks of age (Figure 3.3a). Food consumption during the 53 h and 173 h wheel lock period did not significantly differ from daily average pre-lock values (Figure 3.3b).

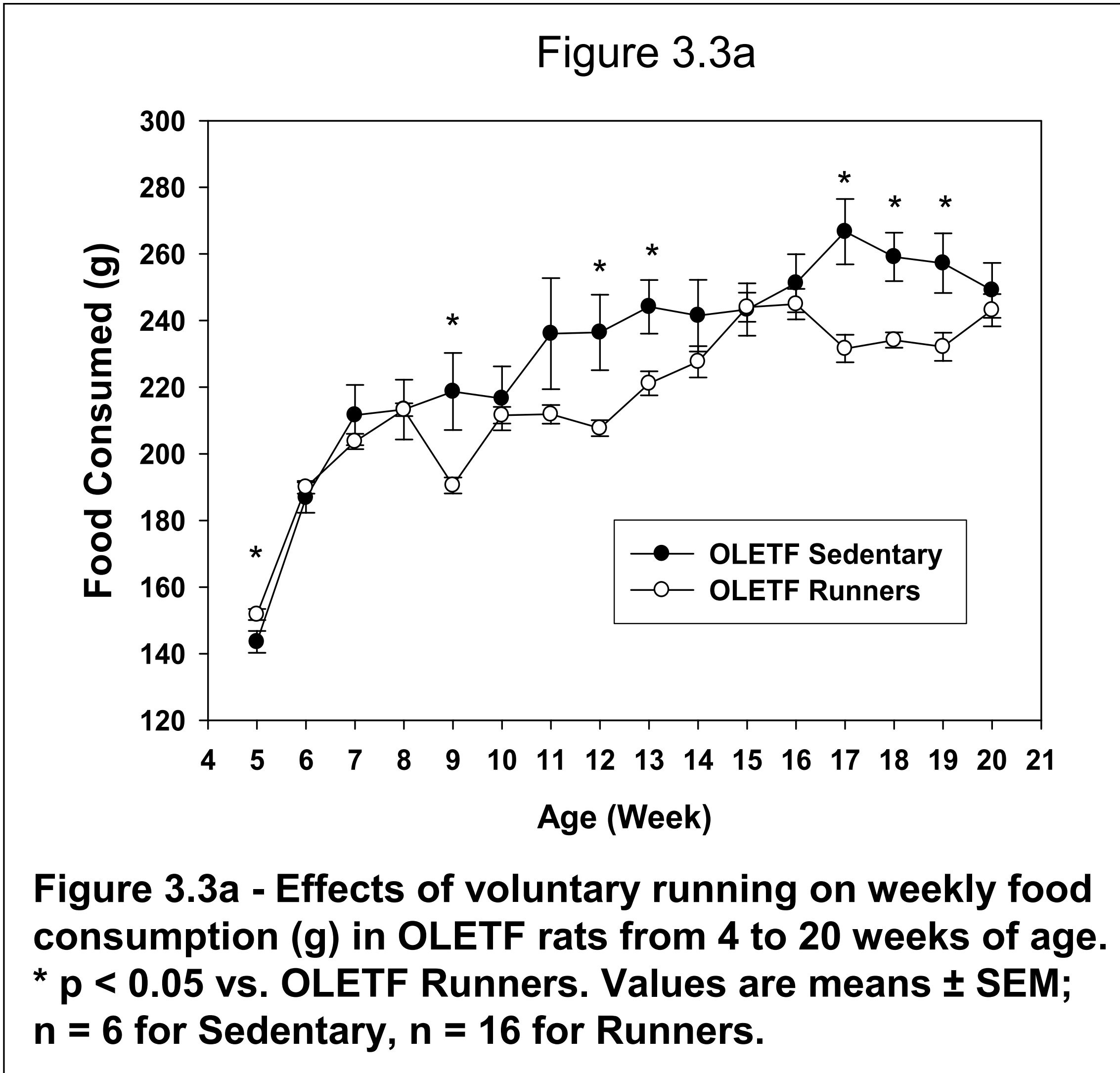


Figure 3.3a - Effects of voluntary running on weekly food consumption (g) in OLETF rats from 4 to 20 weeks of age. * p < 0.05 vs. OLETF Runners. Values are means ± SEM; n = 6 for Sedentary, n = 16 for Runners.

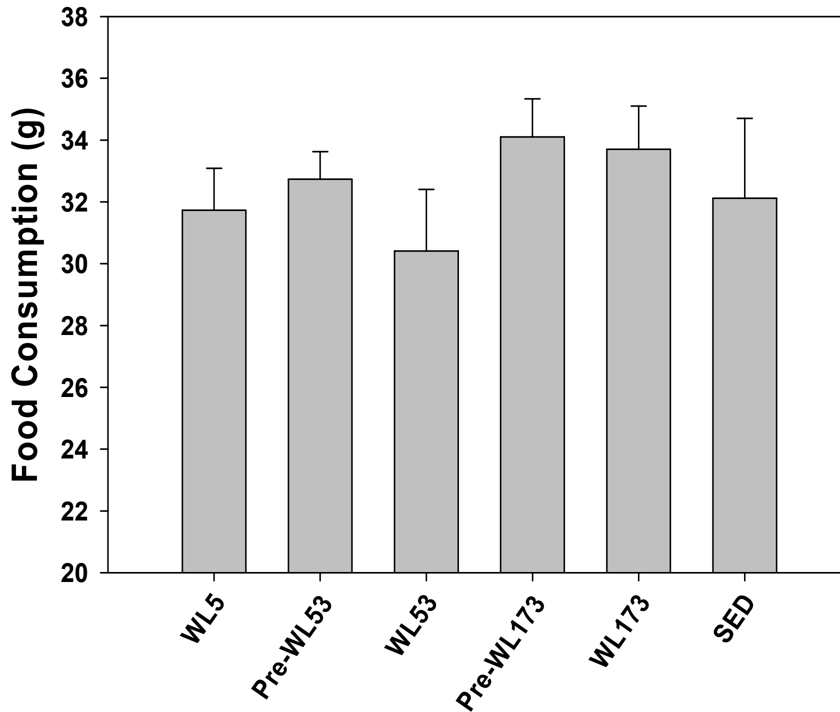


Figure 3.3b - Effects of voluntary running followed by 53 and 173 hrs of no running on daily food consumption (g) in the OLETF rat during the 21st week of age. WL5 = wheels locked for 5 hrs after 16 wks of wheel running; WL53 = wheels locked for 53 hrs after 16 wks of wheel running; WL173 = wheels locked for 173 hrs after 16 wks of wheel running; For each rat, average daily food consumption was obtained for the immediate 4-5 days prior to sacrifice (Pre-WL53, Pre-WL173, and respective SED groups) with the exception of WL5 which is at the time of sacrifice. Values are means ± SEM; n = 5-6 for each group.

Prevention of Weight Gain with Voluntary Running and Maintenance at WL173.

With the exception of weeks 4 through 6, body weights of the SED rats were significantly greater at all weeks of life compared with the voluntary wheel runners ($p < 0.05$). SED average body weight was 6.5% and 30.7% higher at

weeks 7 and 20, respectively (Figure 3.4a). There was no change in body weight from the time the wheels were locked until the animals were sacrificed in the WL53 or WL173 groups. (Figure 3.4b).

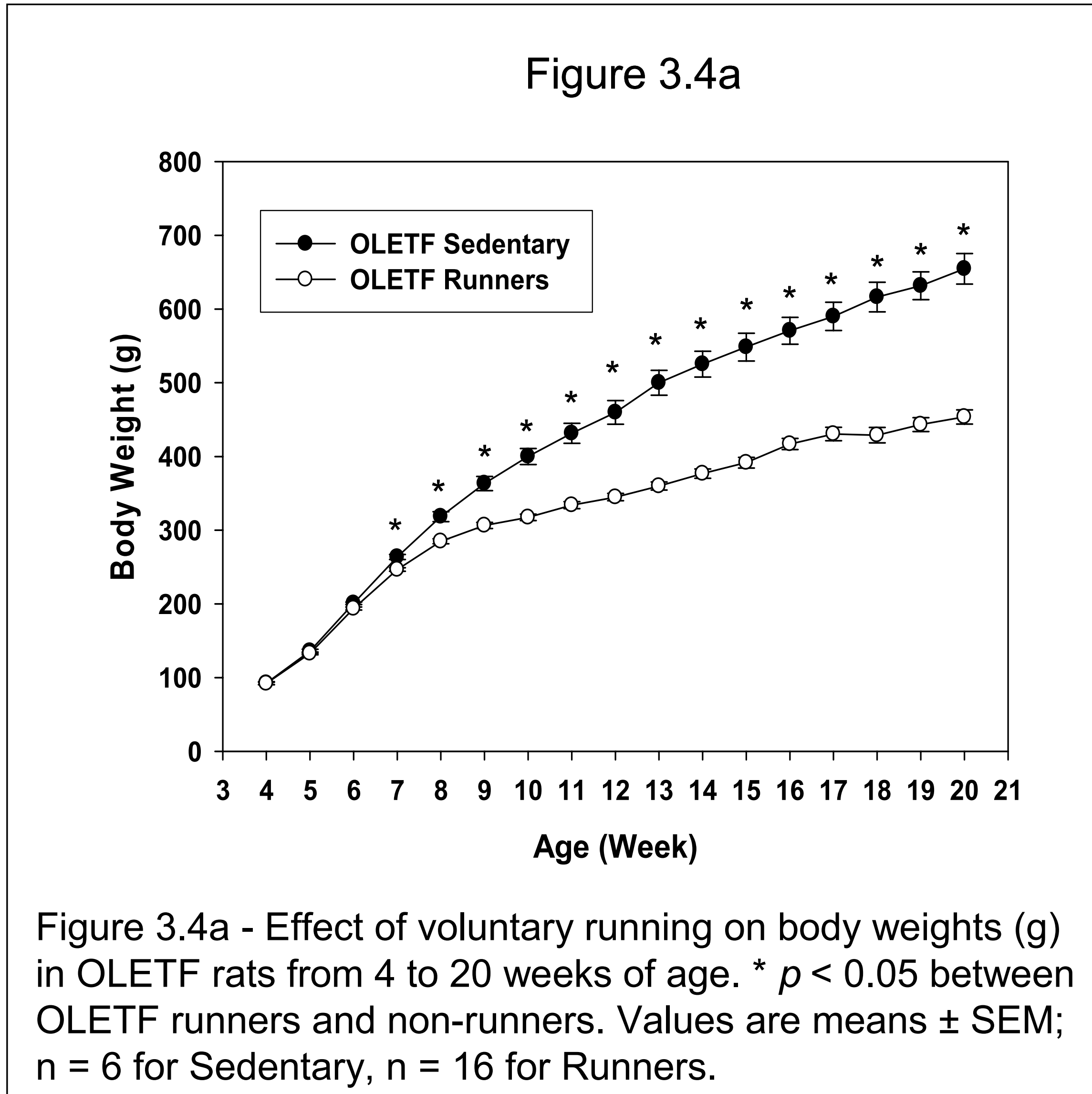


Figure 3.4a - Effect of voluntary running on body weights (g) in OLETF rats from 4 to 20 weeks of age. * $p < 0.05$ between OLETF runners and non-runners. Values are means ± SEM; n = 6 for Sedentary, n = 16 for Runners.

Figure 3.4b

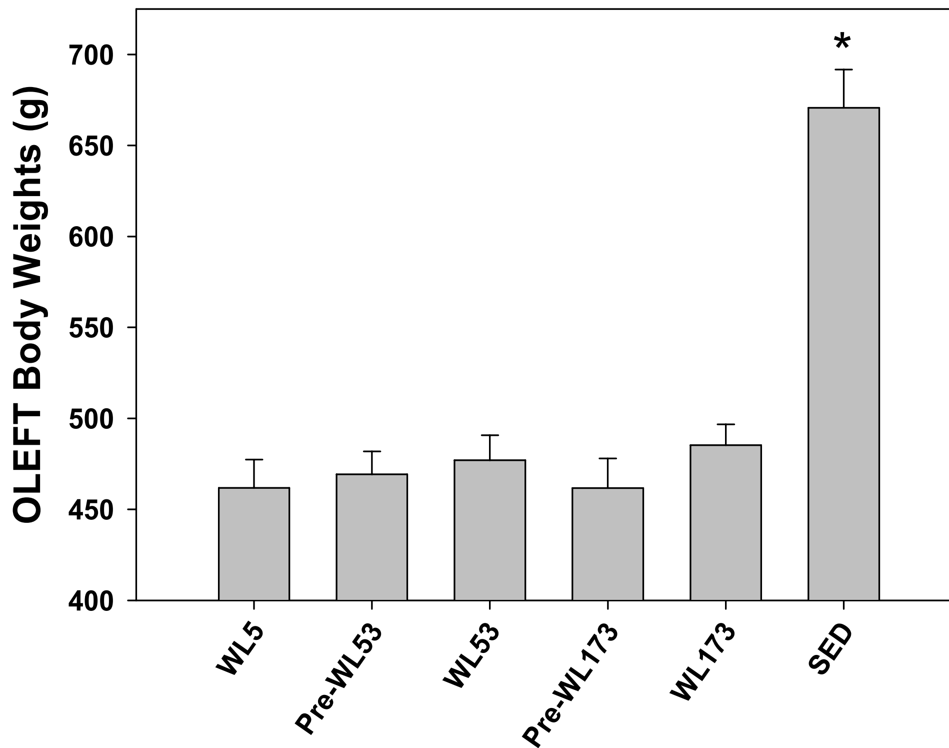


Figure 3.4b - Effects of voluntary running followed by 53 and 173 hrs of no running on body weight (g) in the OLETF rat at 20 weeks of age. Groups were defined figure 3.3b legend. * $p < 0.05$ vs. WL5. Values are means ± SEM; n = 5-6 for each group.

<u>Exercise Prevented Hyperinsulinemia in the OLETF Rat</u>

Serum insulin levels were significantly higher (p<0.05) in the SED OLETF vs. WL5 and were not affected by wheel lock for 173 hours (Figure 3.5).

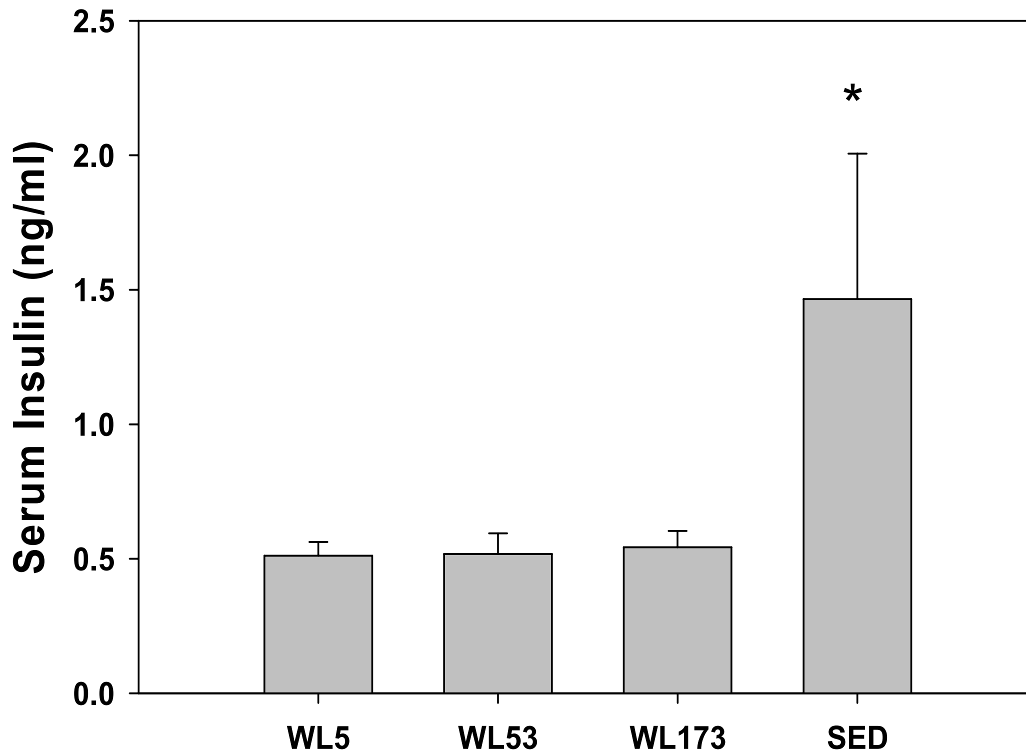


Figure 3.5 - Effect of 16 weeks of voluntary running followed by 53 and 173 hrs of no running on serum insulin levels (ng/ml) of OLETF rats. Groups were defined figure 3.3b legend. Values are means ± SEM; n = 4-8 animals / group.

Intramuscular Lipid Content is Unaffected by Wheel Running.

Oil Red O staining in epitrochlearis muscle was not significantly different among, WL5, WL53, WL173, and SED ($p < 0.077$) (Figure 3.6a). Representative images at 20x magnification are shown in Figure 3.6b. Overlay of Oil Red O staining and succinate dehydrogenase enzyme activity in serial sections revealed

that the predominant accumulation of intramuscular lipids was in highly oxidative fibers (Figure 3.6c).

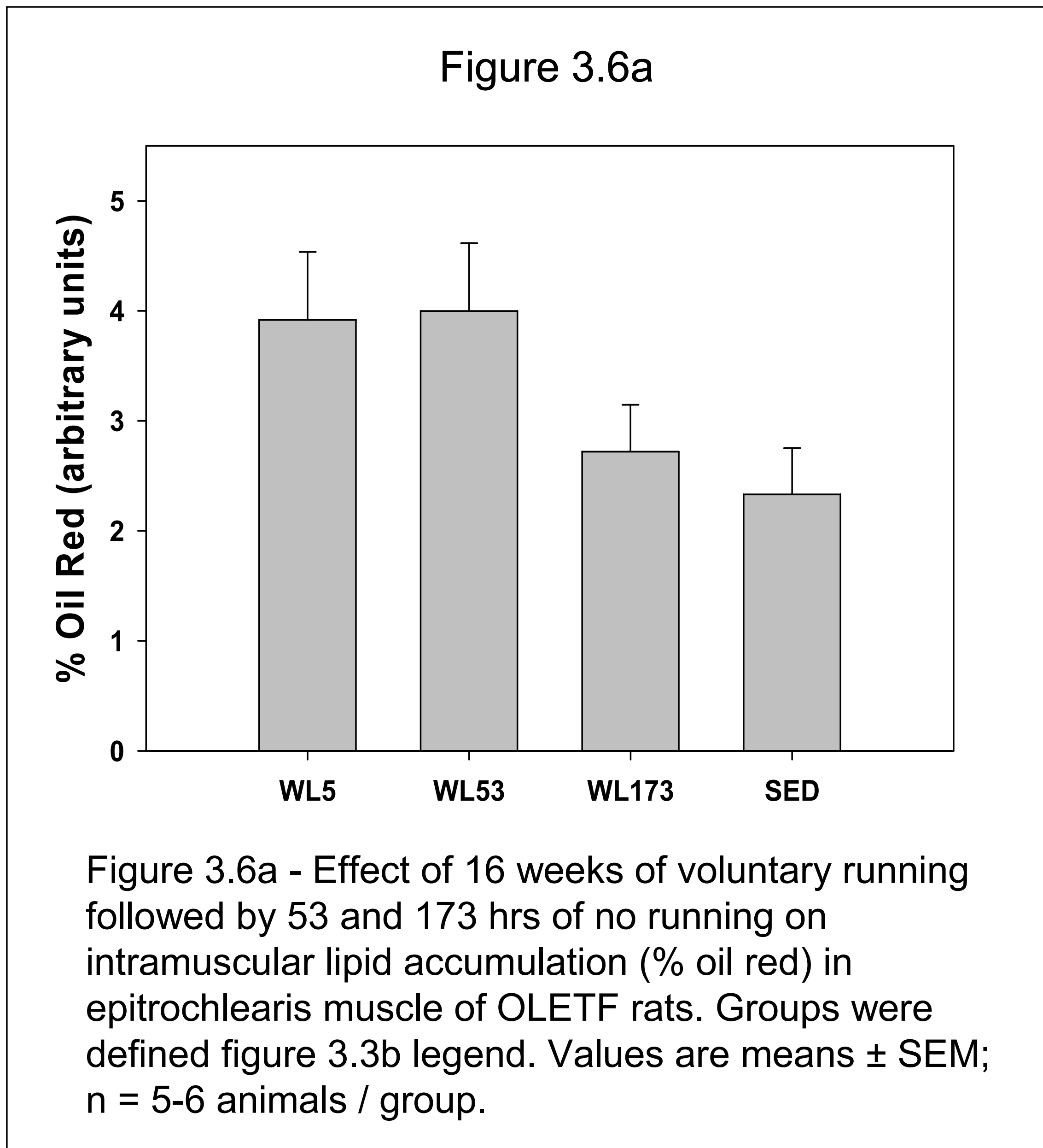
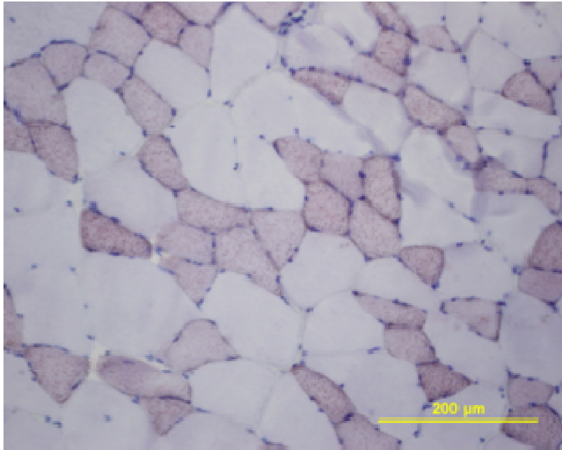


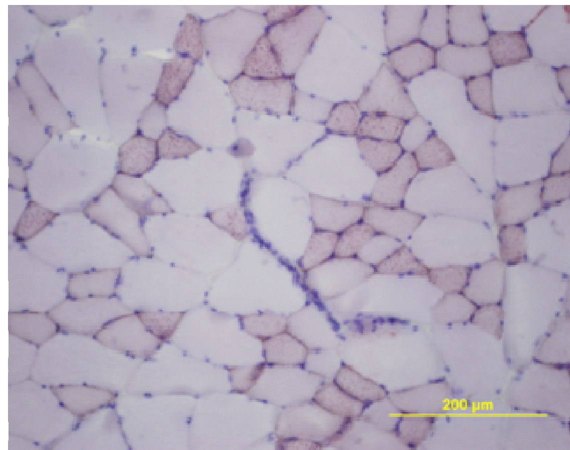
Figure 3.6a - Effect of 16 weeks of voluntary running followed by 53 and 173 hrs of no running on intramuscular lipid accumulation (% oil red) in epitrochlearis muscle of OLETF rats. Groups were defined figure 3.3b legend. Values are means ± SEM; n = 5-6 animals / group.

Figure 3.6b

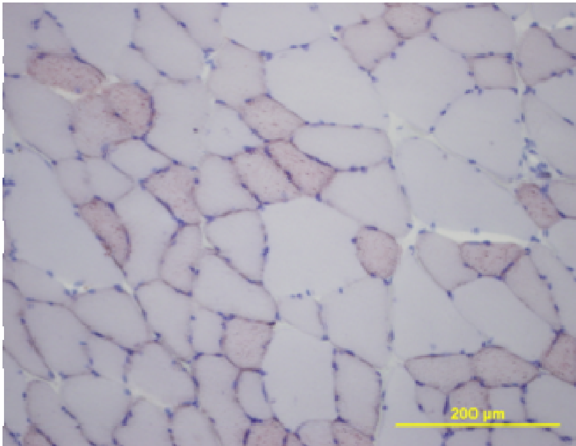
WL5



WL53



WL173



SED

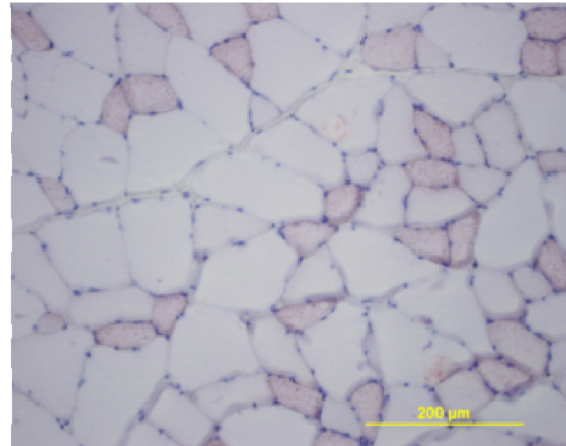
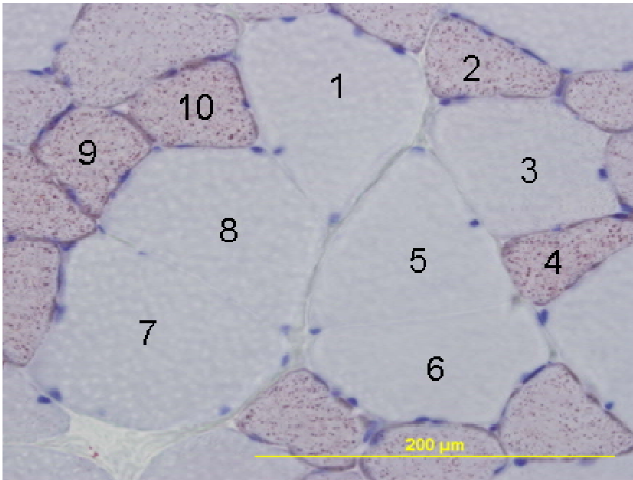


Figure 3.6b - Representative oil red staining of epitrochlearis muscle from WL5 OLETF rats. (20 x magnification).

Figure 3.6c

Oil Red



Succinate Dehydrogenase

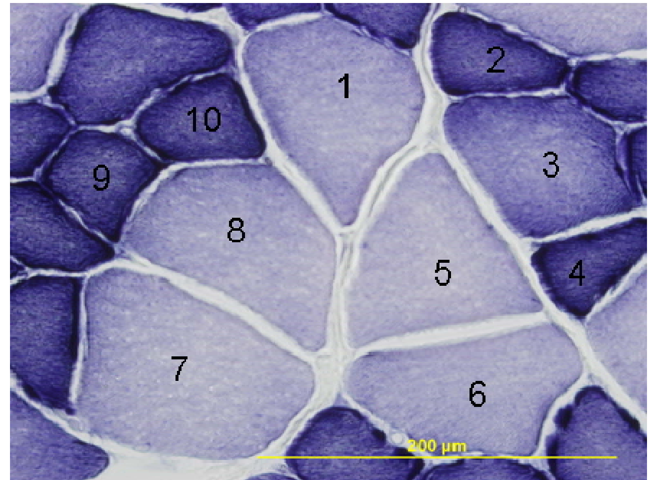


Figure 3.6c - Serial staining of OLETF epitrochlearis muscle (WL5) with oil red and succinate dehydrogenase (40 x magnification)

4-Hydroxynonenal (4-HNE) Levels are Higher in Skeletal Muscle of Sedentary OLETFs.

Lipid peroxidation in epitrochlearis muscle, as determined by levels of 4-HNE, was significantly greater in SED group compared to WL5, WL53, and WL173 ($p < 0.05$) (Figure 3.7a). In contrast in omental adipose tissue, 4-HNE was not different among the four groups (Figure 3.7b). Representative images are displayed for each group. 4-HNE normalized to % Oil Red O staining in epitrochlearis muscle was 394%, 365%, and 199% ($p < 0.05$) greater in SED compared to WL5, WL53, and WL173, respectively (Figure 3.8).

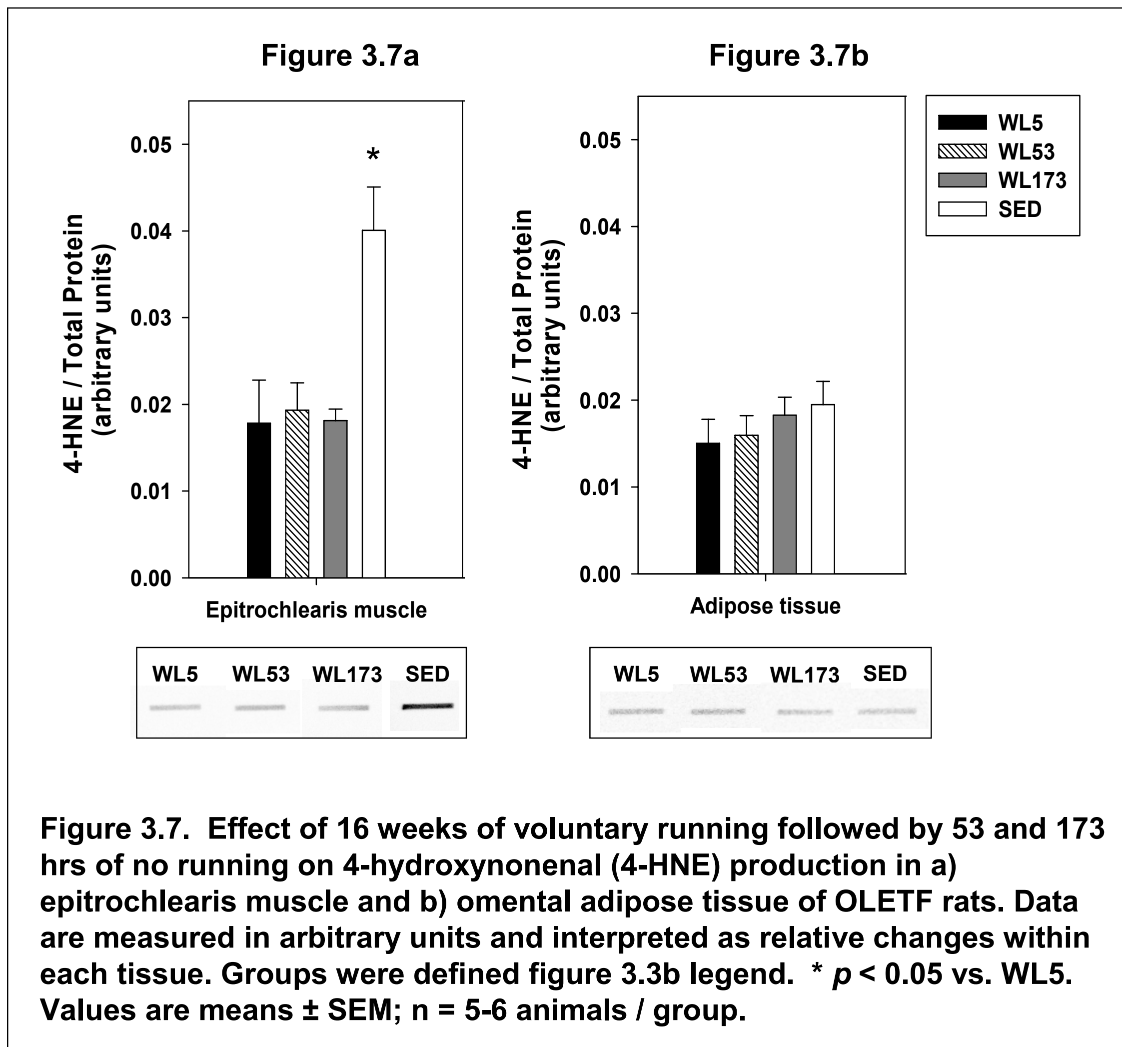


Figure 3.7. Effect of 16 weeks of voluntary running followed by 53 and 173 hrs of no running on 4-hydroxynonenal (4-HNE) production in a) epitrochlearis muscle and b) omental adipose tissue of OLETF rats. Data are measured in arbitrary units and interpreted as relative changes within each tissue. Groups were defined figure 3.3b legend. * $p < 0.05$ vs. WL5. Values are means ± SEM; n = 5-6 animals / group.

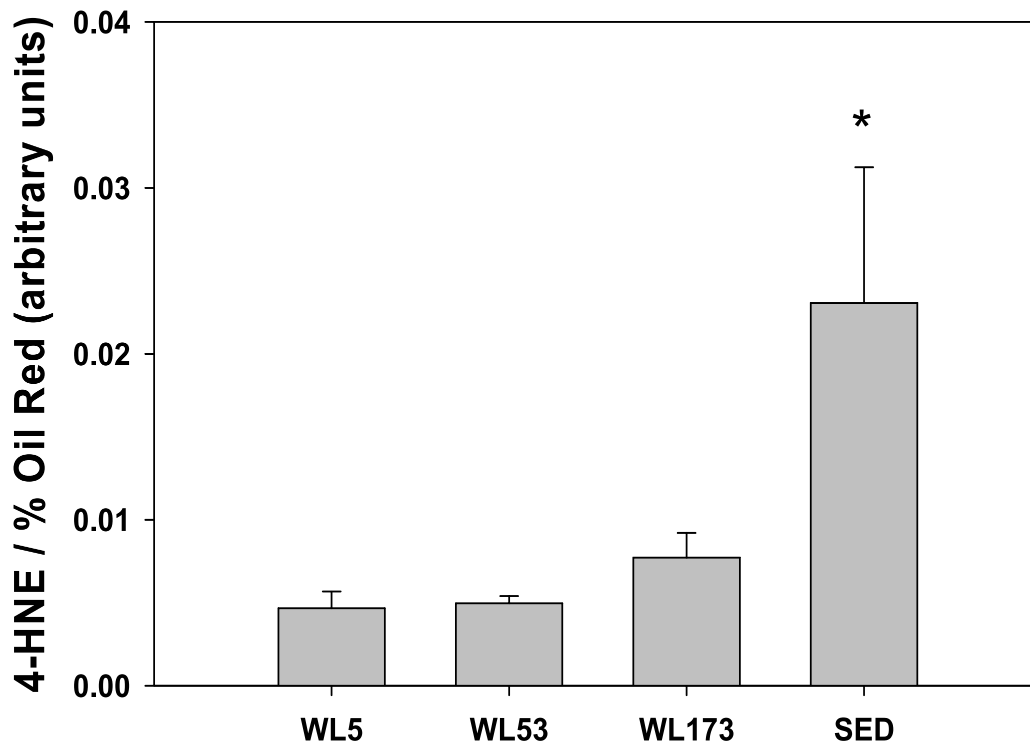


Figure 3.8 - Effect of 16 weeks of voluntary running followed by 53 and 173 hrs of no running on 4-HNE / intramuscular lipid in epitrochlearis muscle of OLETF rats. Groups were defined figure 3.3b legend.
* $p < 0.05$ vs WL5, Values are means ± SEM; n= 5-6 animals / group.

SOD-1 and SOD-2 Protein Expression is Unaltered by Exercise.

No significant difference existed among groups for either SOD-1 (p=0.887) or SOD-2 (p=0.864) protein expression in epitrochlearis muscle following 16 weeks of voluntary running or short term physical inactivity (Figure 3.9a and 3.9b).

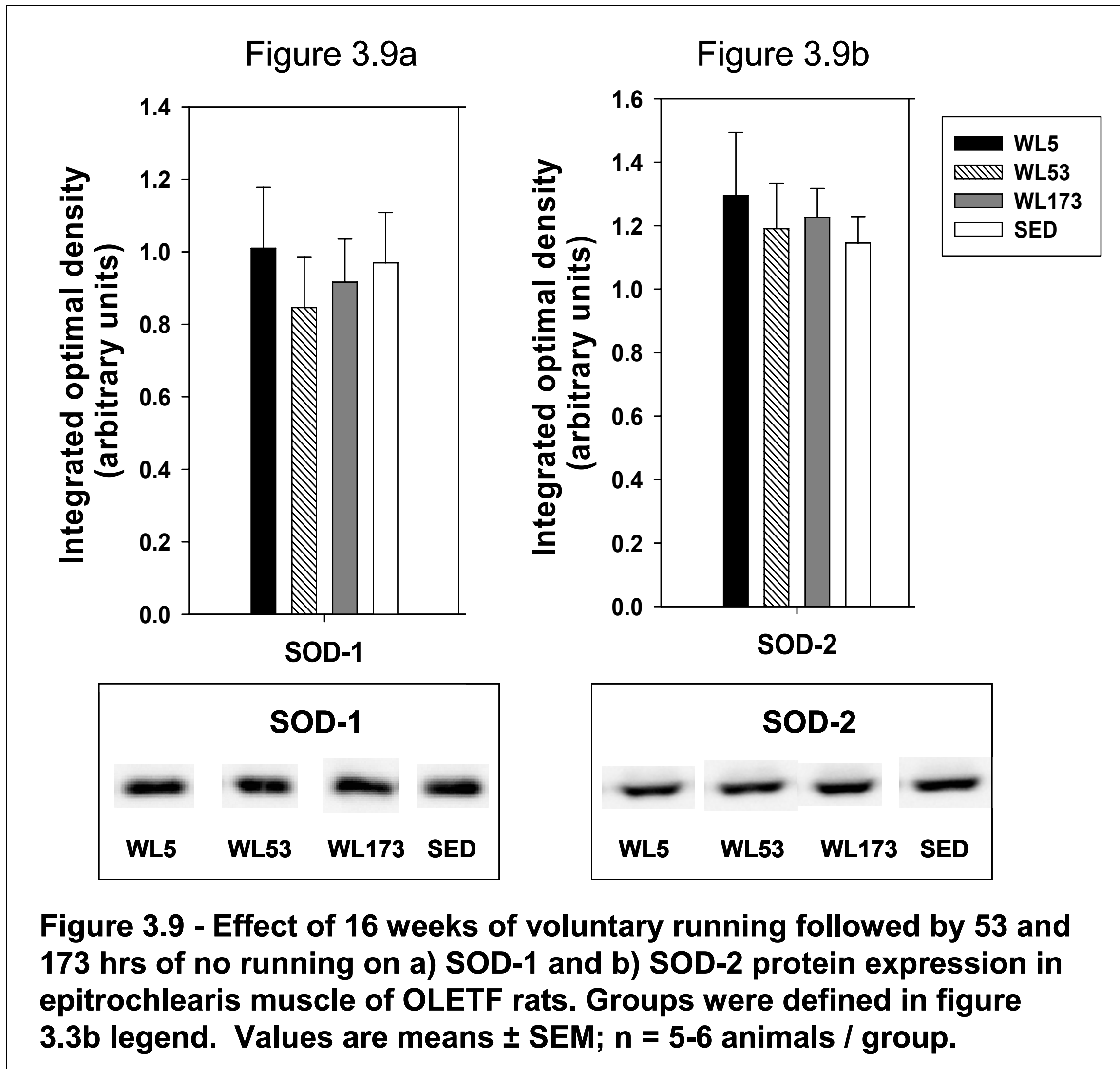


Figure 3.9 - Effect of 16 weeks of voluntary running followed by 53 and 173 hrs of no running on a) SOD-1 and b) SOD-2 protein expression in epitrochlearis muscle of OLETF rats. Groups were defined in figure 3.3b legend. Values are means ± SEM; n = 5-6 animals / group.

No Change in Fatty Acid Oxidation.

Total palmitate oxidation in epitrochlearis muscle was not significantly different among groups. (Figure 3.10)

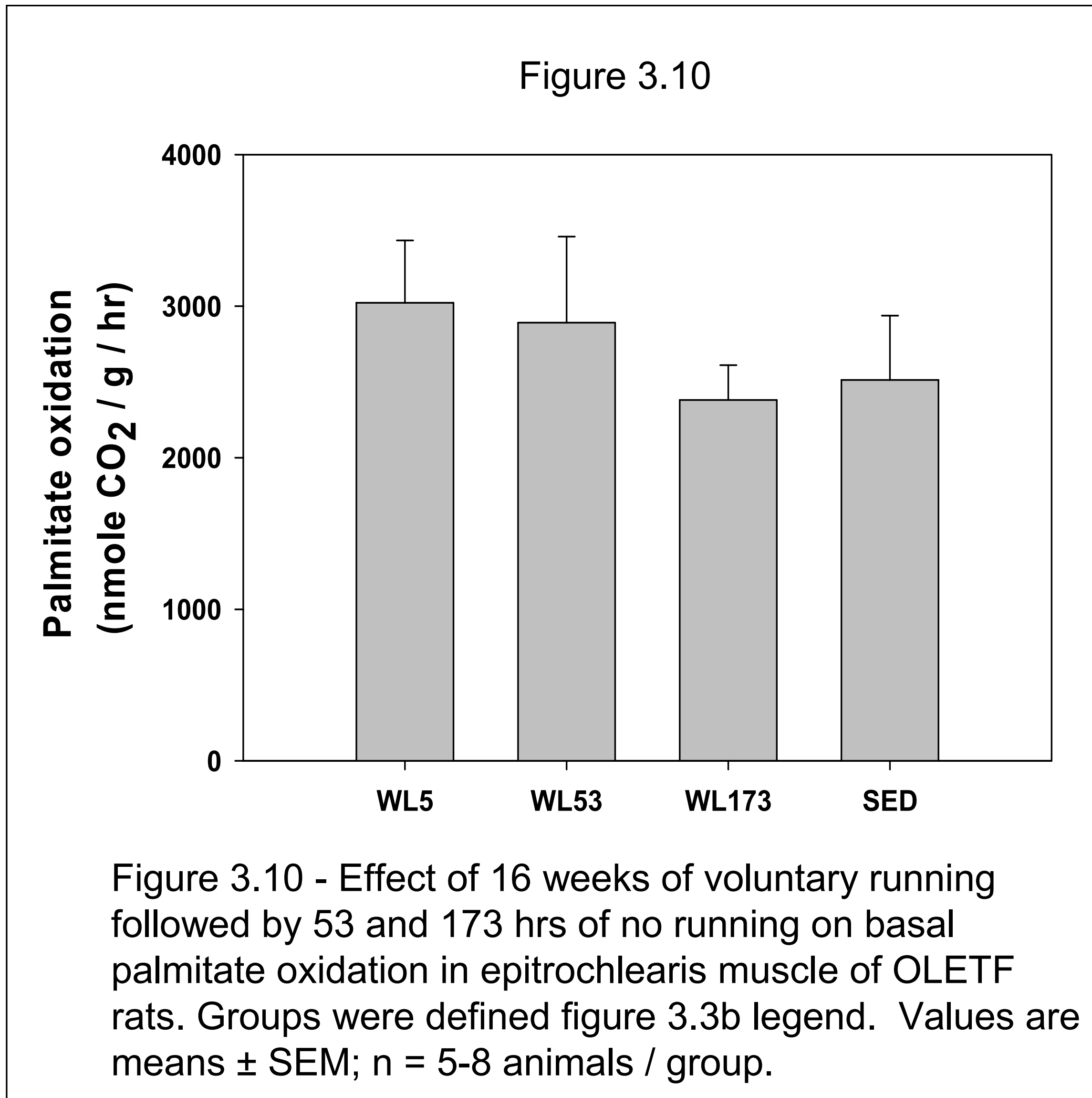


Figure 3.10 - Effect of 16 weeks of voluntary running followed by 53 and 173 hrs of no running on basal palmitate oxidation in epitrochlearis muscle of OLETF rats. Groups were defined figure 3.3b legend. Values are means ± SEM; n = 5-8 animals / group.

GSTα-4 mRNA Levels were Down Regulated in Sedentary OLETFs.

Neither PPAR-δ nor PGC-1α mRNA levels normalized to 18S were significantly different in epitrochlearis muscle among groups (Figure 3.11; PPAR-δ, p = 0.285; PGC-1α, p = 0.363). GSTα4 mRNA was lower in SED and WL173 (p < 0.05) compared to WL53. No statistical difference for GSTα4 mRNA existed between WL5 and SED (p = 0.053).

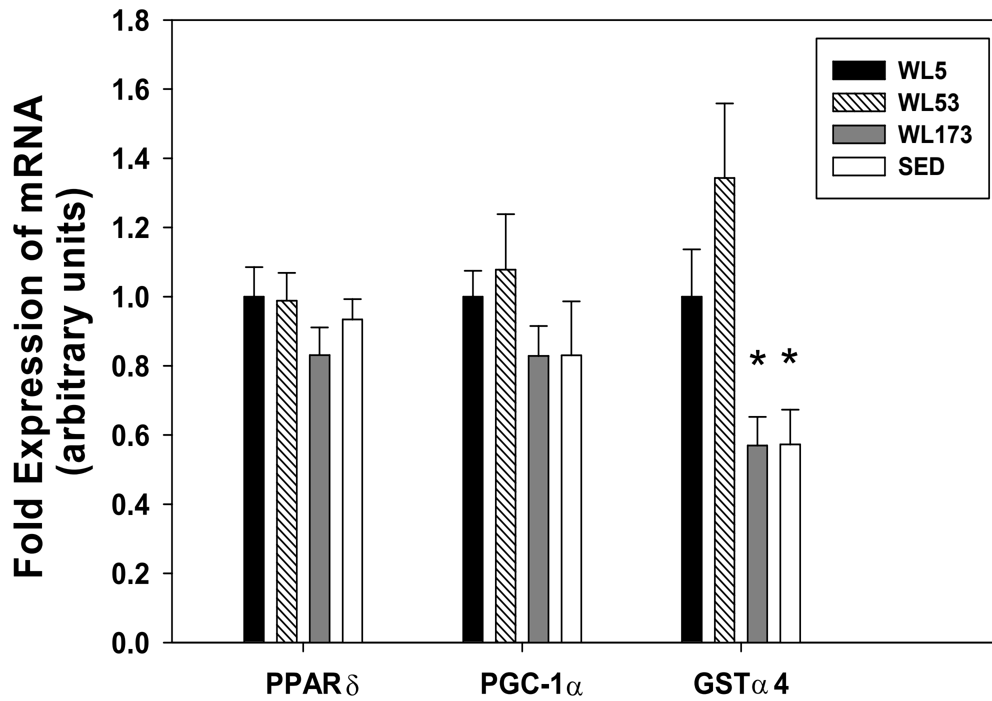


Figure 3.11. Effect of 16 weeks of voluntary running followed by 53 and 173 hrs of no running on PPARδ, PGC-1α, and GSTα4 mRNA levels in epitrochlearis muscle of OLETF rats. WL5 was set to 1 and all other groups normalized to WL5. * $p < 0.05$ vs. WL53. Groups were defined figure 3.3b legend. Values are means ± SEM; n = 5-8 animals / group.

Skeletal Muscle Citrate Synthase Activity was Upregulated at WL53.

Enzyme activity for citrate synthase was greater in the WL53 group compared to all other groups (Figure 3.12; $p < 0.05$).

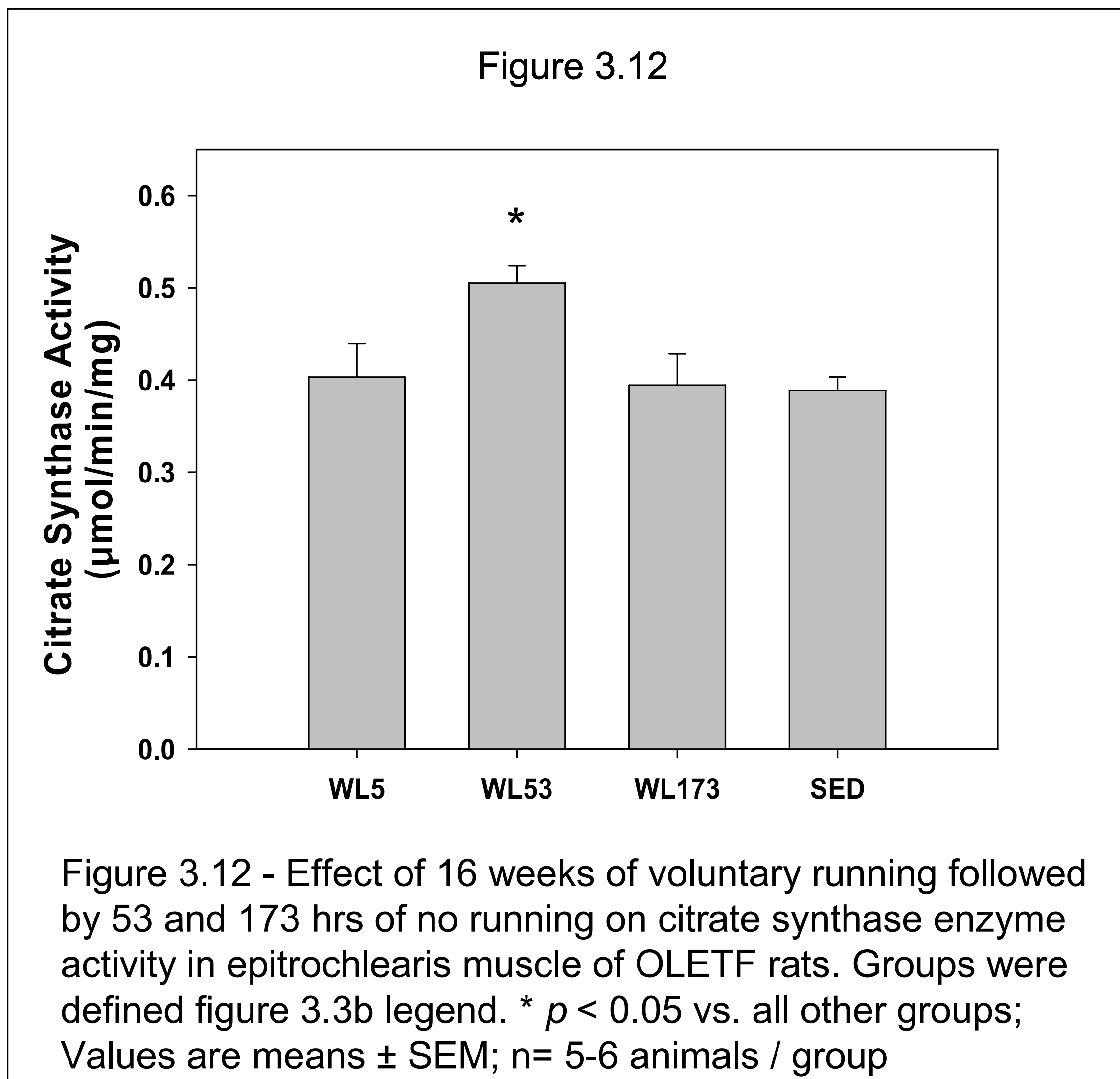


Figure 3.12 - Effect of 16 weeks of voluntary running followed by 53 and 173 hrs of no running on citrate synthase enzyme activity in epitrochlearis muscle of OLETF rats. Groups were defined figure 3.3b legend. * $p < 0.05$ vs. all other groups; Values are means ± SEM; n= 5-6 animals / group

DISCUSSION.

As expected, we found that voluntary running reduced body weight, reduced omental (69% less) and epidydimal (58% less) fat pad weight as well as insulin levels in the OLETF rats when compared to SED. We also found daily voluntary wheel running caused dramatic reductions in lipid peroxidation of the epitrochlearis muscle, despite finding little evidence that basal lipid oxidation of mitochondria was enhanced. Additionally, a number of novel observations were made which included both expected and unexpected responses.

Grimsrud et al (114) found that obesity is accompanied increased carbonylation of a number of adipose-regulatory proteins which may serve as a mechanistic link between increased oxidative stress and the development of insulin resistance. Pro-oxidative conditions lead to production of reactive aldehydes such as trans-4-hydroxy-2-nonenal (4-HNE), an electrophile which has toxic effects on cells and can react with amino acid side chains of proteins (54; 83; 330). Previous reports have shown that protein modification due to 4-HNE can lead to both the loss of functionality as well as activation of cellular signaling pathways (9; 115; 275; 357). Chronic oxidative stress, which is associated with enhanced 4-HNE levels, produces nitrosylation of Akt causing reduced insulin signaling transduction in insulin resistant muscle (211).

Confirming our hypothesis, OLETF runners had ½ as much 4-HNE (an index of lipid peroxidation) in the epitrochlearis muscle as their sedentary cohorts. Further, the ratio of 4-HNE to intramyocellular lipid in epitrochlearis muscle was 80% less in runners than SED. The difference is augmented by the tendency of intramyocellular lipid in the epitrochlearis muscle to be 22% greater (p = 0.077) in runners compared to SED. Our observation that exercise lowers production of 4-HNE in skeletal muscle confirms and extends a human study by Russell et al. (290). They reported that levels of 4-HNE were lower in the vastus lateralis muscle of insulin sensitive, endurance-trained athletes compared to sedentary, obese subjects (289). The current study found that 4-HNE levels in omental adipose was similar between runners and sedentary groups, indicating that the exercise adaptation may only occur in muscle.

The current findings also provide new evidence that physical activity induces preferential increases of glutathione s-transferaseα-4 (GSTα-4) mRNA levels while not changing the expression of SOD-1 or SOD-2 protein in epitrochlearis muscle. Levels of mRNA for GSTα-4 were significantly increased in WL53 vs. both the WL173 and sedentary group (WL5 vs. SED, $p = 0.053$). The GST family of enzymes catalyzes the conjugation of reduced glutathione with electrophilic compounds, such as 4-HNE, via Michael addition (118; 131). This enzymatic reaction serves to reduce 4-HNE levels via GSTα-4. In order to assess the pathophysiological role of 4-HNE *in vivo*, GSTα-4 knockout mice (GSTα-4 -/-) were previously generated and these animals had a reduced ability to conjugate 4-HNE (93). Consequently, GSTα-4 -/- mice had significantly higher steady-state levels of 4-HNE and malondialdehyde (an end-product of lipid peroxidation by reactive oxygen species) in the liver compared to wild type mice at 4 months of age. Also, the survival time of GSTα-4 -/- mice was significantly reduced following intraperitoneal injection of paraquot, a generator of superoxide radicals (92). Although changes in mRNA levels may not reflect functional protein activity, measurements of GSTα-4 mRNA levels in the present study suggest enhanced gene transcription and / or improved mRNA stability for GSTα-4 in response to exercise. Taken together, the above suggests that exercise induced activation of GSTα-4 could reduce 4-HNE levels *in vivo* and thus protect against oxidative stress.

In contrast to the above, other hypotheses within this study were not upheld. The epitrochlearis muscle of physically active OLETF rats did not exhibit

increases in the following: 1) protein levels for SOD-1 or SOD-2, and 2) mRNA levels for PPARδ, and PGC-1α. The lack of a change in SOD-1 and SOD-2 proteins in physically active OLETF rats is surprising. SODs are enzymes that catalyze the conversion of superoxide into hydrogen peroxide and oxygen. Exercise training is a powerful stimulus for increased antioxidant capacity via improvements of reactive oxygen species scavenging (62; 302; 339). For example, treadmill exercise training in young, Sprague Dawley rats for 10 weeks (4 days / week) at 60 min / day significantly enhanced SOD activity in soleus muscle (279). Although skeletal muscle protein expression of SOD-1 or SOD-2 did not change in the current study, a limitation is that SOD activities were not determined and increased SOD enzyme activity may have reduced oxidative damage without changes in protein expression. However, the overexpression of PGC-1α or PGC-1β in C_2C_{12} myotubes has been shown to increase Mn-SOD (SOD-2) mRNA levels (313). Accordingly, our data demonstrate that PGC-1α mRNA levels remained unchanged and SOD protein expression was concomitantly unaltered with exercise in the OLETF rat.

Based upon previous publications and the impaired growth of intra-abdominal fat in OLETF runners, the lack of an adaptive increase in fatty acid oxidation in epitrochlearis muscle was unexpected. Endurance-type training has been shown to increase fatty acid oxidation in the vastus lateralis muscle of obese women (10 days of cycling) (59) and in red and white gastrocnemius muscle of rats ran on a treadmill for 10 weeks (196). Additional studies demonstrate that fatty acid oxidation in skeletal muscle is significantly reduced

with human obesity and negatively associated with body mass index (BMI) (176). The lack of increased fatty acid oxidation in skeletal muscle of OLETF runners was supported by no change in mRNA levels for PPARδ and PGC-1α. The PPARδ and PGC-1α transcriptional pathway is an established stimulus for upregulation of fatty acid oxidation (209). Moreover, several studies have shown that exercise training increases skeletal muscle PGC-1α and PPARδ mRNAs (3; 12; 13; 270).

The failure to observe evidence of upregulation in fatty acid oxidation in OLETF skeletal muscle following 16 weeks of voluntary exercise may have benn due to the following possibilities: First, a defect may exist in the adaptive mechanisms regulating mitochondrial biogenesis following exercise training in obesity. Menshikova et al (235) has recently observed that moderate-intensity physical activity combined with weight-loss, which reduced BMI from 34 to 31 kg/m^2, did not increase mitochondrial DNA (mtDNA) in the vastus lateralis muscle of previously sedentary men and women. However, this intervention resulted in a greater inter-mitochondrial membrane surface area and increased enzymatic capacity for oxidative phosphorylation, including enhanced citrate synthase and succinate dehydrogenase activities. Menshikova et al (234) commented that the pattern of adaptation in obesity may differ from the classic patterns of mitochondrial biogenesis that have been described with adaptation to vigorous exercise. The lack of a mitochondrial adaptation observed in the current study raises the possibility that extreme obesity [OLETF rats are 30-40% heavier than non-obese groups at 20 wks of age (165)] alters the classic pattern of adaptive

mitochondrial biogenesis by exercise. Increases in skeletal muscle citrate synthase activity is a well-established marker of exercise training, but was unchanged in the WL5 group of the hyperphagic, obese OLETF rat. Unexpectedly, citrate synthase activity was increased in the WL53 group compared to all other groups and may have occurred due to a predisposal for obesity in this rat model. Furthermore, abating the molecular signals for fatty acid oxidation and mitochondria biogenesis, such as PGC-1α and PPARδ, may serve to impair this response which typically increases in healthy subjects following exercise training (215). The second possibility to explain lack of upregulated fatty acid oxidation may result from the measurements not being taken during peak running distances. Within this study, skeletal muscle mRNA levels and concurrent palmitate oxidation rates were measured at 20 weeks of age when the average running distance was ~5 km/day. These measurements might have changed due to the exercise if taken following peak running distances (~9 km/day) at 8-11 weeks of age. Future studies will be needed to evaluate these possibilities.

Taken together, this study provides evidence that levels of 4-HNE (a marker of lipid peroxidation) in the epitrochlearis muscle is lower in physically active OLETF rats and were maintained after the cessation of exercise for up to 1 week. In contrast, omental fat did not show the same adaptation to physical activity. This study also indicates that protection against lipid peroxidation in skeletal muscle may, in part, occur through increases in GSTα4. Future studies will be needed to determine the role of exercise in regulating lipid peroxidation in

skeletal muscles of obese subjects and whether this response protects against the development of insulin resistance. Also, the possibility that mitochondrial biogenesis due to exercise is impaired in obesity should be investigated.

Acknowledgements:

Research was supported by anonymous gift (FWB), University of Missouri Life Sciences pre-doctoral fellowship (MJL), Research grant (MHL). We also thank Sarah Borengasser and Scott Naples for help with the data collection.

CHAPTER 4.

DISCUSSION

Physical activity is commonly referred to as “any bodily movement produced by skeletal muscles that results in energy expenditure beyond resting expenditure” (38). Engagement in physical activity is now recommended on a daily basis to help preserve health and promote longevity. Studies have confirmed that 30 minutes of daily physical activity is associated with ~30% reduction in rates of all-cause mortality (171); particularly in industrialized societies. Furthermore, data from epidemiological studies suggest that a sedentary lifestyle increases the incidence of at least 20 pathological conditions, including chronic diseases and risk factors for chronic disease (25).

Health, exercise, and thrifty genes

The benefits of regular exercise in maintaining health are well established within the scientific literature, but the integrated cellular and molecular mechanisms underlying this phenomenon are still largely unknown. However, Neel (1962) previously described the “thrifty genes hypothesis” which provides support for the role of exercise in preserving metabolic function and health (251). The term “thrifty genes” refers to certain genes that evolved during the Late-Paleolithic era to regulate efficient energy consumption and utilization of fuel stores. During this time, food procurement and physical activity were inter-dependent among hunter-gatherer societies. This lifestyle ultimately adapted our genotype to support regular physical activity. Today, humans possess the same “thrifty genes” but have been accommodated with overnutrition, technological

convenience, and an increased sedentary lifestyle. Many have postulated that the metabolic dysfunction derived in type II diabetes and obesity is a direct consequence of impaired regulation of our "thrifty genes" through the lack of sufficient physical activity and energy balance (41).

Factors contributing to the rise of chronic diseases in the 21st century

The incidence of chronic diseases has dramatically increased over the last half of the 20th century and is predicted to further escalate in the future (26). Within the context of the "thrifty gene hypothesis", it seems likely that physical inactivity and poor diet serve as mechanisms for predisposition of chronic disease. Interestingly, only 38.5 % of U.S. adults in 2000 received the daily recommended amount of physical activity (~30 minutes of daily moderate exercise) (307). Similar findings exist in Europe where ~83.4% of adults in Geneva, Switzerland were characterized as being sedentary (<150 kcal/day expended in moderate intensity activity) (233). Secondly, the loss of a more natural diet and the development of unhealthy nutrition through processed foods and high fat diets may promote metabolic derangements. For example, the Mediterranean diet is an eating pattern which has been shown to protect against weight gain and the development of type II diabetes (296). It is modeled after the dietary habits of individuals from the Mediterranean basin and consists of a high consumption of fruits and vegetables as well as the use of olive oil which contains lower saturated fat and higher poly- or mono-saturated fat content. Thus, the advent of a more sedentary lifestyle and an unbalanced diet has likely predisposed us for chronic diseases in modern times.

Counteracting chronic disease through exercise

- In summary, it was the aim of this dissertation to determine whether `exercise can delay and/or counteract the development of specific markers of maladaptation, such as cardiac dysfunction and skeletal muscle lipid peroxidation, in genetically modified rats predisposed for chronic disease.

Part I.

Delayed cardiac maladaptation in the hypertensive, (mREN2)27 rat through low-intensity exercise

It was hypothesized that exercise training would attenuate increases in blood pressure and preserve normal cardiac output and diastolic function. In addition, left ventricular collagen deposition, levels of α- and β-myosin heavy chain (MyHC) mRNA, and B-type natriuretic peptide (BNP) mRNA were assessed to determine if exercise training would delay the appearance of markers associated with maladaptive cardiac hypertrophy. The novel findings are that introduction of low-intensity treadmill running at a young age (3-4 weeks of age) largely prevented a decrease in relative cardiac function at 8 weeks of age. Echocardiography revealed resting cardiac index (↑ 57%) and stroke volume (↑ 43%) to be significantly higher for the EX-Ren2 compared to their SED-Ren2 counterparts. Interestingly, EX-Ren2 and SED-SD had similar values for resting cardiac index and stroke volume, suggesting that exercise can maintain cardiac systolic function during the early development of hypertension in the (mREN2)27 model. In addition, mitral deceleration time slope (m/sec^2), a non-invasive estimate of diastolic function, was significantly reduced in the (mREN2)27 compared to SD rats. Further, diastolic dysfunction was not

prevented by exercise training in the (mREN2)27 rats, indicating that the initial preservation of cardiac index and stroke volume occurred in the presence of diastolic dysfunction. Cardiac fibrosis due to pathological hypertrophy is associated with chronic hypertension and can cause impaired relaxation (stiffness) and filling of the ventricle. This condition can lead to an elevation of ventricular diastolic pressure and produce diastolic dysfunction.

Exercise fails to alter several common markers of cardiac maladaptation

Markers of cardiac remodeling and maladaptation were measured to examine potential factors by which exercise training influenced cardiac function. Although our data indicate impaired diastolic function in (mREN2)27 rat hearts, collagen deposition was not significantly different between (mREN2)27 and SD hearts suggesting that mechanisms other than ventricular wall stiffness due to collagen deposition are contributing to diastolic dysfunction at this stage. Myosin heavy chain (MHC) isoform expression also plays a significant role in the performance of the LV. The data indicate that both α and β MyHC mRNA levels are significantly increased in the (mREN2)27 rat hearts and these increases were not altered by exercise training. MyHC isoform switching might have been more direct if protein expression was measured in addition to mRNA. However, previous studies have suggested that myosin heavy chain isoenzyme transition in the heart occurs through pre-translational mechanisms. Thus, exercise doesn’t appear to maintain cardiac index through the above remodeling events and may improve ventricular performance through alternative factors.

Exercise does not attenuate systolic blood pressure

Finally, results from the current study show that SBP blood pressure in the (mREN2)27 rat did not change in response to 5-6 weeks of low-intensity treadmill exercise. This may be, in part, related to the forced nature of the exercise and a consequent adverse physiological stress. Or, that the total amount (distance ~1 km/d) of running performed at a relatively low intensity (vs. 6-7 km/d voluntary) was insufficient to overcome the Ang II-mediated hypertension. It is possible that exercise may not affect the production of Ang-II due to the high expression of the renin transgene in a number of extrarenal tissues (35). However, our data suggest that improved cardiac function in the (mREN2)27 rat following exercise training occurs via inherent changes in contractility and not a decrease in ventricular afterload.

Supporting Studies and Future Directions:

Low-intensity exercise training improves survival following prolonged hypertension

Exercise training has been shown to delay or reduce the onset of chronic heart failure following prolonged hypertension. For example, 6 months of low-intensity exercise in spontaneously hypertensive heart failure prone rats (SHHF) improved survival ($p<0.01$) independent of any changes in the hypertensive status (86). The exercise training also delayed the $\alpha \rightarrow \beta$ MHC isoform switch which is commonly observed during maladaptation in the left ventricle. Additionally, Libonati et al (216) demonstrated that intervention with low-intensity

exercise training beginning at 8 weeks of age significantly enhanced survival of the hypertensive Dahl Salt sensitive rats. In parallel with the results from Empter et al (87), exercise training had no effect on systolic blood pressure among surviving animals. These studies support our current findings and provide evidence that low-intensity exercise protects against cardiac dysfunction independent of high blood pressure attenuation.

Additional regulatory sites for improved cardiac contractility and function

Due to the significantly improved stroke volume index observed following exercise training in this study, other likely candidates for improved cardiac performance include factors that regulate contractility. It has been shown that Ang II exerts a negative inotropic effect on cardiomyocytes by diminishing calcium transits which, in turn, leads to myocardial dysfunction and remodeling (81; 261). Also, endurance exercise training in rats demonstrates improved cardiac myocyte contractility through increased sensitivity to Ca^{+2}. In response to the same [Ca^{+2}], single cardiac myocytes from exercise trained rats produced a higher level of 50% maximal tension than the sedentary group (75).

Excitation-contraction (EC) coupling in a cardiac myocyte is a complex system with numerous regulatory sites (222). Many of the proteins and transporters regulating this process have been shown to improve the overall performance of the myocardium and are currently being targeted for gene therapy in conditions, such as chronic heart failure (Figure 4.1). After an

Figure 4.1

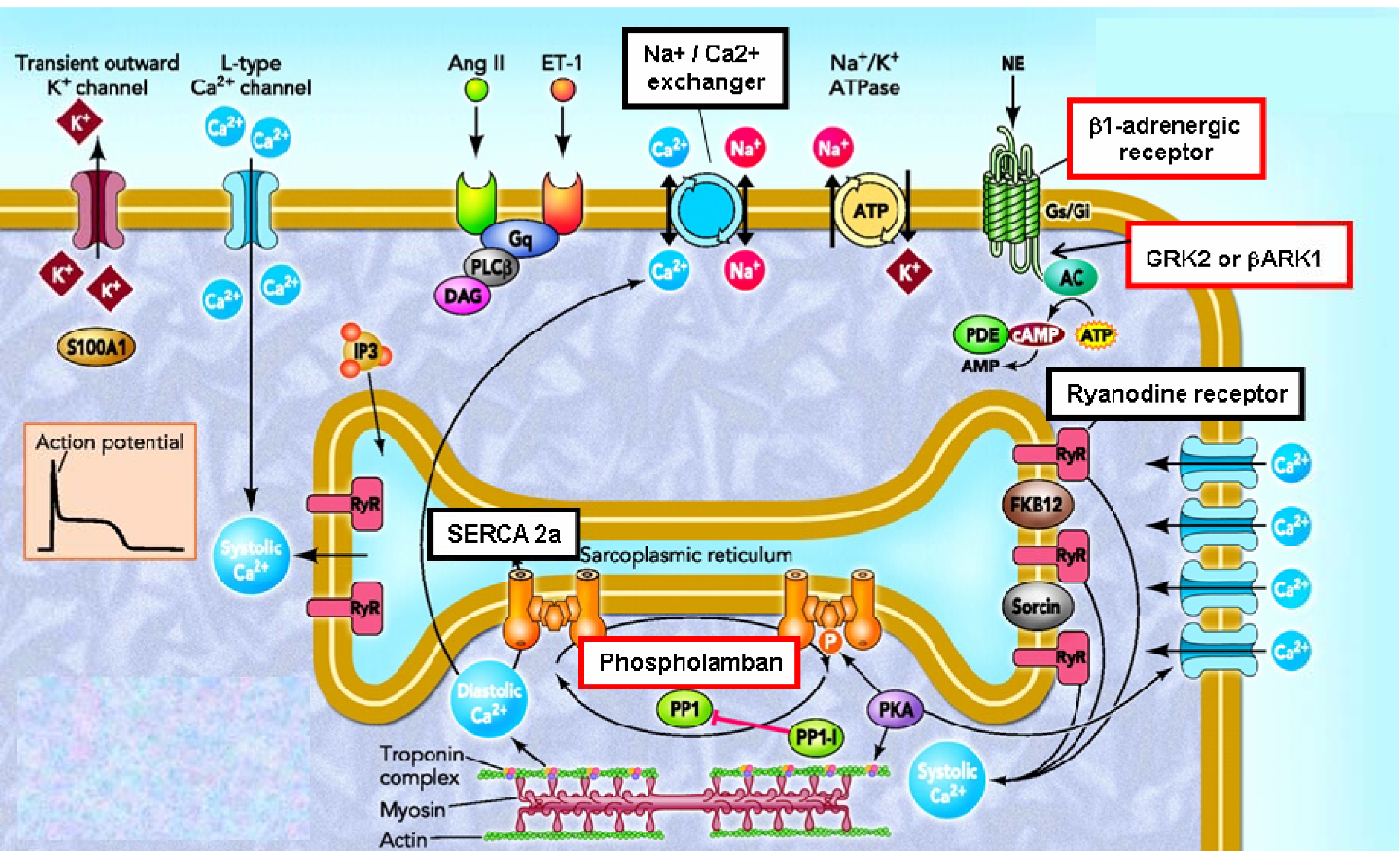


Figure 4.1 – Excitation-contraction coupling in cardiac myoctyes. (Previously studied within the literature- Black boxes: little or no change following exercise in hypertension, Red boxes: marked changes following exercise in hypertension)

(Used with permission from Ly H et al, *Physiology*, 2007)

extensive review of literature, it was determined that many of these regulatory sites have been previously characterized in the left ventricle following exercise training in hypertension (88; 223). Within figure 8, those sites of regulation previously studied with little or no change included the Na^{+} / Ca^{+2} exchanger, the ryanodine receptor, and SERCA2a protein. However, the most striking and attractive targets involved: #1) GRK2 (or βARK1) protein expression of the β_1-adrenergic receptor signaling cascade, and #2) the phosphorylation status of phospholamban (PLB). The following will describe the importance of these regulatory sites following exercise in hypertension.

It is well-established that pressure overload-induced cardiac hypertrophy facilitates negative alterations in contractile function via reduced β-adrenergic receptor responsiveness (8; 248). A study by MacDonnell et al (224), whereby spontaneously hypertensive rats (SHR; age, 4 months) were treadmill exercise trained for 12 weeks, provided evidence that myocardial β-adrenergic responsiveness was improved as assessed by isoproterenol infusions ($1x10^{-10}$ to $1x10^{-7}$ mol/L) in Langendorff preparations. Furthermore, total expression of GRK2 (or βARK1) was significantly upregulated in sedentary SHR vs. normotensive, wild type rats and exercise training of SHR effectively lowered GRK2 expression levels to control value. GRK2, or β-adrenergic receptor kinase, has been previously shown to desensitize cardiac β-adrenergic receptors following pressure overload (47) suggesting the GRK2 protein as a novel regulatory site for improving contractility following exercise in hypertension.

Lastly, MacDonnell et al (225) also demonstrated that the phosphorylation status of PLB is another important potential regulatory site for improving EC coupling and contractile function following exercise training in hypertension. Phosphorylation at amino acids, serine^{16} and threonine^{17}, was significantly decreased in sedentary SHR vs. normotensive, wild type controls, but exercise training in SHR markedly augmented phosphorylation on each of these sites. Unphosphorylated PLB is believed to dock and inhibit SERCA function and thus promoting less Ca^{+2} reuptake in the sarcoplasmic reticulum (SR). However, phosphorylation of PLB relieves this effect resulting in increased SR Ca^{+2} reuptake and subsequent release (221). Interestingly, the β_1-adrenergic receptor signaling cascade has been shown to regulate the activity of PLB through a cyclic-AMP dependent protein kinase A (PKA) pathway (39). Future studies may use these observations to help ascertain important regulatory sites for improving cardiac contractility and function following exercise training in the (mREN2)27 rat.

Part II.

Prevention of obesity via exercise in the hyperphagic, OLETF rat

An important benefit of exercise includes long-term weight control. Individuals who regularly participate in physical activity are more likely to maintain a healthy weight for longer than one year (351). Thus, regular exercise has a fundamental role in counteracting the development of obesity and maintaining long term weight control. In this study, final OLETF body weights at 20 weeks of age in the sedentary group were 45% higher ($p < 0.05$) than the WL5 group following 16 weeks of voluntary running. Short-term wheel lock for

173 hrs did not cause changes in body weight compared to the WL5 group. Also, epididymal and omental fat pad masses in the SED group were 136% and 220% larger ($p < 0.05$), respectively, when compared to the WL5 group. Sedentary epididymal adipocyte volume (pL), diameter (μm), and number (10^6) were all greater as compared to the WL5.

Exercise-induced prevention of obesity reduces skeletal muscle lipid peroxidation

The novel findings of these studies are that voluntary exercise for 16 weeks in the OLETF rat reduces skeletal muscle lipid peroxidation and increases glutathione s-transferase (GST-α4) mRNA levels. Interestingly, levels of 4-HNE normalized to total intramuscular lipid content was decreased in the WL5 (↓ 395%) compared to a SED group. Further, the ratio of 4-HNE to lipid content following short-term wheel lock for 53 and 173 hours did not differ from WL5 suggesting a protective effect in response to exercise in this model.

4-HNE is an electrophile that has toxic effects on cells and can react with amino acid side chains of proteins (53; 82; 331). Previous reports have shown that protein modification due to 4-HNE can lead to both the loss of functionality as well as activation of cellular signaling pathways (10; 113; 276; 357). Exercise training is a powerful stimulus for increased antioxidant capacity and may serve to reduce the peroxidation of lipids in skeletal muscle via improvements of reactive oxygen species scavenging, specifically through super oxide dismutase (SOD) (63; 301; 338). Our results demonstrate no difference in skeletal muscle protein expression of SOD-1 or SOD-2, but increases in SOD activity could potentially influence the production of 4-HNE (not tested). Furthermore, the lack

of changes in protein expression may have occurred due to the type exercise used in this study. Voluntary exercise, compared to treadmill exercise training in rats, occurs at a substantially lower intensity and may have influenced these results.

Additionally, mRNA levels for glutathione s-transferaseα-4 (GSTα-4) were significantly increased in WL53 vs. both WL173 and SED groups (WL5 vs. SED p=0.053). The GST family of enzymes catalyzes the conjugation of reduced glutathione with electrophilic compounds, such as 4-HNE, via Michael addition (120; 129). In order to assess the pathophysiological role of 4-HNE *in vivo*, GSTα-4 knockout mice (GSTα-4 -/-) were previously generated and resulted in a reduced ability to conjugate 4-HNE (91). Thus, it seems likely that skeletal muscle GST-α4 is sensitive to the effects of exercise and may serve to reduce 4-HNE levels in this model.

Exercise-induced prevention of obesity in the OLETF rat does not alter skeletal muscle lipid content

A common phenotype associated with obesity includes augmented levels of intramuscular lipid content. For example, the amount of Oil red O staining for lipid content was significantly greater for all 3 muscle fiber types (I, IIa, IIb) in both obese and type II diabetic subjects compared to a lean group (133). Also, Goodpaster et al (103) hypothesized that skeletal muscle lipid content is associated with insulin sensitivity. Measurements of muscle lipid accumulation and insulin sensitivity via hyperinsulinemic / euglycemic clamping were determined in human subjects characterized as lean, obese, type II diabetic, and exercise trained. Interestingly, the results demonstrate that intramuscular lipid

content was elevated in both type II diabetic and exercise trained groups compared to lean subjects. This observation suggests a paradox between skeletal muscle lipid content and insulin sensitivity (104). Our data demonstrate that skeletal muscle lipid content of exercised OLETF rats does not change compared to sedentary animals. These results also correspond with a study by Russell et al (285) in which intramuscular lipids were similar between exercise-trained athletes and obese patients. Additionally, cessation of voluntary running for 53 and 173 hours in the present study did not affect lipid content.

Skeletal muscle fatty acid oxidation does not change in response to voluntary exercise in the OLETF rat

Previous studies indicate that fatty acid oxidation is significantly reduced with human obesity and positively associates with Body Mass Index (BMI) (177). Other studies suggest that decreased fatty acid oxidation may contribute to augmented skeletal muscle lipid content in obesity. Interestingly, exercise-induced weight loss in obese subjects can effectively increase fatty acid oxidation and is significantly correlated with improvements in insulin sensitivity (108) Furthermore, insulin sensitivity has been shown to positively correlate with fatty acid oxidation. To our knowledge this is the first study to evaluate the effect of exercise-induced prevention of obesity and short term physical inactivity on skeletal muscle fatty acid oxidation. The results demonstrate that basal palmitate oxidation rates in the epitrochlearis muscle did not change among groups in this model. In support of this finding, PPARδ and PGC-1α mRNA levels within the same muscle were not different among the treatment groups. The PPARδ and

PGC-1α transcriptional pathway is an established mediator of gene expression for lipid oxidation. Several studies have shown that exercise increases skeletal muscle PGC-1 and PPARδ transcriptional activity (4; 11; 14; 271). Within this study, skeletal muscle mRNA levels and concurrent palmitate oxidation rates were measured at 20 weeks of age when the average running distance was ~5 km/day. These measurements might have been different if taken following peak running distances (9 km/day) at 8-11 weeks of age. Future studies will be needed to evaluate this possibility.

Additionally, exercise training in obese subjects may not cause similar mitochondrial adaptations as observed in healthy, lean counterparts. Menshikova et al (236) has recently observed that moderate-intensity physical activity combined with weight-loss, which reduced BMI from 34 to 31 kg/m^2, did not increase mitochondrial DNA (mtDNA) in the vastus lateralis muscle of previously sedentary men and women. However, this intervention resulted in a greater inter-mitochondrial surface area and increased enzymatic capacity for oxidative phosphorylation, including enhanced citrate synthase and succinate dehydrogenase activities. Menshikova et al (237) commented that the pattern of adaptation may differ from the classic patterns of mitochondrial biogenesis that has been described with adaptation to vigorous exercise. The impaired mitochondrial adaptation observed in this study raises the possibility that extreme obesity alters the classic pattern of adaptive mitochondrial biogenesis by exercise.

Supporting Studies and Future Directions:

Lipid peroxidation in other tissues in obesity and type II diabetes

The potential lipotoxic effect of intramuscular lipids in development of insulin resistance is poorly understood. However, many studies have associated lipid peroxidation in the obese and diabetic state. For example, products of lipid peroxidation, such as 4-HNE, are elevated in the liver of diabetic rats (324). Also, subcellular compartments of the liver from diabetic rats were found to accumulate lipoperoxides, such as 4-HNE and malondialdehyde, at a higher rate in response to a peroxidative stimulus (325). Furthermore, blood-derived lipid peroxide levels in humans have been measured in obese (BMI > 30 kg/m^2) and age-matched, non-obese controls (358). Malondialdehyde levels in the blood were significantly higher ($p<0.0001$) in the obese group. Finally, Russell et al (286) has shown that levels of lipid peroxidation, or 4-HNE, in skeletal muscle are higher in obese subjects compared to insulin sensitive, endurance-trained athletes with both groups having comparable levels of accumulated intramuscular triglycerides (IMTG). Taken together, these observations suggest that obesity and diabetes can promote augmented 4-HNE levels in a variety of tissues, such as liver, blood, and skeletal muscle. However, it was demonstrated in this study that physical activity lowers 4-HNE levels in skeletal muscle. Also, 4-HNE levels remained unchanged in adipose in OLETF rats suggesting a tissue specificity of this adaptation and a novel contribution from this study.

Additionally, 4-HNE has been shown to regulate the function of specific proteins via carbonyl formation. A study by Grismund et al (116) demonstrated

that diet-induced obesity of C57Bl/6J mice resulted in carbonylation of the adipose fatty acid-binding protein with 4-HNE (*in vivo*). Carbonylation of the adipocyte fatty acid-binding protein with 4-HNE was mapped to Cys-117 *in vitro* and significantly reduced the ability of the protein to bind fatty acids by ~10 fold. These observations suggest that 4-HNE carbonylation can regulate the activity of specific proteins and could serve as a mechanistic link between lipid peroxidation and impaired insulin sensitivity. Few studies exist that investigated the effects of 4-HNE on glucose transport and insulin signaling pathways in skeletal muscle.

In conclusion, the data presented in this dissertation support the overall hypothesis that physical activity has a significant role in delaying and/or counteracting development of specific markers of maladaptation, such as cardiac dysfunction and skeletal muscle lipid peroxidation, in genetically modified rats predisposed for chronic disease. Further investigation is needed to determine the cellular and molecular mechanisms regulating this response.

APPENDIX 1

The presence of β-Mercaptoethanol (BME) in or during tissue homogenation is recommended to reduce excessive oxidative stress and to preserve *in vivo* conditions (1). For Figure A1, we homogenized rat epitrochlearis muscle with zero, low (88.8 mM), and high (178 mM) concentrations of BME. The protein concentration was determined via Bradford assay and the homogenate was used to measure 4-HNE levels via slot blot using increasing protein levels (5, 10, 15, and 20ug). The results demonstrate that 4-HNE production is increased without the addition of BME relative to low and high BME. This suggests that the addition of BME (88.8 mM or 178 mM) during homogenization of muscle tissue reduces the amount of 4-HNE produced for each amount of protein loaded and preserves a linear dose response. Since the addition of high BME did not appear to further reduce 4-HNE levels, low BME was used for the homogenization of muscle tissue.

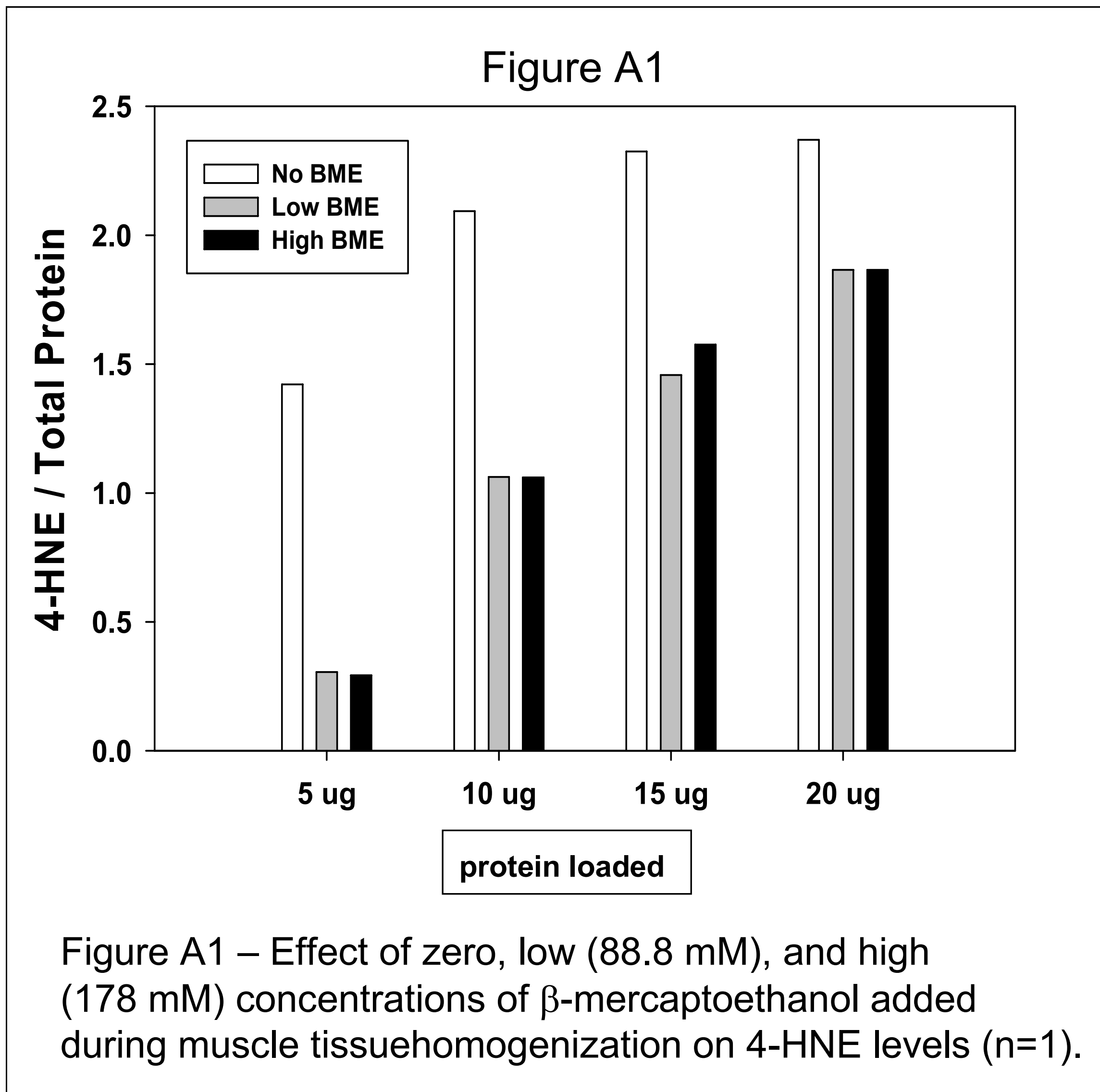


Figure A1 – Effect of zero, low (88.8 mM), and high (178 mM) concentrations of β-mercaptoethanol added during muscle tissuehomogenization on 4-HNE levels (n=1).

APPENDIX II

Because p38 signaling has been shown to regulate activity of PGC-1α in muscle (314; 353), we determined if voluntary exercise and short term physical inactivity regulates the expression and phosphorylation of p38 in the OLETF epitrochlearis muscle. However, the results demonstrate no significance difference in this study (Figures A2.1, A2.2, and A2.3).

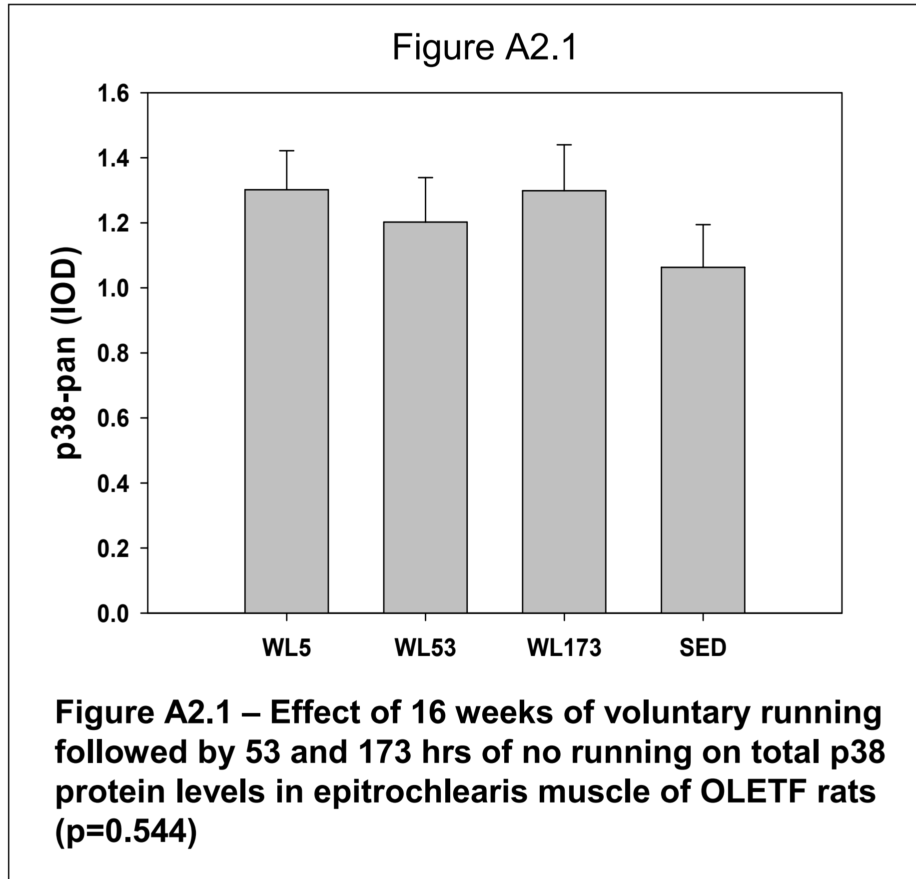


Figure A2.1 – Effect of 16 weeks of voluntary running followed by 53 and 173 hrs of no running on total p38 protein levels in epitrochlearis muscle of OLETF rats (p=0.544)

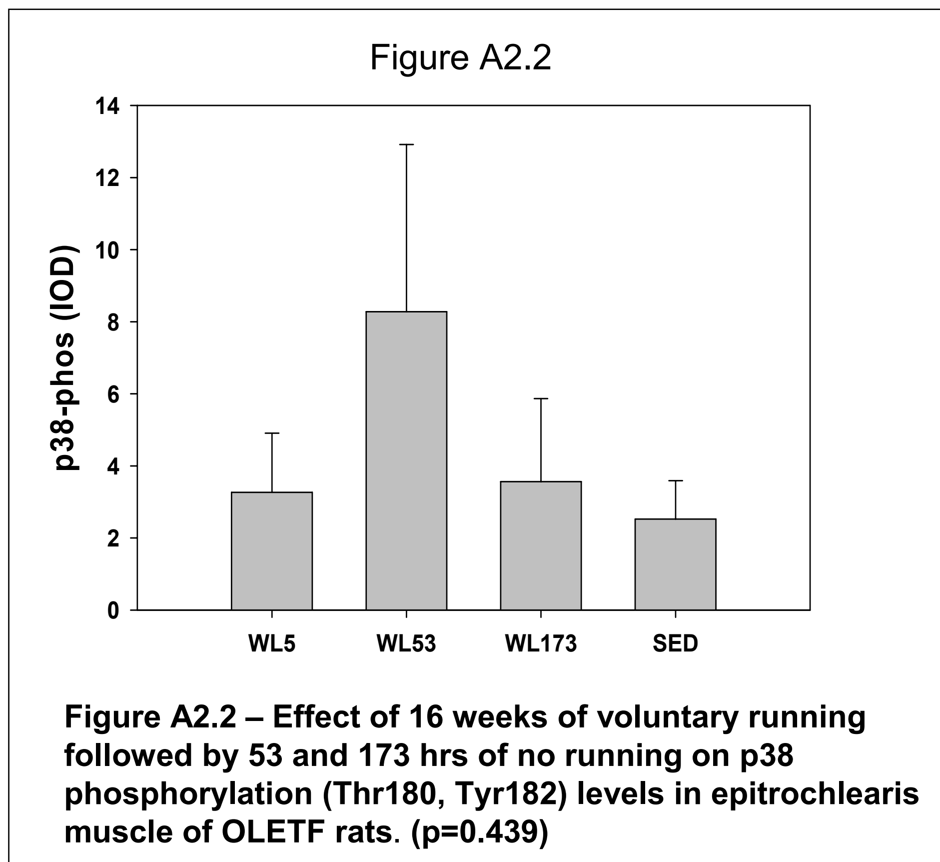


Figure A2.2 – Effect of 16 weeks of voluntary running followed by 53 and 173 hrs of no running on p38 phosphorylation (Thr180, Tyr182) levels in epitrochlearis muscle of OLETF rats. (p=0.439)

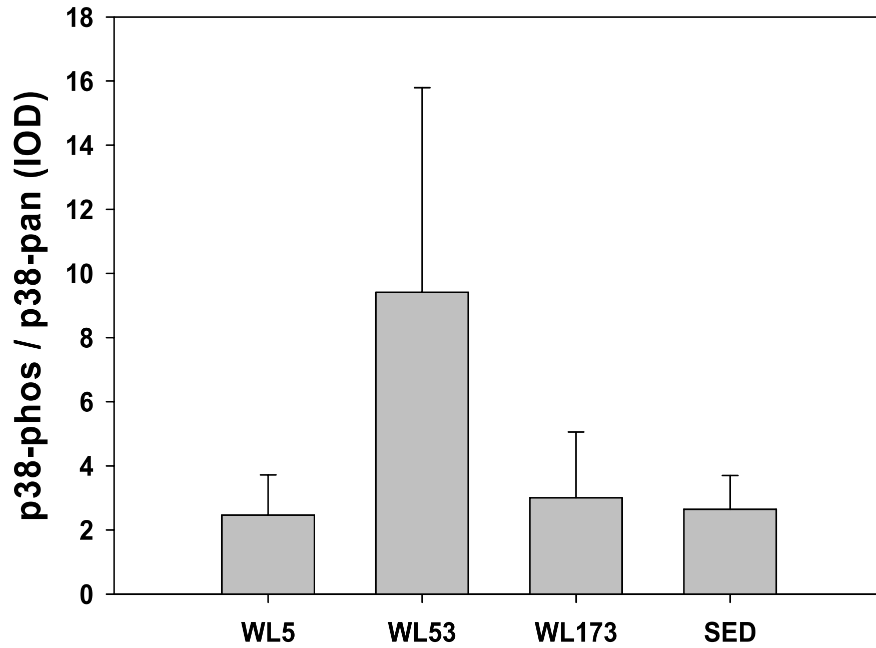


Figure A2.3 – Effect of 16 weeks of voluntary running followed by 53 and 173 hrs of no running on p38 phosphorylation / p38 total protein in epitrochlearis muscle of OLETFrats. (p=0.401)

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VITA

Tyler Morris was born on December 2, 1978 in Seymour, Indiana to Bob Morris and April Herrin. He attended public schools in Harrison, Arkansas, and graduated from Harrison High School in 1997. Tyler has earned the following degrees: Bachelor of Arts in Biology from Drury University in Springfield, Missouri (2001); Master of Science in physiology from the University of Missouri in Columbia, Missouri (2003); Ph.D. in physiology from the University of Missouri in Columbia, Missouri (2007). He also worked in the laboratory of Dr.James Carrington, Ph.D., at Oregon State University in Corvallis, Oregon during the summer of 2001. In the future he plans to teach biology, anatomy & physiology, and exercise physiology at the college level.