

November 2015

## The effect of exercise and caloric restriction on cardiac NF-kB signaling and inflammation in Otsuka Long-Evans Tokushima Fatty (OLETF) rats

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<https://doi.org/10.7275/7591639> [https://scholarworks.umass.edu/masters\\_theses\\_2/262](https://scholarworks.umass.edu/masters_theses_2/262)

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THE EFFECT OF EXERCISE AND  
CALORIC RESTRICTION ON CARDIAC  
NF- $\kappa$ B SIGNALING AND  
INFLAMMATION IN OTSUKA LONG-  
EVANS TOKUSHIMA FATTY (OLETF)  
RATS

A Thesis Presented

By

ANGEL EXPEDITO BAEZ

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfillment  
Of the requirements for the degree of

MASTER OF SCIENCE

September 2015

Department of Kinesiology

THE EFFECT OF EXERCISE AND  
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# ABSTRACT

## THE EFFECT OF EXERCISE AND CALORIC RESTRICTION ON CARDIAC NF- $\kappa$ B SIGNALING AND INFLAMMATION IN OTSUKA LONG-EVANS TOKUSHIMA FATTY (OLETF) RATS

SEPTEMBER 2015

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**Introduction:** Cardiometabolic syndrome is considered a chronic low-grade inflammatory condition that affects various organs and tissues. Individuals with type 2 diabetes mellitus (T2DM) and obesity are at an increased risk for developing the cardiometabolic syndrome and have greater rates of cardiovascular disease (CVD). These conditions are also associated with increased systemic and local inflammation and greater expression of pro-inflammatory markers such as monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin 1 $\beta$  (IL-1 $\beta$ ) in many tissues. The heart is adversely affected by the inflammation and metabolic changes induced by diabetes and obesity. Nuclear transcription factor kappa B (NF- $\kappa$ B) activity is known to be related to inflammation and cytokine production. However, there is limited information on whether NF- $\kappa$ B signaling and inflammation play a role in early cardiac pathogenesis related to obesity and diabetes and whether lifestyle changes known to prevent or treat these diseases are effective in the heart. **Purpose:** Therefore, the purpose of this study was to compare the effect of exercise (EX) and caloric restriction (CR) to alter NF- $\kappa$ B signaling, inflammation, and markers of cardiac dysfunction in the heart of

20-week old Otsuka Long Evans Tokushima (OLETF) rats. **Methods:** Hearts of male 20 week old OLETF rats from a previous study (Crissey et al., 2014) were collected for gene expression (RT-PCR), NF- $\kappa$ B activity, and markers of inflammation and immune cell infiltration. **Results:** There were no significant differences detected in markers of cardiac dysfunction including,  $\alpha$ -MHC,  $\beta$ -MHC, ANP, BNP, COL1, COL3 (all  $p > 0.05$ ). Second, 1-way ANOVA showed that there was trend for an overall effect of group ( $p = 0.07$ ) on NF- $\kappa$ B activation where CR tended to be greater compared to SED and WR ( $p = 0.06$ ). Finally, there were no significant differences between groups in inflammatory and immune cell markers; CD4, F4/80, CD68, IL-1 $\beta$ , MCP-1, TGFB<sub>1</sub>, and TNF- $\alpha$  (all  $p > 0.05$ ). **Conclusion:** This study shows that at 20 weeks, a time when OLETF animals exhibit characteristics of the metabolic syndrome such as hypertension, mild obesity, and increased insulin resistance, EX and CR do not reduce markers of cardiac dysfunction and inflammation, potentially because inflammation does not influence the heart at this early time period in the development of the disease. Further, the trend of greater NF- $\kappa$ B activity in CR compared to EX and SED, needs further exploration.

## TABLE OF CONTENTS

	Page
ABSTRACT.....	iii
LIST OF TABLES.....	vii
LIST OF FIGURES .....	viii
CHAPTER	
1: INTRODUCTION .....	1
2: LITERATURE REVIEW .....	3
2.1 Introduction.....	3
2.1.1 Cardiometabolic Syndrome .....	3
2.1.2 Obesity, Type 2 Diabetes Mellitus and Insulin Resistance.....	5
2.1.3 Inflammation, Insulin Resistance and Obesity .....	8
2.1.4 Myocardial Fat, Lipotoxicity, and Inflammation in the Heart.....	11
2.1.5 Role of NF- $\kappa$ B .....	16
2.1.6 Detection of Molecular Pathology in the Heart .....	21
2.2 Role of Exercise and Caloric Restriction.....	25
2.2.1 Exercise.....	25
2.2.2 Caloric Restriction .....	28
2.2.3 Comparative Effectiveness of Exercise and Caloric Restriction .....	30
2.2.4 Specific Aims and Hypotheses .....	34
3: METHODS .....	36
3.1 OLETF Animal Model and Characteristics .....	36
3.1.2 Animal Set Up.....	37
3.1.3 Gene Expression/RNA Extraction .....	38
3.1.4 NF- $\kappa$ B ELISA Assay .....	41
3.2 Preliminary Data .....	42
4: RESULTS .....	45
4.1 Reference Gene Quality Control.....	45
4.1.2 Cardiac Dysfunction: Fetal Gene Expression and Fibrosis.....	45
4.1.3 NF- $\kappa$ B Activation in Cardiac Tissue of OLETF rats .....	48

4.1.4 Markers of Immune Cells and NF- $\kappa$ B activity.....	48
5:DISCUSSION.....	51
REFERENCES .....	58

# LIST OF TABLES

Table	Page
1. NAME OF GENES INVOLVED IN FETAL GENE PROGRAM.....	39
2. GENES INVOLVED IN INFLAMMATION .....	40
3. GENES INVOLVED IN FIBROSIS.....	40
4. GENES RELATED TO MONOCYTES AND MACROPHAGES .....	40
5. FASTING CHARACTERISTICS OF OLETF ANIMALS .....	44



# LIST OF FIGURES

Figure	Page
1. REFERENCE GENE CONTROL.....	45
2. EXPRESSION OF GENES IN CARDIAC DYSFUNCTION.....	46
3. NF-KB ACTIVATION .....	48
4. EXPRESSION OF IMMUNE GENES .....	49

# CHAPTER 1

## INTRODUCTION

Public health currently faces two epidemics; obesity and type 2 diabetes mellitus (T2DM). Worldwide obesity has nearly doubled since 1980. Approximately 1.6 billion adults are overweight with close to 500 million being considered obese (WHO, 2011). About two-thirds of adults in the United States are considered overweight or obese. Along with the rise of adult obesity, childhood obesity in the US has tripled over the past three decades (Flegal et al., 2010; Kalaupahana et al., 2012). According to the World Health Organization, 382 million people worldwide are afflicted with diabetes and in the United States 18 million people suffer from this disease (WHO, 2011; CDC, 2009). In addition, 90% of individuals suffering from type 2 diabetes mellitus are obese or overweight and are at an increased risk for cardiovascular morbidity and mortality. Cardiovascular disease is the number 1 cause of death in obese and diabetic individuals (Ogden, 2009).

Obesity and T2DM are both chronic low-grade systemic inflammatory diseases. These inflammatory responses are due to changes in adipose tissue depots and immune cell infiltration that lead to expression of pro-inflammatory cytokines. These pro-inflammatory cytokines are believed to play a role in inducing insulin resistance, lipid accumulation in non-adipose sites, and metabolic changes in various organs such as the liver, skeletal muscle, and the heart (Kalupahana et al., 2012; Kahn et al., 2006). These pro-inflammatory cytokines are detrimental to the myocardium and can lead to the development of cardiac dysfunction, fibrosis, cardiomyopathies, and eventually to heart

failure. However, lifestyle interventions, such as exercise or caloric restriction, may be able to improve these conditions and induce an anti-inflammatory state. Though there is limited information on the effect of these two treatments.

In this study, the inflammatory-related changes that occur in the myocardium of overweight and insulin resistant rats in response to exercise and caloric restriction were explored. To determine if either intervention attenuated myocardial dysfunction and inflammatory signaling, an evaluation of activity of a major inflammatory transcription factor, nuclear factor kappa B (NF- $\kappa$ B) and inflammation-related gene expression in the heart was completed.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Introduction

This literature review will detail cardiometabolic syndrome and two of the main factors affecting it: obesity and insulin resistance. Specifically, the role of inflammation on the myocardium will be highlighted. Nuclear factor kappa B (NF- $\kappa$ B) will be introduced for its role in inflammation related to cardiometabolic syndrome and lifestyle interventions of exercise and caloric restriction will be reviewed as methods to improve inflammatory-related myocardial dysfunction as defined by fibrosis and tissue damage.

##### 2.1.1 Cardiometabolic Syndrome

Cardiometabolic syndrome, commonly referred to as metabolic syndrome or Syndrome X, is a cluster of multiple cardiovascular, metabolic, pro-thrombotic, and inflammatory abnormalities, which cause disruptions in energy storage and utilization. These cardiovascular and metabolic disruptions, individually and together, lead to an increased risk in the development of cardiovascular disease (CVD) and stroke (Castro et al., 2003). Cardiometabolic syndrome is becoming a growing concern. Globally, an estimated 25% of the world population is believed to suffer from cardiometabolic syndrome. In the United States, this syndrome affects nearly 44% of the elderly population over the age of 50. Approximately one-third of the US population age 20 and above already exhibit the criteria for cardiometabolic syndrome (Ford ES, 2005).

Cardiometabolic syndrome is diagnosed when a patient exhibits three out of five co-occurring risk factors: 1) abdominal adiposity (obesity), 2) hypertension, 3) insulin resistance, 4) dyslipidemia, and 5) reduction in high density lipoprotein (HDL) (Hertle et al., 2014; Kaur, 2014). Individuals suffering from these risk factors, especially individuals suffering from obesity as well as insulin resistance, are more prone to manifest other components of the cardiometabolic syndrome such as: an increased inflammatory state, increased oxidative stress, microalbuminuria, an increased risk of developing type 2 diabetes mellitus (T2DM) and endothelial dysfunction (Isomaa et al., 2001).

Aside from physiological risk factors, environmental, personal, and genetic traits can also contribute to cardiometabolic syndrome. Indeed, sedentary lifestyle, excess energy intake, genetic susceptibility, and stress play a role in the development of cardiometabolic syndrome (Cameron et al, 2004). When genetic or environmental factors, such as physical inactivity, advancing age, and high saturated fat-simple carbohydrate diet (Western Diet) concomitantly occur alongside obesity, the cardiometabolic syndrome is exacerbated (Grundy, S 2006). These individuals are three times more likely to develop coronary heart disease (CHD) and stroke and have two times the risk of dying from a cardiovascular event (Kaur, 2014). Thus, it is important to identify individuals at risk and establish early interventions that prevent the development of CVD, stroke and CHD.

Cardiometabolic syndrome is a progressive disorder that worsens over time (Grundy, S 2006). The pathogenesis of cardiometabolic syndrome is known to include two related conditions; insulin resistance and obesity (Grundy et al., 2005). It is well

known that insulin resistance and obesity induce dysfunction and physiological damage to the myocardium and various tissues/organs. The cause of the dysfunction in multiple tissues and organs may be due to inflammation from excess adipose tissue, endothelial inflammation, and alterations in the metabolism of these various tissues/organs due to insulin resistance and adipose tissue (Romacho et al., 2014).

Most studies evaluating the relationship between obesity, insulin resistance and myocardial dysfunction have used dramatic models of the disease or condition. It is unknown what changes occur to the myocardium in early stages of obesity (overweight) or insulin resistance. Understanding the early effects of obesity and insulin resistance on the heart and the influence of early lifestyle or pharmacological interventions can help prevent patients from developing T2DM and CVD.

### 2.1.2 Obesity, Type 2 Diabetes Mellitus and Insulin Resistance

Although there are many factors that influence cardiometabolic syndrome, obesity and insulin resistance (which progresses to T2DM) are considered the main underlying causes and greatly influence changes in the heart. Obesity is defined as excess or increase in adiposity that is detrimental to an individual's health and resulting in a body mass index (BMI)  $\geq 30 \text{ kg/m}^2$  (WHO, 2011). Obesity is a growing epidemic that is afflicting millions of people in the world and United States. Worldwide obesity has nearly doubled since 1980. Approximately 1.6 billion adults are overweight with close to 500 million being considered obese (WHO, 2011). About two-thirds of adults in the United States are considered overweight or obese. Along with the rise of adult obesity, childhood obesity in the US has tripled over the past three decades (Flegal et al., 2010; Kalaupahana et al., 2012), highlighting the need to generate and evaluate effective therapies for the

condition. Obesity is considered the 5th leading cause of mortality worldwide (WHO, 2011). In the United States alone, the estimated annual healthcare cost of treating obesity is approximately \$147 billion dollars (CDC, 2011). Obese individuals are at an increased risk of non-communicable diseases such as various forms of cancer, diabetes, kidney diseases, and cardiovascular disease (WHO, 2011). Indeed, 70% of all CVD causes are related to obesity and 112,000 deaths each year are associated to obesity and CVD (NIH, 2013). Therefore, prevention of obesity can help reduce the mortality and morbidity both of obesity and CVD and reduce the expense associated with treating these diseases.

As with obesity, type 2 diabetes mellitus (T2DM) is another growing epidemic in the United States and around the world. Type 2 Diabetes Mellitus (referred to previously as non-insulin dependent diabetes or adult-onset diabetes) is a progressive metabolic disorder that is characterized by insulin resistance and dysfunction of pancreatic  $\beta$ -cells (Beck-Nielson et al., 1994). According to the World Health Organization, 382 million people worldwide are afflicted with diabetes and it is projected to be the 7<sup>th</sup> leading cause of death by the year 2030 (WHO, 2013). In the United States, the number of individuals suffering from diabetes have tripled between 1980 and 2008, increasing from 5.5 million to 18 million (CDC, 2009; Kalupahana et al., 2012). Cardiovascular disease (CVD) is the leading cause of death in individuals with type 2 diabetes mellitus (WHO, 2013; Kalupahana et al., 2012; Kahn et al., 2006).

Both obesity and T2DM are chronic and complex diseases whose causes can also be attributed to genetic and environmental factors (Wang, Z & Nakayama, T., 2010). Both diseases are linked to one another as 35% of obese individuals suffer from diabetes while about 90% of diabetics are overweight or obese. Further, individuals who suffer

from T2DM and are obese or overweight are two to four times more likely to exhibit cardiovascular disease (Kalupahana et al., 2012). Insulin resistance appears to be the main pathophysiological link between obesity and diabetes.

In the normal insulin response, during a fasting state, the liver produces glucose *via* gluconeogenesis and glycogenolysis as a means to maintain normal blood glucose concentrations. During the consumption of a meal, the intestines become a major site of glucose utilization, leading to increases in glucose availability to the pancreas. This in turn causes the pancreas to secrete insulin (Kalupahana et al., 2012). Insulin allows many tissues and organs in the body to transport and utilize glucose for metabolism. Insulin helps maintain normoglycemia, especially in liver, cardiac, adipose, and skeletal tissues. After insulin is secreted, it plays an important role in inhibiting gluconeogenesis and glucogenolysis from the liver. This mechanism in turn causes the liver to undergo glycogen synthesis. In skeletal and cardiac tissue, insulin increases glucose uptake and utilization. In adipose tissue, it inhibits lipolysis and increases the up-regulation of lipogenesis.

Insulin resistance is defined as an impaired biological response of insulin-sensitive tissues in the body to normal circulation of insulin in the blood, leading to uncontrollable blood sugar levels. When insulin resistance occurs, the normal tissue response to insulin is blunted (i.e. decreased insulin sensitivity) leading to relative hyperglycemia and an increase in plasma levels of free fatty acids (Olefsky and Glass, 2010). To compensate for the resulting hyperglycemia, the  $\beta$ -cells of the pancreas secrete more insulin. The increased secretion of insulin alleviates the relative hyperglycemia inducing systemic normoglycemia. However, over time, as insulin resistance in tissues



progresses, it can lead to pancreatic  $\beta$ -cell dysfunction and failure. With more circulating insulin, lipogenesis continues, leading to increases in fat tissue. Another consequence of insulin resistance is propagation to a pro-inflammatory state (Stumvoll et al., 2005). Early studies found that obesity and the release of cytokines from adipose tissue plays a major role in the development of insulin resistance, known as obesity-related insulin resistance.

### 2.1.3 Inflammation, Insulin Resistance and Obesity

Previously thought to be inert and passive depots for lipid storage, an energy-rich substrate, adipose tissue and adipocytes were discovered to be a major active endocrine organ responsible for secreting bioactive factors called adipokines (Trayhurn & Wood, 2004). In 1994, Zhang et al., cloned and sequenced the mouse *ob* gene and human homologue, leading to the discovery that this gene encoded a 4.5 kilobase (kb) adipose tissue messenger RNA that would become known as leptin, thereby identifying adipose tissue as a major endocrine organ. Adipokines are cell signaling proteins released from adipose tissue, consisting of classical cytokines and chemokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukins (eg., IL-6, IL-1 $\beta$ , etc.), vasoactive and coagulation factors, and regulators of lipoprotein metabolism, among others (Mohamed-Ali et al., 1998). It was through this adipokine secretion, that adipocytes and adipose tissue were discovered to play a central role in a complex and multidirectional network of autocrine, paracrine, and endocrine crosstalk between organs and tissues such as liver, skeletal muscle, pancreas, and heart (Romacho et al., 2014). Adipokines can act locally through autocrine and paracrine signaling within the adipose tissue as a way to regulate local metabolism. They can also have downstream effects through systemic circulation *via* endocrine signaling. Adipokines play a vital role in maintaining insulin sensitivity,

inflammation, regulation of food intake and body weight, and coagulation or vascular function (Trayhurn & Wood, 2004; Guzik et al., 2006).

The hypertrophy and hyperplasia of the visceral adipose tissue network due to insulin resistance and obesity leads to localized inflammatory responses. Inflammation is defined as a series of cellular and humoral reactions meant to defend the body from various insults such as infections or tissue damage. The ultimate goal of inflammation is to restore function and morphological integrity of the affected tissues (Lee & Lee 2014; Cildir et al., 2013). Inflammation is characterized by increases in local and systemic cytokine levels along with increased number of infiltrating immune cells. These immune cells are the first responders in such events. Neutrophils play an important role during acute phases of inflammation by initiating the immune response, while macrophages take the stage in more chronic conditions (Lee & Lee, 2014; Mraz & Haluzik, 2014).

Adipose tissue inflammation in obesity has been characterized by macrophage infiltration, which influences the changes in secretion of pro- and anti-inflammatory cytokines. Weisburg et al. (2003) used 24 mice with varying degrees of adiposity due to sex, diet, and obesity-related mutations such as agouti ( $A^y$ ) and obese ( $Lep^{ob}$ ) to assess gene expression in perigonadal, perirenal, mesenteric, and subcutaneous adipose tissues and the potential points of origin. In addition, human abdominal subcutaneous adipose tissue samples of lean, overweight, and obese subjects were collected. The researchers used microarrays, PCR, and immunohistochemistry and found over 1,304 transcripts that were correlated significantly with body mass, an indirect indicator of adiposity. A large portion of these transcripts encode proteins that are characteristic of macrophages. These results were further supported by histology data showing macrophage and immune cell

infiltration both in mice and human adipose tissues. These data confirmed that macrophage infiltration correlates with both BMI and adipocyte size and that adipose tissue macrophages are the primary source of pro-inflammatory adipokines.

Inflamed adipose tissues, especially visceral and subcutaneous, increase their pro-inflammatory adipokine secretion. These adipokines then leak into the circulation and potentially alter the microvasculature, various organs, and tissues. Free fatty acids are released from adipose tissue during this inflammatory response. These free fatty acids lead to intracellular accumulation of triglycerides, the development of more ectopic fat (fat that surrounds the organs), and lipid-derived metabolites, exacerbating the inflammatory response (Unger 2002). This imbalanced adipokine production, especially the increased secretion of pro-inflammatory adipokines, is observed in metabolic conditions such as obesity, insulin resistance, and T2DM (Romacho et al., 2014).

Tumor necrosis factor-alpha (TNF- $\alpha$ ), an adipokine involved in systemic inflammation and regulation of immune cells, has been identified as a pro-inflammatory adipokine important to the development of insulin resistance and obesity. Early research found that TNF- $\alpha$  was highly expressed in obese and diabetic rodents and that there was a correlation between the expression of TNF- $\alpha$  and peripheral insulin resistance (Hotamisligil & Spiegelman, 1993). Translating these findings from rodents to humans, a follow up study conducted by Hotamisligil et al. (1995), showed that in 19 obese premenopausal women there was a 2.5 fold increase in TNF- $\alpha$  mRNA expression in subcutaneous adipose tissue compared to their lean counterparts. They also reported a positive correlation established between the TNF- $\alpha$  mRNA expression levels in the adipose tissue and the level of hyperinsulinemia (an indirect measure of insulin

resistance). Additionally, obese females were studied before and after a weight loss intervention targeting a 17% reduction in BMI. These patients exhibited an improvement in insulin sensitivity and a reduction in TNF- $\alpha$  mRNA expression in adipose tissue. This study suggests that abnormal cytokine and adipokine production in adipose tissue plays a role in the pathogenesis of obesity-induced insulin resistance (Hotamisligil et al., 1995) and that it may be reversible.

#### 2.1.4 Myocardial Fat, Lipotoxicity, and Inflammation in the Heart

Although cardiometabolic syndrome is associated with peripheral fat deposition and inflammation, it is also associated with adipose accumulation in and around the heart as epicardial adipose tissue (EAT) and intra-myocardial fat. This fat deposition localized to the heart likely has a direct effect on cardiac function and may be a major contributing factor to the high prevalence of heart disease in patients with diabetes and obesity.

In a study by Iacobellis et al. (2003), the researchers were interested in estimating the volume of epicardial adipose tissue relative to anthropometric, metabolic, and cardiac parameters of cardiometabolic syndrome using echocardiography. Seventy-two subjects (36 males and 36 females) were recruited with BMIs ranging from 22 to 42 (median 34). The subjects were identified as exhibiting cardiometabolic syndrome based on how many risk factors were present. The researchers found that subjects with predominant visceral fat accumulation and at least two clinical and metabolic parameters of metabolic syndrome, showed higher epicardial adipose tissue compared to individuals with predominant peripheral fat distribution and no clinical parameters. In addition, data showed that EAT had a strong positive correlation with BMI, waist circumference, diastolic blood pressure, fasting plasma insulin, LDL cholesterol and plasma adiponectin.

It was further suggested that individuals exhibiting impaired insulin sensitivity, independent of BMI, showed the highest epicardial adipose tissue thickness (Iacobellis et al., 2003). In all, this study positioned cardiac fat as a new cardiovascular risk factor since it is associated with increased insulin resistance, visceral fat and, in general, with the metabolic syndrome.

However, the location of cardiac fat may play a pivotal role in its relationship with obesity, T2DM, cardiometabolic syndrome, and inflammation. EAT comprises approximately 30% of the intrathoracic fat while the majority is present as extra-pericardial fat (PAT); (Sironi et al., 2011). EAT is concentrated in the atrioventricular and interventricular grooves and along the branches of the coronary artery. EAT is close to the myocardium and has no real physical barrier to the coronary arteries. PAT is EAT that is located in these areas along with paracardial fat. Paracardial fat is situated on the external surface of the parietal pericardium within the mediastinum (Williams, 1995; Iacobellis et al., 2005; Wheeler et al., 2005; Sironi et al., 2004). This is important since the amount of fat that can be deposited into the EAT sac is extremely limited, thus, the majority of fat begins to accumulate in the PAT (Sironi et al., 2012). This has led some researchers to consider PAT as being more vital in cardiovascular risk management compared to EAT.

A study conducted by Sironi et al. (2012), using magnetic resonance imaging (MRI), tested whether EAT or extra-pericardial fat had similar clinical relevance using a large number of subjects, including patients with T2DM. One hundred thirteen individuals were recruited (94 men and 19 women) with a mean age of 52 and BMI ranging from 18 to 40 and 21 had T2DM. The authors looked at all four fat depots:

visceral, subcutaneous, epicardial, and extra-pericardial fat. The authors found that all fat depots increased in proportion to the degree of obesity. EAT and PAT were significantly correlated with BMI. Visceral fat and PAT were inversely correlated with insulin sensitivity but not with EAT. In addition, when relating fat depots to CVD risk and cardiometabolic risk, visceral, PAT, and intrathoracic fat were significantly associated with three or more cardiometabolic risk factors compared to subcutaneous or EAT. Further, in a stepwise multiple regression model adjusting for gender and BMI, blood pressure was only significantly associated with PAT only while insulin resistance and triglyceride concentrations were best associated both with PAT and visceral fat but not EAT (Sironi et al., 2012). The authors concluded that visceral fat was the best marker for altered CVD risk profile while increased PAT was associated with coronary heart disease risk.

PAT has also been shown to have a high capacity for non-esterified free fatty acid release and is proposed to be a preferred metabolite source for the myocardium (Marchington et al., 1989). With the close proximity to the myocardium and the changes, especially the inflammatory response, that occur with obesity and T2DM, the PAT and EAT exhibit the same inflammatory changes as observed in visceral adipose tissue. Indeed, Baker et al. investigated the expression profile of EAT from human EAT of patients suffering from coronary artery disease. Some of these patients (n=10) exhibited T2DM but were untreated while also gathering omental, abdominal subcutaneous, thigh adipose tissues from individuals with no T2DM and CAD. Using PCR and gene expression assays, Baker et al. (2006) established from their results that EAT exhibits metabolic risk markers and pro-inflammatory agents, such as TNF- $\alpha$ , IL-6, plasminogen

activator inhibitor-1 (PAI-1), were similar to omental adipose tissue (visceral) in that of non-CAD patients. The authors proposed that this pro-inflammatory profile of EAT and its close proximity to the myocardium may have direct influence on myocardial metabolism, cardiac dysfunction, lipid accumulation, and macrophage infiltration that leads to more inflammation (Baker et al., 2006).

The heart is not a major site for lipid storage but fatty acids in the form of triacylglycerols and phospholipids can be easily stored within the cardiomyocytes. This is evident in disease states as cardiometabolic syndrome where circulating fatty acids are high (Gastaldelli et al., 2012). The accumulation of these lipids deposits changes cardiac metabolism. Normally, the mammalian heart is able to obtain its energy from a balance of fatty acid and carbohydrate oxidation. Under the conditions of inflammation, lipid accumulation and insulin resistance, the heart begins to lose the ability to oxidize carbohydrates and then relies on fatty acids. This in turn causes the heart to rely on  $\beta$ -oxidation, then glucose oxidation is downregulated and this leads to the production of damaging intermediates such as reactive oxygen species (ROS), ceramides, and diacylglycerols that advance cardiac insulin resistance and promotes lipotoxicity (Kok et al., 2012; Lewin et al., 2008). Lipotoxicity is defined as the alteration to the intracellular signaling within metabolic tissues due to lipids and lipid utilization (Wende et al., 2012). Lipotoxic cardiomyopathy is a disorder of the myocardium induced by the damaging effects of free fatty acids and inflammation and is characterized by mitochondrial dysfunction, cardiomyocyte apoptosis, and contractile dysfunction. Further, lipotoxicity influences sarcoplasmic reticular  $\text{Ca}^{2+}$  stores to propagate contractile dysfunction while

increasing oxygen demand in the heart, exacerbating myocardial damage (Turer et al., 2012).

This lipotoxic heart has been witnessed in individuals diagnosed with T2DM. Indeed, an autopsy study conducted by Nakanishi & Kato (2014) investigating fatty hearts and changes in adipose triglyceride lipase of seventy-three hearts found that patients with diabetes mellitus were more likely to exhibit fatty hearts. These seventy-three hearts were randomly collected and tissues were sectioned from the anteroseptal area or from a different part of the left ventricle if any tissues scars were present. The tissue sections were subjected to immunohistochemical staining and tissue lipid content was extracted. The authors found that seven out of seventy-three hearts were fatty hearts. These seven hearts were identified as positive for lipid deposition and these individuals were clinically diagnosed with T2DM. Compared to the non-lipid deposited hearts, patients with these fatty hearts had significantly higher incidences of myocardial infarctions and heart failure. In addition, the fatty hearts from the diabetic cases exhibited significantly elevated triglyceride content in the myocardium compared to non-T2DM cases. Interestingly, the adipose triglyceride lipases were intact and no changes were noted in all cases. It can be postulated that these changes in the myocardium were not due to over-activation or changes to adipose triglyceride lipases, thus, it is possible that diabetes is associated with myocardial lipid accumulation and inflammation may play a role in this accumulation process.

The authors of this study found the hearts of patients from the diabetes cases experienced more severe histological damage than non-diabetes mellitus cases. Due to this higher incident, it appears that individuals with T2DM are more susceptible to



cardiomyopathies. Further, the authors believe that this histological damage may not be due to lipid deposition alone, but may be correlated more with the factors secreted from epicardial adipose tissue. Indeed, individuals with T2DM have increased EAT depots and the factors released from the EAT are associated with the development of cardiomyocytes dysfunction. EAT is known to be metabolically active and produces adipokines such as TNF- $\alpha$ , IL-1, adiponectin, IL-6, and free fatty acids (Mazurek et al., 2003). Thus, an interaction between lipid accumulation, epicardial adipose tissue, and secreted factors likely plays a role in the development of cardiomyopathies and cardiac dysfunction in diabetic cases, though the role of inflammation in early stages of the disease progression needs further investigation.

#### 2.1.5 Role of NF- $\kappa$ B

Inflammation is defined as a series of cellular and humoral reactions meant to defend the body from various insults such as infections or tissue damage. The ultimate goal of inflammation is to restore the function and morphological integrity of the affected tissues (Lee & Lee 2014; Cildir et al., 2013). Nuclear factor kappa B (NF- $\kappa$ B) represents one of the many key systems that mediate both neurohormonal and pro-inflammatory signals, especially in hypertrophy and fibrosis of the heart (Pechanova & Simko, 2010). NF- $\kappa$ B initiates the coordinated expression of inflammatory responses in the myocardium, which includes the increased expression of pro-inflammatory cytokines, chemokines, and cell adhesion molecules (Hernandez-Presa et al., 1997; Luft, 2001; Sekiguchi et al., 2004). This pathway is not only responsible for inducing a pro-inflammatory response but it also can exhibit protective responses.

In mammals, the NF- $\kappa$ B super family of transcriptional factors consist of at least five genes that encode the members RelA (p65), RelB, c-Rel, p50, and p52 (Hayden & Ghosh, 2008). All of these members are conserved throughout evolution and share a Rel homology domain (RHD), allowing DNA binding and dimerization between members. Transcription by NF- $\kappa$ B only works when two members form a heterodimer. In the heart the most predominant heterodimer is p50/p65 complex. With all transcription factors, nuclear localization is necessary for down-stream gene transcription. Indeed, if no signals are active, NF- $\kappa$ B exists as an inactive dimer in the cytoplasm of cells bound to its inhibitor (I $\kappa$ B) proteins (I $\kappa$ B $\alpha$ , - $\beta$ , - $\epsilon$ ) (Gordon et al., 2011).

To induce activation of NF- $\kappa$ B, phosphorylation-dependent degradation of I $\kappa$ B proteins *via* a proteasomal regulated pathway must occur. This phosphorylation is a key step in NF- $\kappa$ B activation and is mediated by I $\kappa$ B-kinase (IKK). Liberating NF- $\kappa$ B from its inhibitory complex causes NF- $\kappa$ B to become active and translocate to the nucleus, where it begins to bind to the promoters or enhancer regions of target genes containing a consensus motif 5'-GGGRNWYYCC-3' (R=any purine, N=any nucleotide, W=adenine or thymidine, Y=any pyrimidine) and commencing transcription (Gordon et al., 2011; Pechanova & Smiko, 2010). Indeed, NF- $\kappa$ B transcription and activation has been found in various models of cardiac hypertrophy, hypertension, myocardial ischemia, and cardiac remodeling (Pechanova & Smiko, 2010; Kupatt et al., 1999).

Li et al. (2004) was one of the first groups to show that NF- $\kappa$ B activation is required for the development of pathological cardiac hypertrophy *in vivo*. The researchers used male Sprague-Dawley rats and separated them into two groups: an aortic band group to elicit hypertrophy and a sham operated group (n=8 -10 in each group). The aortic band

group had binding of the ascending aorta with silk sutures while the sham group had small incisions made to mimic the surgery but no actual banding occurred. In a similar group, the researchers used transfection of an I $\kappa$ B $\alpha$  dominant negative mutant super repressor of NF- $\kappa$ B activation and PDTC, an antioxidant that has been shown to inhibit NF- $\kappa$ B activation, to determine if these interventions can attenuate hypertrophy in these animals. First, aortic banding led to hypertrophy in the hearts of the aortic band group compared to the age-related sham group. To verify, real time PCR was used to examine gene expression of two genes related to cardiac hypertrophy, ANP (atrial natriuretic peptide) and BNP (brain natriuretic peptide). ANP and BNP mRNA gene expression levels were increased in the aortic band group compared to the sham, showing that cardiac hypertrophy did occur. Using electrophoretic mobility shift assays (EMSA), they found increased NF- $\kappa$ B binding activity in hypertrophied hearts. Along with the increase in NF- $\kappa$ B binding activity, IKK $\beta$  activity was also increased. In addition, using their transfection of I $\kappa$ B $\alpha$  and PDTC, they found that they could attenuate the development of cardiac hypertrophy *in vivo*. This study demonstrated that NF- $\kappa$ B activation is required for the development of cardiac hypertrophy and that inhibiting NF- $\kappa$ B attenuates this detrimental hypertrophy. Interestingly, the researchers stated that ANP & BNP gene expression requires NF- $\kappa$ B activation and that the increased expression was representative of cardiac hypertrophy (Li et al., 2004; Liang & Gardner, 2001; Purcell et al., 2001). However, in conditions such as obesity and diabetes, inflammation may play a role in the activation of NF- $\kappa$ B in the heart.

Inflammation and microbial products can also activate NF- $\kappa$ B. Pro-inflammatory markers such as TNF- $\alpha$  or IL-1 $\beta$  can activate NF- $\kappa$ B through what is considered to be the

“canonical pathway”. In this pathway, the IKK dimer that consists of IKK $\alpha$  and IKK $\beta$  are activated by TNFR (TNF- $\alpha$ ) receptor 1, interleukin-1 receptors (IL-1R) and other pro-inflammatory cytokine receptors. The binding at these locations causes the recruitment of signal adaptors like myeloid differentiation primary response gene 88 (MyD88) that result in the activation of tumor necrosis factor receptor-associated factor (TRAF6). TRAF6 allows for auto-ubiquitination to occur which, in turn, allows for the recruitment of other intermediates that phosphorylate IKK. Indeed, TRAF6 along with receptor interacting protein (RIP) help create a scaffold for binding of pro-inflammatory kinases TAK1 that phosphorylate IKK (Van der Heiden et al., 2010). Majority of the literature that focuses on the non-canonical pathway has been on lymphocytes and information on other cell types is limited. Thus we will mainly focus on the canonical pathway.

In a recent study by Thomas et al. (2014), the 3M mouse was used to study the protective effects that NF- $\kappa$ B suppression would have in diabetes-induced cardiac dysfunction. The 3M animal model is a cardiac-specific transgenic mouse model that overexpresses I $\kappa$ B $\alpha$  with a triple mutation that prevents its phosphorylation, thus suppressing the canonical NF- $\kappa$ B pathway. The authors used C57B1/6J wild type mice as a control group and another group of these same mice as diabetic models. 3M mice and some of the wild-type C57B1/6J mice were given streptozotocin (STZ) to induce diabetes while animals in the control groups were given a placebo vehicle. Animals underwent echocardiography to determine cardiac function and after 24 weeks were sacrificed and heart tissues extracted. Western blots and real-time PCR were conducted using RNA extracted from heart tissues. The researchers found that in the hearts of the diabetic wild-type mice, through DHE staining, an increase in oxidative stress compared to the 3M

diabetic and control group was witnessed. The 3M control mice initially exhibited oxidative stress but greater levels were observed in the wild-type diabetic mice after 24 weeks. Molecular markers such as  $\beta$ -myosin heavy chain ( $\beta$ -MHC), ANP, and BNP were increased in wild-type diabetic mice and not in the 3M diabetic mice. As stated previously, ANP and BNP need NF- $\kappa$ B activation to increase their expression. Lastly, compared to wild-type diabetic mice, the 3M diabetic mice lacked an increase in the p65 subunit of NF- $\kappa$ B in the nuclei, which correlated with decreased NF- $\kappa$ B activity in these mice.

The 3M mouse mutation is cardiac-specific. Thomas et al. (2014) observed that lack of NF- $\kappa$ B activation protects animals from developing diabetic cardiomyopathy. Canonical NF- $\kappa$ B activation in the diabetic myocardium is required for cardiac remodeling. As evidenced by their results, genetic silencing of NF- $\kappa$ B, specifically in the heart, helps prevent diabetic cardiomyopathies. In addition, the levels in plasma and expression in tissues of pro-inflammatory cytokines have been shown to change with time in T1DM and T2DM but the authors observed no increase in expression of inflammatory cytokines such as IL-1 $\beta$ , IL-6, or TNF- $\alpha$ . This is supported by studies in streptozotocin treated rats.

Other stimuli can affect the canonical NF- $\kappa$ B pathway. Hypoxia, ROS, and mechanical stretch are known to be effective activators of the canonical pathway. Indeed, in conditions like diabetes and obesity, where states of inflammation are chronic, NF- $\kappa$ B may be overly stimulated (Arkan et al., 2005; Bierhaus et al., 2001). This overt stimulation promotes more NF- $\kappa$ B transcription of inflammatory markers. Some of these pro-inflammatory markers such as matrix metalloproteinases, TNF- $\alpha$ , IL-1 $\beta$ , and MCP-1

that are active during cardiomyopathy are regulated by NF- $\kappa$ B (Van der Heiden et al., 2010).

In conclusion, the NF- $\kappa$ B family controls multiple processes, including immunity, inflammation, cell survival, differentiation, and proliferation. Pro-inflammatory cytokines, reactive oxygen species, and microbial products can induce NF- $\kappa$ B signaling, resulting in transcriptional regulation of pro-inflammatory genes such as TNF- $\alpha$ , IL-8, IL-1 $\beta$ , MCP-1, and many more, which promote the inflammatory process. Indeed, diabetes and obesity are both conditions with chronic subclinical inflammation that affects NF- $\kappa$ B. Individuals suffering from diabetes and obesity are more likely to exhibit over-activation of NF- $\kappa$ B. NF- $\kappa$ B has been shown to play a role in the development of cardiac hypertrophy and cardiac dysfunction in these individuals. However, there currently exists little data on the effects of NF- $\kappa$ B in the earlier stages of disease (overweight and insulin resistance) in the heart and whether interventions such as exercise or caloric restriction can alter or prevent pathology.

#### 2.1.6 Detection of Molecular Pathology in the Heart

When pathophysiological stresses are introduced in the heart, certain genes are activated as a way to compensate and remodeling of the heart occurs. A well-characterized adaptation of the heart to stress is the fetal gene program or the activation of genes that are expressed during early fetal circulation but become silent as the mammalian heart ages. The expression of genes in this group play an important role in cardiovascular diseases such as cardiac hypertrophy (Kuwahara et al., 2012; Bernado et al., 2010) and may be useful in characterizing cardiac dysfunction due to obesity and diabetes or insulin resistance. The genes involved in this program are: atrial and brain

natriuretic peptide (ANP and BNP, respectively), fetal isoforms of contractile proteins (skeletal  $\alpha$ -actin and  $\alpha$ -,  $\beta$ -myosin heavy chain), fetal-type cardiac ion channels (SERCA2a), and smooth muscle genes (smooth muscle  $\alpha$ -actin); (Kuwahara et al., 2012; Bernado et al., 2010).

ANP & BNP are peptide hormones that are encoded by the *Nppa* and *Nppb* genes, respectively. These hormones are secreted in the adult heart in response to cardiac wall stretch and strain (Dietz, 2005). ANP is released by atrial cardiomyocytes while BNP is released from ventricular cardiomyocytes. Both hormones respond to high blood pressure (cardiac wall stretch and strain) and act to reduce water, sodium, and adipose loads to induce a decrease in blood pressure. However, both ANP and BNP have been discovered to antagonize cardiac hypertrophy, fibrosis, and to stimulate lipolysis (breakdown of lipids into fatty acids/triglycerides); (Cox et al., 2014; Rosenkranz et al., 2003; Franco et al., 2004; Wang et al., 2003).

The sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase 2 (Serca2a) is another important member for the fetal gene program. Serca2a is responsible for the re-uptake of calcium into the sarcoplasmic reticulum following the contraction of the sarcomere, allowing muscular relaxation. Serca2a expression levels are maintained through adulthood but a decrease is observed in the diabetic heart and may play a role in the diastolic dysfunction that leads to diabetic cardiomyopathy (Cox et al., 2014).

The myofilament proteins  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) and  $\beta$ -myosin heavy chain ( $\beta$ -MHC) exhibit different properties and their ratio is changed during cardiac hypertrophy.  $\alpha$ -MHC, which has the highest ATPase activity and contractile velocity, is highly-expressed during adulthood while  $\beta$ -MHC, which has the lowest ATPase activity

and lowest contractile velocity, is highly-expressed during fetal development. A ratio between  $\alpha$ -MHC and  $\beta$ -MHC exists which is maintained in adulthood. However, during cardiac hypertrophy, the ratio decreases. It is important to note that humans exhibit more  $\beta$ -MHC than  $\alpha$ -MHC. Rodents exhibit the  $\alpha$ -MHC/ $\beta$ -MHC ratio as described previously (Gustafon et al., 1987; Lyons et al., 1990; Reiser et al., 2001; Miyata et al., 2000). In addition to the myosin cytoskeletal proteins, skeletal  $\alpha$ -actin changes in the hypertrophied heart. Skeletal  $\alpha$ -actin is highly-expressed in the fetal heart and is replaced by cardiac  $\alpha$ -actin in adulthood. During cardiac hypertrophy and cardiac dysfunction, there is a re-expression of and conversion to skeletal  $\alpha$ -actin (Driesen et al., 2009; Ren et al., 2012).

Some evidence suggests that the fetal gene program is activated in diabetes. In a study conducted by Depre et al. (2000), Wistar rats were injected with  $\beta$ -cell toxin streptozotocin (STZ) to induce T2DM while another group of Wistar rats was injected with vehicle only to serve as a control group. The animals were then sacrificed and RNA was extracted from the animals' heart tissue. The authors used polymerase chain reaction (PCR), to assess the gene expression of a myriad of contractile proteins and ion pumps. The researchers found that the gene expression of many of the contractile proteins and ion pumps (e.g. SERCA2a,  $\beta$ -/ $\alpha$  MHC) were decreased in the diabetic hearts and exhibited the same gene expression as what is observed in the hypertrophied heart.

In addition to the fetal gene program, cardiac pathology related to diabetes and obesity may be evident as fibrosis. Yagi et al. (1997), characterized the changes in the myocardium of the Otsuka Long-Evans Tokushima Fatty rat (OLETF), a genetic animal model of mild obesity and non-insulin dependent T2DM, evaluating fetal gene expression and genes involved in cardiac fibrosis as markers for changes in cardiac performance. In



this study, the Long-Evans Tokushima-Otsuka (LETO) rats were used as a genetic control for the OLETF rats. LETO rats are lean rats and are from the same colony that the OLETF come from, thus having the same genetic background. The hearts of animals were characterized at week 14 (a pre-diabetic state), week 30 (non-insulin dependent diabetes mellitus phase), and week 54 (insulin dependent diabetes mellitus phase) by left ventricle mRNA expression. The researchers found that although there were no differences between fetal gene expression program markers at 14 weeks, at 30 weeks, OLETF left ventricle expression of  $\beta$ -MHC was greater than LETO. mRNA levels for contractile proteins  $\alpha$ - $\beta$ -MHC and ANP, and were significantly different between groups at 54 weeks. Specifically,  $\alpha$ -MHC mRNA levels were lower and ANP mRNA levels were 1.3-fold higher in the OLETF rat compared to the LETO at 54 weeks. This data indicates that  $\beta$ -MHC expression differences appear early in the progression of the OLETF disease phenotype whereas other fetal gene program indicators of dysfunction do not appear until later in this model.

There is evidence that cardiac fibrosis occurs at early time points in the OLETF model and that it may be related to inflammation. Yagi et al. (1997), reported that at 14 weeks of age, the left ventricular gene expression of collagen types I, III and IV, along with laminin, were significantly enhanced in OLETF compared with LETO rats of the same age. Collagen types III and IV and laminin mRNA levels remained elevated in OLETF rats at week 30 compared to LETO. Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) is a pleiotropic cytokine that can regulate fibrogenesis and is released from pro-inflammatory cells (e.g. macrophages). The authors showed that mRNA levels of 14-, 30-, and 54-week-old OLETF rats were 1.5-, 1.6-, and 1.3-fold greater, respectively, than in the

LETO rats examined at those same time points. Thus, markers of fibrosis are evident at early stages in the OLETF model when animals are overweight and insulin resistant.

Importantly, although OLETF rats develop hypertension, authors reported no alterations in cardiac TGF- $\beta$  or  $\beta$ -MHC in genetically hypertensive rats (SHR) suggesting different regulation of cardiac pathology between hypertension and diabetes/obesity (Yagi et al., 1997). The involvement of TGF- $\beta$  in cardiac fibrosis in this pre-diabetic stage is still unclear. Inflammation may play a role in linking TGF- $\beta$  and cardiac fibrosis in the OLETF rat. Exploring cardiac fibrosis gene expression in young OLETF rats and its interaction with inflammation may provide further insight into the genetic and molecular pathways that may exacerbate the risk of CVD in obese and diabetic individuals.

## 2.2 Role of Exercise and Caloric Restriction

### 2.2.1 Exercise

Exercise is well-known to be a powerful treatment for obesity-related metabolic complications, including insulin resistance, hypertension, impaired glucose tolerance or diabetes, hyperinsulinemia, and dyslipidemia that are characterized by elevated adipose accumulation (Kim et al., 2008; Hu et al., 2001; Tuomilehto et al., 2001). A study by Hu et al. (2001), that followed approximately 85,000 female nurses for 16 years, found that adopting a healthy diet and physical activity reduced obesity, risks for cardiovascular disease, and chances of developing cancer and other chronic diseases. In addition, physical activity improves glucose homeostasis and insulin sensitivity, coronary blood flow and cardiac function, enhances endothelial function, and reduces blood pressure

(Murphy et al., 2009). These improvements in cardiac function may be related to a reduction in overall fat mass and pro-inflammatory state, systemically and at the level of the myocardium. However, understanding the independent effect of exercise from weight or fat loss is difficult given that many studies do not control fat loss.

Trachta et al. (2014) investigated the effects of regular physical activity on improving systemic sub-clinical inflammation and other obesity-related pathologies through the modulation of inflammatory profile of adipose tissue. Fifteen non-diabetic obese females with arterial hypertension were recruited for the study and were placed in a three-month aerobic exercise program consisting of 30 minutes of aerobic exercise for three times a week. Fifteen healthy lean women that were free of any cardiovascular and metabolic parameters were selected to serve as a control group. Subcutaneous adipose tissue samples were collected from the abdominal region and RNA was extracted and used in PCR to examine pro-inflammatory gene expression. After their intervention, Trachta et al. (2014) found that regular aerobic exercise significantly decreased body weight and body fat, systemic sub-clinical inflammation, and insulin resistance. Though the exercise intervention did not have much effect on the endocrine function, there was an improvement in the pro-inflammatory gene expression such as chemokine C-C motifs 2, 3, 4, IL-1 $\beta$ , IL-6, for example, in subcutaneous adipose tissue. The obese non-diabetic women exhibited a decrease in a myriad of pro-inflammatory cytokines though not statistically significant. In addition, the authors noted that though the subcutaneous adipose tissue is the largest of the fat depots, it is not a major player in systemic inflammation compared to visceral adipose tissue. Since the non-diabetic obese participants had a reduction in waist circumference, it can be postulated that the reduction

in visceral adipose tissue due to aerobic exercise lead to improvements in systemic inflammation.

Longitudinal studies have shown that daily physical activity helps combat inflammation by reducing adiposity and thus diminishing the macrophage infiltration. In studies that looked at 12-week interventions of sedentary males with metabolic syndrome, they found that moderate-intensity exercise reduced inflammatory markers such as MCP-1 and IL-8. Similar results in other studies investigating individuals with heart failure found a reduction in pro-inflammatory markers and an increase in anti-inflammatory proteins (Goldhammer et al., 2005). Markers such as IL-1, MCP-1, and C-reactive protein (CRP), which is released from the liver, were all significantly reduced compared to levels in individuals who did not exercise. Importantly, these benefits were evident even in the absence of significant weight loss (Goldhammer et al., 2005). In addition, exercise is able to attenuate insulin resistance and improve insulin sensitivity in many different tissues, including the heart. Thus, exercise can be an effective treatment for obesity and T2DM, especially if they both exist concomitantly (Dalzell et al., 2014).

Kim et al. (2008) conducted a study to determine whether three-months of aerobic exercise training without caloric restriction (by having the participants maintain a food log and normal eating habits) produced changes in EAT and its relation to abdominal visceral adipose tissue changes. Twenty-four obese middle age Japanese males were recruited. The participants were supervised by an exercise physiologist for compliance and aerobic exercise intensity was based on the individual's maximal heart rate attained from an initial aerobic fitness test. The researchers found that EAT thickness (measured by echocardiography) was reduced in obese men after a three month aerobic exercise

intervention, compared to pre-intervention. Interestingly, the percent change in EAT was twice as high compared to changes in waist, BMI, and visceral adipose tissue. In addition, the reduction in EAT was accompanied by a reduction in the visceral adipose tissues. Some participants in this study exhibited the cardiometabolic syndrome according to the Japan Society for the Study of Obesity criteria and exhibited improvement in insulin sensitivity and fat reduction both in visceral and epicardial adipose tissue. This study was the first study to show that exercise has a potent effect in reducing EAT thickness and improving biological markers associated with obesity and cardiometabolic syndrome.

Kim et al. (2008) published one of the first studies investigating the effects of exercise on the heart, especially on EAT, and it did not include caloric restriction as part of their intervention. Many studies use a combination of exercise and calorie restriction in their interventions and the outcomes that are presented cannot be attributed either to exercise or caloric restriction alone. Many agree that weight loss improves many of the outcomes in obesity and diabetes. The mechanisms behind the direct effects of exercise on the heart and other tissues are still heavily investigated areas. In all, exercise may prove to be an effective intervention for improving inflammation and cardiac dysfunction in individuals with cardiometabolic syndrome.

### 2.2.2 Caloric Restriction

Caloric restriction (CR) is classified as a state in which the energy intake of an individual or animal is minimized to low-normal levels while maintaining a balance of macronutrients like protein and carbohydrates. CR usually consists of about 30-50% reduction in energy intake that is required to maintain normal body weight and adiposity, leading to a leaner phenotype. In animal studies, it is usually administered early in the

animal's life so that there is no weight loss and the changes are attributed to the condition of CR and not weight loss. In humans, it is administered in adulthood, where the results can be contributed to weight loss or the caloric restriction itself. Therefore, in humans, determination of the independent effects of CR versus weight loss is difficult (Weiss & Fontana, 2011).

With respect to the heart, CR may prove to be an effective intervention for individuals who are obese and/or diabetic. Hammer et al. (2008) studied the effects of prolonged caloric restriction in 12 obese patients with T2DM on myocardial triglyceride content and cardiac function. The intervention lasted for 16 weeks and the participants had their myocardial triglyceride content and left ventricle function measured through photon magnetic resonance spectroscopy. The authors found that the caloric restriction had reduced the BMI of the patients and improved many of their metabolic parameters. Indeed, the participants had improved glycemic control (decreases in fasting plasma glucose levels), and hemoglobin A1c levels, plasma non-esterified fatty acids, and liver enzymes such as CRP (inflammatory biomarker), were all reduced after prolonged caloric restriction. In addition, myocardial triglyceride content and left ventricular mass decreased while improving cardiac output and both systolic and diastolic blood pressure.

The effects of caloric restriction on EAT were further supported by the work of Iacobellis et al. (2008). In this study, 20 severely obese subjects ( $BMI \geq 40 \text{ kg/m}^2$ ) underwent a very low calorie (900 calories) diet weight loss program for 12 weeks. Anthropometric measures were taken and epicardial adipose tissue was measured using echocardiograms. Iacobellis et al. found that severely obese subjects had significantly higher epicardial fat thickness compared to lean subjects. After the intervention, the

subjects decreased their body weight and BMI by approximately 20%, had a 23% reduction in their waist, and a 32% reduction in epicardial fat thickness. In addition, left ventricular mass (LVM) was significantly reduced and improvements in diastolic function were witnessed. These changes all correlated strongly with the decrease in epicardial fat thickness. Thus caloric restriction, even for a short period, can elicit powerful changes and improvements in the myocardium, especially in severely obese individuals.

Though there may be some differences in administration of CR, evidence shows that in animals and humans, CR is able to increase longevity and reduce death due to chronic diseases such as cancer, T2DM, obesity, and cardiovascular disease. Indeed, research has shown that CR is able to attenuate the production of ROS and oxidative damage by activation of SIRT1 and transcription factor NF-E2-related factor (Nrf2). In addition, CR is able to attenuate age-related vascular inflammation by improving the NF- $\kappa$ B inhibition of pro-inflammatory gene transcription, thereby causing NF- $\kappa$ B to become active in anti-inflammatory gene transcription and reducing a pro-inflammatory state. Lastly, like exercise, CR is able to reduce adiposity and inflammatory markers, especially in the cardiovascular system, leading to healthy cardiovascular function and can even decrease the adverse effects of cardiomyopathy (Weiss & Fontana, 2011; Kemi et al., 2000).

### 2.2.3 Comparative Effectiveness of Exercise and Caloric Restriction

Comparative effectiveness studies are novel ways to inform health-care decisions by providing evidence of the effectiveness, benefits, and/or detriments of different treatment options. Few studies have looked at comparative effectiveness of exercise or

caloric restriction on pathologies of the heart, especially in early overweight and insulin resistance. Both exercise and caloric restriction are beneficial for improving cardiovascular health in individuals who suffer from cardiometabolic syndrome, however, no studies have been conducted to determine which is more beneficial.

A report by Crissey et al. (2014) is one of the few that has compared exercise to caloric restriction for changes related to cardiometabolic syndrome. The researchers used OLETF rats to study the changes in the various adipose tissue depots either through exercise or caloric restriction in relation to vascular insulin resistance and inflammation. The study was conducted using 30 male OLETF rats that were purchased and randomized into 3 groups: sedentary (SED), caloric restriction (CR), and exercise (wheel running; WR). The animals in the caloric restriction were fed 70% *ad libitum*, exhibiting a modest 30% caloric reduction. The OLETF rats were placed in each intervention for 8 weeks and then sacrificed at 20 weeks, at which time the animals present with an early insulin resistant and overweight phenotype. Perivascular adipose tissue from the thoracic aorta, retroperitoneal white adipose tissue, inguinal subcutaneous adipose tissue and brown interscapular adipose tissue were excised from the animals. A portion of the perivascular adipose tissue was used for *in vitro* assessment of cytokine secretion. RNA was extracted from the various adipose tissues and aortic samples. Real-Time PCR was used to determine gene expression.

The researchers found that the exercise (wheel running; WR) and caloric restriction (CR) groups had improved body composition, exhibiting lower body fat percentages and less visceral adipose tissue mass compared to the sedentary group. Both exercise and caloric restriction groups had improved fasting total cholesterol, LDL



cholesterol, leptin, glucose, non-esterified fatty acids (NEFA), and triglycerides (Table 4). In addition, the exercise group exhibited improvements in HOMA-IR, lower plasma insulin, and lower circulating MCP-1. In the retroperitoneal adipose tissue, both WR and CR groups had lower expression of leptin, MCP-1, TNF- $\alpha$ , IL-6, PAI-1, and intercellular adhesion molecule-1 (ICAM-1) compared to SED. Expression of these inflammatory markers were higher in brown adipose tissue, in the WR group only. In the periaortic adipose tissue, both CR and WR exhibited lower expression of leptin, MCP-1, TNF- $\alpha$ , with the WR group exhibiting a lower PAI-1 expression compared with the SED group. In the CR group, the periaortic fat exhibited lower IL-6, E-selectin, and ICAM expression. Subcutaneous adipose tissue had lower leptin and MCP-1 expression. Insulin-stimulated aortic relaxation was significantly greater in WR rats relative to SED and CR rats (Crissey et al., 2014).

In addition, Crissey et al. (2014) investigated gene expression indicative of immune cell infiltration into peripheral adipose tissue depots, periaortic adipose, and aorta to determine if WR or CR was able to attenuate their expression. Markers for immune cell infiltration, such as cluster of differentiation 4 (CD4), cluster of differentiation 8 (CD8), and EGF-like module containing mucin-like hormone receptor-like 1 (F4/80), revealed that WR and CR reduced expression of these three markers in the aorta and retroperitoneal adipose tissue compared with the SED group. Interestingly, WR and CR both reduced leptin, IL-6 and MCP-1 secretion from periaortic adipose tissue indicating that immune cells and cytokine release from adipose may contribute to tissue inflammation in OLETF rats. CD4 and CD8 are markers commonly expressed in macrophages, monocytes, and T-cells while F4/80 is another macrophage glycoprotein

receptor that is expressed in high levels in various macrophages and more importantly can be expressed in macrophages of connective tissue such as the heart (Heidt et al., 2014; Weisberg et al., 2003). EX and CR were able to attenuate their expression in adipose tissue and may be able to induce similar changes in the immune cell infiltration within myocardial tissue.

Overall, these results show the improvements on adipose tissue and inflammation that can occur either with exercise or caloric restriction. Both can reduce the inflammatory markers that are detrimental in obesity and insulin resistance that lead to cardiovascular morbidity and mortality, especially since adipose tissue inflammation plays a pivotal role in all of these conditions. Indeed, the changes in the periaortic adipose tissue has huge implications for cardiovascular and myocardium health due to its close proximity. In addition, the researchers' data on the effects of exercise and not caloric restriction on insulin-stimulated vasodilation of the aorta shows that exercise is able to induce insulin-sensitizing effects on vascular tissue while caloric restriction may not.

Exercise and caloric restriction both exhibit cardiovascular benefits and decrease the risks associated with CVD, inflammation, and other chronic conditions. However, it is unknown which intervention can improve cardiac dysfunction and inflammation associated with the cardiometabolic syndrome. Investigating the comparative effectiveness of each of these interventions can elucidate more effective interventions during early pathology or provide new knowledge on the role of exercise or caloric restriction in heart health. Our study is novel as there are no studies to date that have

compared the effectiveness of each of these two interventions in an early overweight and insulin-resistant animal model, relative to changes in the myocardium.

#### 2.2.4 Specific Aims and Hypotheses

Cardiometabolic syndrome is becoming a worldwide epidemic, affecting about 25% of the world population. Two of the underlying causes of cardiometabolic syndrome are obesity and insulin resistance. Cardiometabolic disease, along with obesity and insulin resistance, increases an individual's risk of developing cardiovascular disease and dying from a cardiovascular event. Preventive care and lifestyle modifications such as diet and exercise are known to alleviate the rate of insulin resistance and obesity, thus diminishing the number of individuals suffering from cardiometabolic syndrome. Many of the adverse effects of cardiometabolic syndrome in the heart are due to the chronic inflammatory state of the disease. There exists a gap in the literature as to whether there are alterations to the myocardium in early stages of cardiometabolic syndrome and whether lifestyle interventions, in the form of exercise or caloric restriction, delay/prevent the development of cardiometabolic syndrome and CVD.

**AIM 1:** To determine the effect of exercise and caloric restriction to change markers of cardiac dysfunction in a model of overweight and insulin resistance.

Methods: OLETF rats will be separated into three groups (SED, EX, & CR). After 20 weeks in each condition, heart tissue will be isolated and RNA will be extracted.

Expression of genes related to the fetal gene program (i.e.  $\alpha$ -MHC,  $\beta$ -MHC) and fibrosis (i.e. TGF $\beta$ <sub>1</sub>, Col-1, and COL-3) will be examined in each group.

Hypothesis 1: Animals in the CR and EX group will exhibit lower expression of cardiac dysfunction genes compared with the SED group signifying CR and EX as effective countermeasures for pathological alterations in the heart in young OLETF rats.

**AIM 2**: To determine the effect of exercise and caloric restriction to alter NF- $\kappa$ B activity in the heart in an early model of overweight and insulin resistance.

Methods: Nuclear and cytosolic fractions of OLETF heart tissue will be separated. An NF- $\kappa$ B DNA-binding activity assay will be used to determine NF- $\kappa$ B activation in SED, CR and EX groups.

Hypothesis 2: Sedentary animals will exhibit a greater activation of NF- $\kappa$ B in the heart compared to the CR and EX groups.

**AIM 3**: To determine the effect of exercise and caloric restriction on gene expression of inflammatory markers related to NF- $\kappa$ B activity and monocyte/macrophage infiltration in the heart in an animal model of early insulin resistance and obesity.

Methods: Using RNA extracted in Aim 1, Polymerase Chain Reactions (PCR) will be conducted using primers for inflammatory genes such as TNF- $\alpha$ , IL-1 $\beta$ , MCP-1, in addition to monocyte and macrophage makers such as CD4, CD68, and F4/80, and determine their relative gene expression in the three conditions.

Hypothesis 3: Exercise and Caloric restriction should exhibit lower expression of inflammatory genes that are related to NF- $\kappa$ B signaling and immune cell infiltration compared with the sedentary group.

## CHAPTER 3

### METHODS

#### 3.1 OLETF Animal Model and Characteristics

Otsuka Long-Evans Tokushima Fatty (OLETF) rats are spontaneous diabetic rats exhibiting polyuria (excessive urine production), polydipsia (excessive thirst), and mild obesity. This strain of rats was first discovered in 1984 from an outbred colony of Long-Evans rats that was purchased from Charles River, Canada in 1982. The Tokushima Research Institute (Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) has maintained this particular diabetic strain of Long-Evans rat through selective breeding and named it Otsuka Long-Evans Tokushima Fatty rats. Long-Evans Tokushima Rats (LETO) are the lean, non-diabetic counterparts to the OLEFT rats obtained through different original mating. However, both rats come from the same strain of Long-Evans rats. The LETO rat is commonly used as a genetic control group to the OLETF (Kawano et al., 1994; ChengD & Wang, M-W., 2005).

Otsuka Long-Evans Tokushima Fatty (OLETF) rats exhibit the following characteristics: 1) late onset hyperglycemia after 18 weeks of age, 2) chronic course of the disease, 3) mild obesity, 4) clinical onset of diabetes that is exhibited mostly in males, 5) changes to pancreas and kidneys. Beginning at 5 weeks, the OLETF rat begins to rapidly gain weight, compared to their LETO counterparts. OLETF rats begin showing high blood glucose concentrations at 18 weeks and impaired glucose intolerance starting at 24 weeks of age. Plasma triglycerides concentrations start increasing at 8 weeks of age but cholesterol concentrations slightly increase after 40 weeks. Along with these

changes, OLETF rats exhibit increased blood pressure at 18 weeks of age compared to LETO rats (Panchal et al., 2011).

The clinical and pathological features of the OLETF rat closely resembles those exhibited in humans with Type 2 Diabetes Mellitus (ChengD & Wang, M-W., 2005). For our study, the OLETF rats were maintained until 20 weeks of age. At this time, the OLETF rat exhibits an overweight phenotype and early insulin resistance thus representing a pre-diabetic state. Since the animal model exhibits the clinical and chronic pathology of type 2 diabetes with mild obesity, this early time point is crucial in determining if there are early changes in the heart. Other animal models exhibit severe obesity with diabetes that may not be representative of what is exhibited in humans who may just be mildly obese and with insulin resistance.

### 3.1.2 Animal Set Up

All animal protocols were approved by the University of Missouri Institutional Animal Care and Use committee. For this experiment, male Otsuka Long-Evans Tokushima Fatty Rats (OLETF) were used and obtained at four weeks of age (Japan SLC Inc. 3371-8, Kotoh-Cho, Hamamatsu, Shizuoka, Japan) and were individually caged and maintained in temperature controlled (21°C) with light cycles from 6:00 to 18:00 hour and dark cycles from 18:00 to 6:00 hour. At 12 weeks, the rats were randomized into three groups: (I) sedentary (SED; n=10), (II) voluntary wheel running (WR; n=10) and (III) sedentary + diet restriction (DR, fed 70% of *ad libitum*-feed SED animals; n=10). Animals in the wheel running (WR) group were housed with running wheels connected to Sigma Sport BC 800 bicycle computer (Cherry Creek Cyclery, Fosters Falls, VA, USA) to determine daily running distance. All animals were provided with standard rat

chow (Formulab 5008, Purina Mills, St. Louis, MO) that consisted of approximately 26% protein, 18% fat and 56% carbohydrates. Sedentary and WR groups had *ad libitum* access to food. Body weights and food intakes were recorded on a weekly basis. At 20 weeks of age, the rats were sacrificed and anesthetized using intraperitoneal injection of pentobarbital sodium (50 mg/kg). Tissues were harvested after the animals were euthanized by exsanguination in full compliance to American Veterinary Medical Association Guidelines on Euthanasia. Hearts were frozen and stored at -80°C until analysis. The wheels of the WR group were locked and food was removed from the cages of all groups approximately 14 hours before the rats were sacrificed (Crissey et al., 2014).

### 3.1.3 Gene Expression/RNA Extraction

RNA was extracted from frozen cardiac tissue and homogenized using TRIzol reagent. The isolated RNA was then quantified using the Nanodrop 1000 spectrophotometer and used to make 0.2 ug of cDNA using 5X iScript (BioRad). The primers used for the gene expression were designed and purchased from IDT. These primers were optimized using EvaSsofast (BioRad) to an efficiency of >90%. All gene expression analyses were ran in triplicate on the CFX96 RT-PCR machine with *Rpl10a* serving as control gene. *18S* was originally tested as a house-keeping gene but was found to be expressed differently between our groups in the heart (figure 1A). We have found that *Rpl10a* is a suitable house-keeping/reference gene when performing RT-PCR with rat myocardium as it remains stable under the experimental conditions for these animals. mRNA expression values were analyzed via the  $\Delta$ CT method whereby  $\Delta$ CT = *Rpl10a* CT - gene of interest CT (Padilla et al., 2013). mRNA levels are normalized to the SED group of rats, which was always set at 1 (Chrissy et al., 2014; Brattlelid et al., 2010).

The genes that will be analyzed to determine cardiac dysfunction include: *ANP*, *BNP*,  $\beta$ -*MHC*, and  $\alpha$ -*MHC* in OLETF rats. *ANP*, *BNP*,  $\beta$ -*MHC* and  $\alpha$ -*MHC* are members of the fetal gene program and their re-emergence in adult cardiac gene expression represents a pathological sign of cardiac dysfunction. *CD4*, a general T-cell marker is used to identify potential immune cell infiltration and genetic expression in the heart. *CD68*, a general marker of macrophages, was also used for this similar task. *F4/80* is a marker for M1 polarized macrophages that are involved in secreting pro-inflammatory biomarkers. *COL1*, *COL3*, and *TGF $\beta$ <sub>1</sub>* are genes involved in the development of cardiac fibrosis. Lastly, *MCP-1* *IL-1 $\beta$*  and *TNF- $\alpha$*  are genes involved in pro-inflammation and are known to have an effect in NF- $\kappa$ B activity. *Rpl10a* (F:5'-GAGGCGCATCTGATCCTAATC-3', R:5'-ATTCTGCCCCGCTGTCTATC-3') was used as the reference gene for RT-PCR.

The full name and primers for the genes used are listed in table 1. Tables 2-4 include the genes that were studied, including primer sequences, as markers for inflammation, inflammatory cell infiltration, and fibrosis.

**Table 1. Names of Genes involved in Fetal Gene Programming**

Gene Name	Full Name	F (Forward) & R (Reverse) Primer sequence (5'-3' direction)
ANP	Atrial Natriuretic Peptide	F: TGCCGGTAGAAGATGAGGTC R: AGCCCTCAGTTTGCTTTTCA
BNP	Beta (Brain) Natriuretic Peptide	F: CCCTTGTTTTGCTCTCAGAC R:AGGGGAGATGCTCAGAGTGA
$\alpha$ -MHC	Alpha Myosin Heavy Chain	F: GCACTGAGGGGATAAGGTGA R: TTAGCCCAACCCAAAGTGTC
$\beta$ -MHC	Beta Myosin Heavy Chain	F:ACAAACCATGACCAACAGCA R: GGCTGTGGGGTTACTTCAGA



**Table 2. Genes involved in inflammation**

Gene Name	Full Name	F (Forward) & R (Reverse) Primer sequence (5'-3' direction)
MCP-1	Macrophage Chemo-attractant Protein-1	F:AGCTGGGCATGACTGACATCT R:AGCCGACTCATTGGGATCAT
IL-1 $\beta$	Interleukin 1 $\beta$	F:GACCTGTTCTTTGAGGCTGACA R:CTCATCTGGACAGCCCAAGTC
TNF- $\alpha$	Tumor Necrosis Factor-alpha	F: AACACACGAGACGCTGAAGT R: TCCAGTGAGTTCCGAAAGCC

**Table 3. Genes involved with Fibrosis**

Gene Name	Full Name	F (Forward) & R (Reverse) Primer sequence (5'-3' direction)
COL1	Collagen Type 1	F:CTCTTTTAGGGACCCCAAGG R:GCTCCTCTCCCACTATGCAG
COL3	Collagen Type 3- $\alpha$	F:GGGATCCAATGAGGGAGAAT R:TCCTGCTTCCAATCGACTTT
TGF- $\beta_1$	Transforming Growth Factor $\beta_1$	F: AAAGAATTCGGGGTTCGCGCTCTCCGAAG R:AAACTCGAGTTATCAGCTGCACTTGCAGGAG

**Table 4. Genes involved with monocytes and macrophages**

Gene Name	Full Name	F (Forward) & R (Reverse) Primer sequence (5'-3' direction)
CD4	Cluster of Differentiation 4	F: ACCCTAAGGTCTCTGACCCC R: TAGGCTGTGCGTGGAGAAAG
CD68	Cluster of Differentiation 68	F:CTTGGCTCTCTCATTCCCTTAC R:CTGGTAGGTTGATTGTCTGCTC
F4/80	EGF-like module-containing mucin-like hormone receptor-like	F: GCCATAGCCACCTTCCTGTT R: ATAGCGCAAGCTGTCTGGTT

### 3.1.4 NF- $\kappa$ B ELISA Assay

NF- $\kappa$ B analyses were performed on all three groups: caloric restriction (CR), wheel running (WR) and sedentary (SED) groups for a total of 30 subjects (n=10 in each group). Nuclear extractions were prepared by homogenizing tissue samples using a commercial nuclear extraction kit (Nuclear Extraction Kit, Active Motif) according to the manufacturer's instructions. Briefly, about 20-50  $\mu$ g of frozen tissue was collected and crushed. While on ice, the crushed tissue was placed in a pre-chilled Dounce homogenizer and 400  $\mu$ L of ice-cold 1X Hypotonic buffer containing phosphatase and protease inhibitors supplemented with DTT and detergent was added. Then a large-clearance (A) pestle was used to disrupt tissue for about 2 minutes. The samples were incubated for 15 minutes on ice and centrifuged for 10 minutes at 850 x g at 4°C. Next the single cell slurry is subjected to a cell lysis protocol consisting of 200  $\mu$ L 1X Hypotonic buffer was added to the slurry and allowed 15 minute incubation on ice to allow the cells to swell. 15  $\mu$ L of detergent was then added and the cells were vortexed for 10 seconds at the highest setting. The suspension is then centrifuge for 30 seconds at 14,000 x g in a microcentrifuge pre-cooled at 4°C. The supernatant was then transferred off to pre-chilled tubes (this supernatant contains the cytoplasmic fractionation) and the nuclear pellet is resuspended in 50  $\mu$ L of Complete Lysis Buffer by pipetting up and down. The suspension is then incubated for 30 minutes on ice on a rocking platform set at 150 rpm. Suspension was then vortexed for 30 seconds at the highest settings and centrifuged for 10 minutes at 14,000 x g in a 4°C pre-chilled microcentrifuge. The supernatant, containing the nuclear protein fraction, is pipetted off and stored at -80°C

and quantified. Total nuclear protein content was determined using a BSA-based protein quantification assay (ProStain; ActiveMotif, Carlsbad, CA, USA).

NF- $\kappa$ B activation was determined using the nuclear extracts, as described above, and an ELISA-based Trans AM NF- $\kappa$ B p65 assay kit (ActiveMotif, Carlsbad, CA, USA) according to the manufacturer's directions. In short, 15  $\mu$ g/ $\mu$ L of nuclear extract were added to wells coated with a consensus binding sequence for NF- $\kappa$ B (5'-GGGACTTTCC-3') and incubated for 1 hour at room temperature. Wells were then washed, and a primary antibody directed at the p65 subunit was added and left to incubate for 1 hour. This is followed by treatment of all wells with a secondary antibody conjugated to horseradish peroxidase. A subsequent colorimetric reaction is initiated with the addition of a developing solution for 5 min followed by the application of a stop solution. The absorbance of the plate is then read at 450 nm on a multiwell microplate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany). Wild-type and mutated consensus oligonucleotides were used as competitors for NF- $\kappa$ B binding to ensure specificity of the reaction as per the manufacturer's instructions. All samples were run in duplicate, and the average value used for data analysis (LaBarbera et al., 2015; Hyldahl et al., 2011).

## 3.2 Preliminary Data

Data has been published previously on this cohort of animals by Crissey, et al (2014). Figure 1 represents the OLETF rat characteristics at 5 weeks and at the end of 20 weeks when the animals were sacrificed. Changes in body composition in WR and CR groups were significant compared to the sedentary group, exhibiting lower percent body

fat and less visceral adipose tissue mass. Food intake increased from five weeks for all animal groups. The caloric restriction and sedentary group exhibited no changes in food intake after 12 weeks during the intervention period. The wheel running group exhibited fluctuations in food intake, with a decrease occurring around 14 weeks, which may be due to exercise effects. The relative food intake was approximately the same for all groups until during the intervention phase, where the WR group exhibited an increase relative to body weight. In addition, heart weight for both WR and CR were significantly different compared to the SED group. Heart weight to body weight ratio (HW/BW) was significantly different in the WR compared to SED while CR HW/BW ratio was significantly different compared to WR. Lastly, percent body fat for the WR was significantly different from the SED group and CR percent body fat was significantly different from the WR group.

As summarized in table 5 (table 4 from Crissey et al., 2014) and described above, compared to the sedentary group, WR and CR plasma had lower triglycerides, total fasting cholesterol, LDL cholesterol, NEFAs, glucose and leptin. Lastly, the WR group exhibited improvements in HOMA-IR, lower plasma insulin and lower circulating MCP-1 compared with sedentary rats ( $P < 0.05$ ) (Crissey et al., 2014). Fasting plasma levels of IL-6, a marker of inflammation, were not different between SED, WR, and CR. However, TNF- $\alpha$  fasting plasma levels was statistically different between WR and CR ( $P < 0.05$ ), with CR exhibiting higher fasting plasma level of TNF- $\alpha$ . Overall, the data from Crissey et al. shows that exercise and a 30% diet restriction regimen for 8 weeks was related to reduced adiposity, improved blood lipid profiles and systemic markers of insulin resistance in 20-week-old obese OLETF rats.

Fig. 1. Body composition and food intake in sedentary (SED), wheel running (WR), and diet restriction (DR) Otsuka Long-Evans Tokushima fatty (OLETF) rats. Values are expressed as means  $\pm$  SE;  $n = 10$ /group. Body fat, heart weights, and fat pad weights were obtained at 20 wk (time of death). \*Significant difference ( $P < 0.05$ ) from SED rats. #Significant difference ( $P < 0.05$ ) from WR rats.

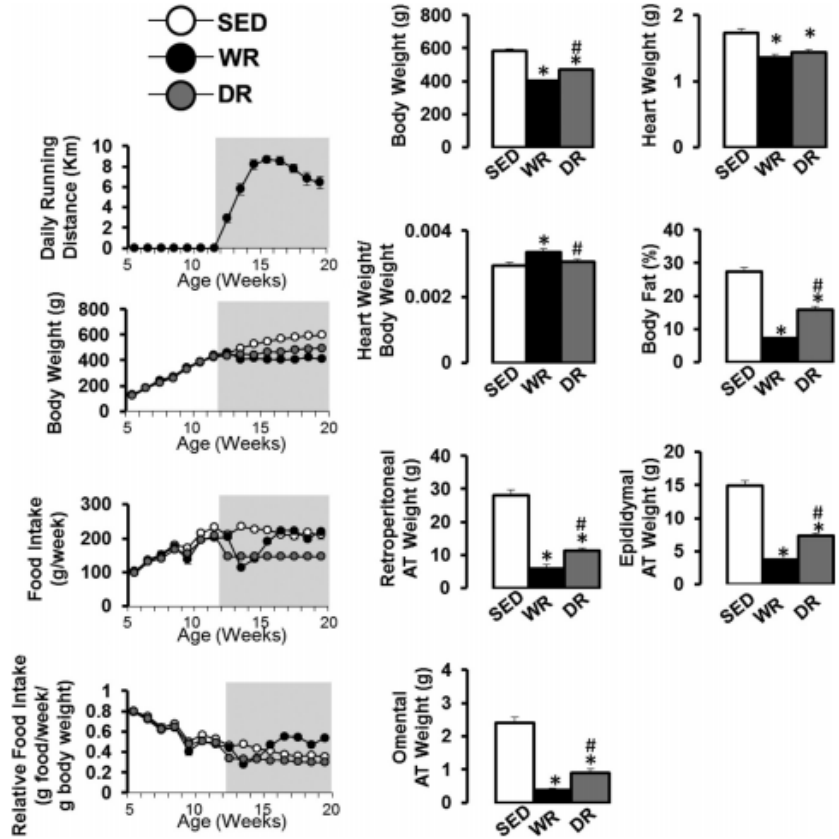


Table 5. Fasting plasma characteristics in sedentary, wheel running, and diet restriction OLETF rats

Variable	SED	WR	DR
Total cholesterol, mg/dl	110.7 $\pm$ 4.4	69.7 $\pm$ 2.8*	78.0 $\pm$ 1.1*
LDL cholesterol, mg/dl	46.4 $\pm$ 3.9	29.6 $\pm$ 1.8*	34.5 $\pm$ 0.8*
HDL cholesterol, mg/dl	33.2 $\pm$ 0.7	29.9 $\pm$ 1.3*	29.1 $\pm$ 0.5*
Triglycerides, mg/dl	155.0 $\pm$ 6.5	51.1 $\pm$ 2.7*	71.9 $\pm$ 4.0*#
NEFA, mmol/l	0.57 $\pm$ 0.05	0.19 $\pm$ 0.01*	0.31 $\pm$ 0.03*#
Insulin, ng/ml	30.8 $\pm$ 10.1	6.9 $\pm$ 0.7*	19.5 $\pm$ 3.2
Glucose, mg/dl	311.6 $\pm$ 9.5	196.1 $\pm$ 4.9*	223.1 $\pm$ 10.6*#
HOMA-IR index	24.1 $\pm$ 8.2	3.4 $\pm$ 0.4*	11.1 $\pm$ 2.2
Leptin, ng/ml	260.1 $\pm$ 39.0	2.2 $\pm$ 0.3*	63.9 $\pm$ 24.7*
MCP-1, pg/ml	197.1 $\pm$ 8.5	144.3 $\pm$ 9.4*	194.4 $\pm$ 26.4#
TNF- $\alpha$ , pg/ml	6.5 $\pm$ 0.3	5.3 $\pm$ 0.5	6.6 $\pm$ 0.5#
IL-6, pg/ml	204.4 $\pm$ 66.0	173.6 $\pm$ 56.7	194.5 $\pm$ 66.3

SED, sedentary; WR, wheel running; DR, diet restriction; LDL, low-density lipoprotein; HDL, high-density lipoprotein; NEFA, nonesterified fatty acids; HOMA-IR, homeostasis model assessment of insulin resistance; MCP-1, monocyte chemoattractant protein-1; TNF- $\alpha$ , tumor necrosis factor alpha; IL-6, interleukin 6. \*Significant difference ( $P < 0.05$ ) from SED rats. #Significant difference ( $P < 0.05$ ) from WR rats.

## CHAPTER 4

### RESULTS

#### 4.1 Reference Gene Quality Control

18S and RPL10a were evaluated for their appropriateness as control gene for RT-PCR. Figure 1A and 1B show the average Ct values of 18S and RPL10a, respectively. 1-way ANOVA revealed that 18S Ct values were significantly different between groups. Post-hoc analysis showed that there were significant differences between CR vs. SED and CR vs. WR ( $p < 0.001$ ) but no differences between SED vs. WR. RPL10a Ct values showed no difference between groups as depicted in figure 1B and was therefore used as the reference gene for all RT-PCR gene expression analyses.

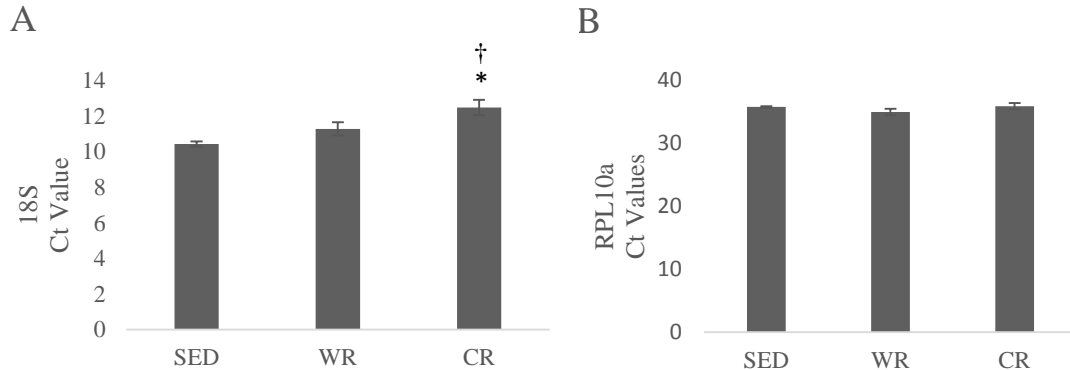
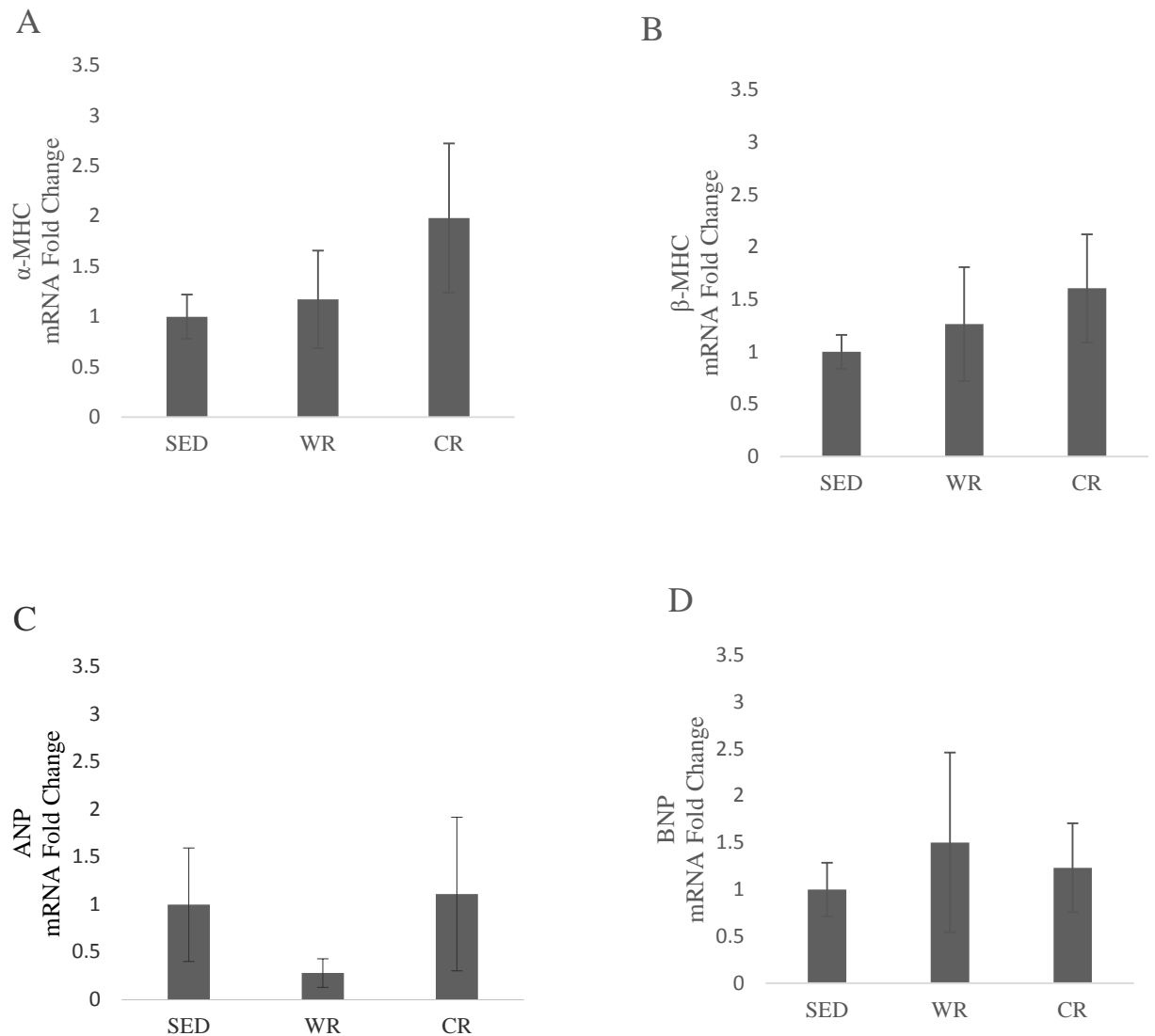
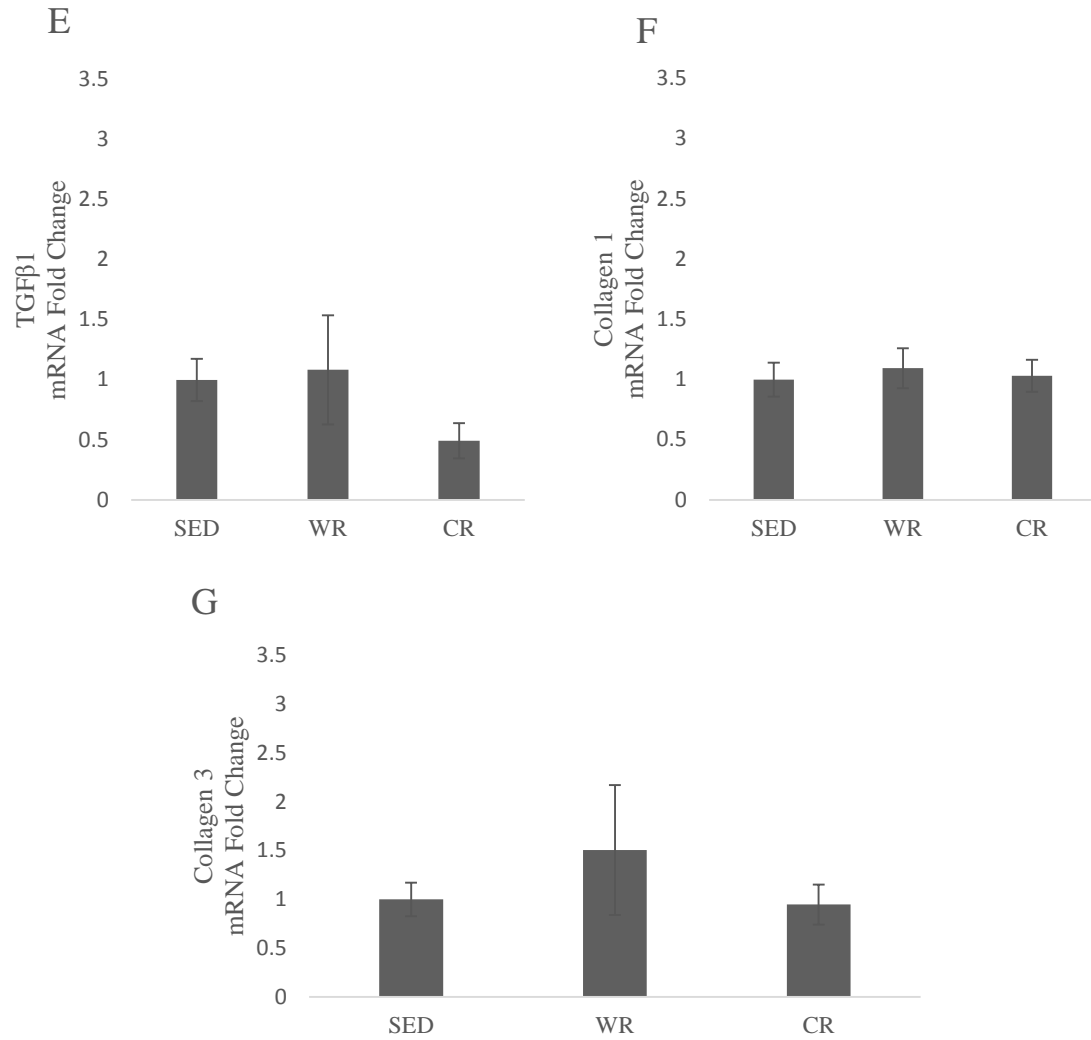


Figure 1. Ct values of RPL10a and 18S mRNA expression in cardiac tissue from caloric restriction (CR), wheel running (WR), and sedentary (SED) groups. (A) 18S Ct values from the means of 10 different runs. (B) Rpl10a Ct values from one run. Values are expressed as means  $\pm$  SE ( $n=10$ /group). \* Denotes difference ( $p < 0.05$ ) from SED; † Denotes difference ( $p < 0.05$ ) from WR.

#### 4.1.2 Cardiac Dysfunction: Fetal Gene Expression and Fibrosis

To assess cardiac dysfunction in heart tissue of OLETF rats, we examined the pattern of  $\alpha$ -MHC,  $\beta$ -MHC, ANP, BNP, TGF $\beta$ -1, collagen 1, and collagen 3 mRNA expression in the three different conditions (CR, WR, and SED). As shown in Fig. 2, there were no significant differences between groups in the mRNA expression of genes involved in the fetal gene program and in fibrosis; Fig. 2A,  $\alpha$ -MHC ( $p=0.385$ ); Fig. 2B,  $\beta$ -MHC ( $p=0.63$ ); Fig. 2C, ANP ( $p=0.56$ ); Fig. 2D, BNP ( $p=0.857$ ); Fig. 2E, TGF $\beta$ <sub>1</sub> ( $p=0.323$ ); Fig. 2F, COL1 ( $p=0.9$ ), and Fig. 2G, COL3 ( $p=0.581$ ).





**Figure 2. Expression of genes related to cardiac dysfunction in caloric restriction (CR), wheel running (WR), and sedentary (SED) groups.**  $\alpha$ -MHC (A),  $\beta$ -MHC (B), ANP (C), BNP (D), TGF $\beta$ -1 (E), Collagen 1 (F), and Collagen 3 (G) mRNA expression was measured by RT-PCR. Values are expressed as means  $\pm$  SE (n=10/group). For each gene, SED is used as the reference group and set at 1.



#### 4.1.3 NF- $\kappa$ B Activation in Cardiac Tissue of OLETF rats

Nuclear extractions were performed on the cardiac tissue of OLETF rats in the WR, CR, and SED groups and used to determine p65 NF- $\kappa$ B activity. Figure 3 shows the values for the NF- $\kappa$ B activity of the WR, CR, and SED groups. There were no significant differences in NF- $\kappa$ B activity between groups. 1-way ANOVA showed that there was a trend for an overall effect of group ( $p=0.07$ ) on NF- $\kappa$ B activation, with CR exhibiting greater NF- $\kappa$ B activation compared to SED ( $P=0.06$ ).

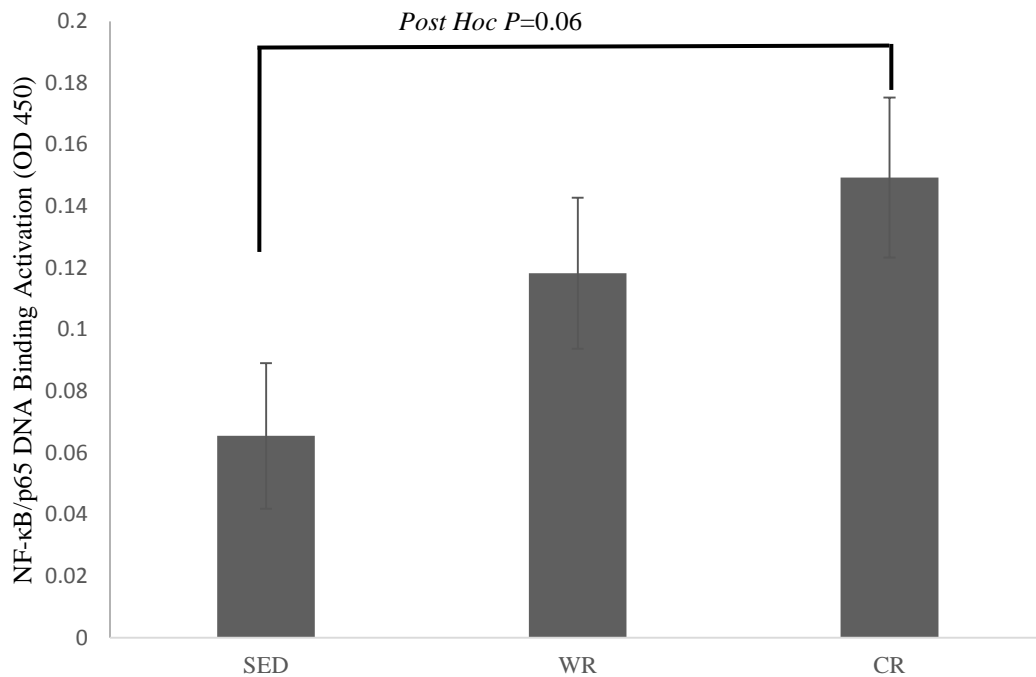
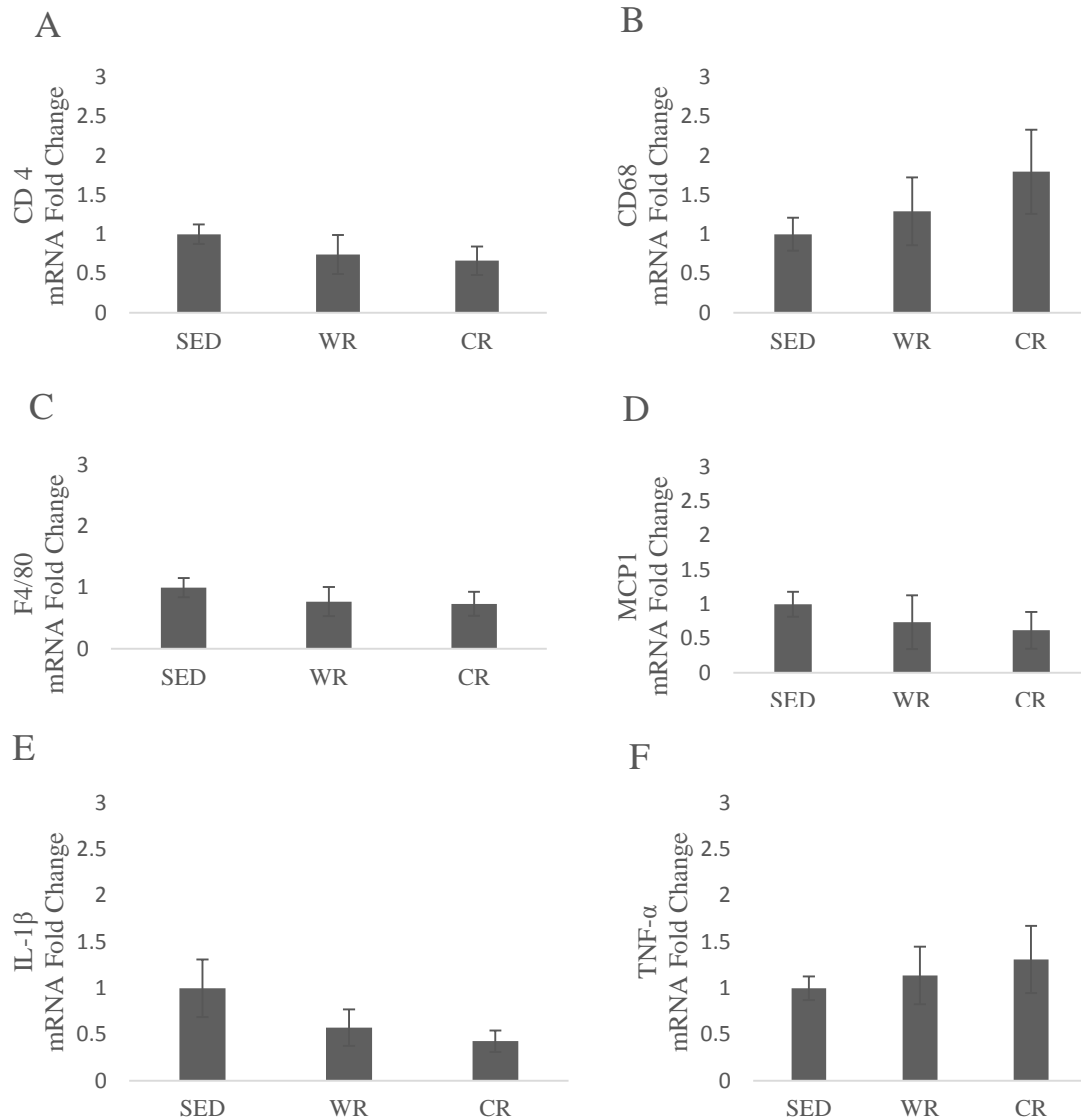


Figure 3. **NF- $\kappa$ B activation in cardiac tissue in caloric restriction (CR), wheel running (WR), and sedentary (SED) groups.** Values are expressed as means  $\pm$  SE (n=10/group).

#### 4.1.4 Markers of Immune Cells and NF- $\kappa$ B activity

We examined the pattern for CD4, CD68, F4/80, MCP-1, TNF- $\alpha$ , and IL-1 $\beta$  mRNA expression in cardiac tissue of the CR, WR, and SED groups. As shown in Fig. 4, there were no differences in the mRNA levels of immune cell and NF- $\kappa$ B related target genes in the three different groups. Fig. 4A, CD4 ( $p=0.436$ ); Fig. 4B, CD68 ( $p=0.402$ ); Fig. 4C, F4/80 ( $p=0.603$ ); Fig. 4D, MCP-1 ( $p=0.649$ ); Fig. 4E, IL-1 $\beta$  (0.188) and Fig. 4F, TNF- $\alpha$  ( $p=0.745$ ).



**Figure 4. Expression of immune cell related and pro-inflammatory NF- $\kappa$ B genes of caloric restriction (CR), wheel running (WR), and sedentary (SED).** (A) mRNA expression of CD4. (B) mRNA expression of CD68. (C) F4/80 mRNA expression. (D) MCP-1 mRNA expression. (E) IL-1 $\beta$  mRNA expression. (F) TNF- $\alpha$  mRNA expression. Values are expressed as means  $\pm$  SE (n=10/group). For each gene, SED is used as the reference group and set at 1.

## CHAPTER 5

### DISCUSSION

In this study, we aimed to determine effects of WR and CR to reduce markers of cardiac dysfunction, NF- $\kappa$ B activity, and gene expression related to NF- $\kappa$ B and immune cell infiltration compared with SED controls in 20 week old OLETF rats. Our major findings were: 1) there were no significant differences in markers of cardiac dysfunction between the groups, 2) there was a trend for increased NF- $\kappa$ B activity in the CR compared to the SED group and 3) there were no significant differences in the gene expression of markers related to immune cell infiltration and NF- $\kappa$ B target genes in the 20 week old OLETF rats.

Our data on cardiac dysfunction in OLETF rats complements and extends that of Yagi and colleagues (1997) in which cardiac dysfunction of OLETF rats were characterized over 54 weeks. In this study, investigators examined pathological features of the heart and markers of cardiac complications in OLETF rats from the pre-diabetic state (14 weeks), non-insulin dependent diabetes mellitus (30 weeks) and insulin dependent diabetes mellitus (54 weeks), evaluating fetal gene expression and genes involved in cardiac fibrosis as markers for changes in cardiac performance. In addition, the Long-Evans Tokushima-Otsuka (LETO) rats were evaluated as the genetic control animals for the OLETF rats. The researchers found that there were no differences in fetal gene expression markers in OLETF rats between 14 weeks, at 30 weeks and that while a small difference in  $\beta$ -MHC was observed between OLETF and LETO at 30 weeks, larger

differences between OLETF and LETO in  $\alpha$ -MHC,  $\beta$ -MHC, and ANP expression were only observed at 54 weeks. This data indicates that changes in cardiac fetal gene program expression do not appear until later in the OLETF model. Therefore, although we did not have a LETO group for comparison, it is possible that we did not observe any differences between the groups because they were only 20 weeks old, which would not be long enough for SED animals to develop a fetal gene signature. Further, fetal gene expression should not change with exercise, as it is not activated in normal physiological hypertrophy (Maillet et al., 2013). To our knowledge, there are no studies that have investigated the effects of CR on the fetal gene program. However, since CR is known to attenuate insulin sensitivity in the heart of OLETF rats (Park et al., 2005) and improve cardiac energy metabolism (Dolinsky et al., 2010; Kemi et al., 2000), we would not anticipate that CR would also help maintain proper cardiac function and prevent an emergence of the fetal gene program.

Interestingly, according to data from the same study (Yagi et al., 1997), there is evidence that cardiac fibrosis occurs at early time points in the OLETF model and that it may be related to inflammation. Investigators reported that at 14 weeks of age, the left ventricular gene expression of collagen types I, III and IV, along with laminin, were significantly enhanced in OLETF compared with LETO rats of the same age. Collagen types III and IV and laminin mRNA levels remained elevated in OLETF rats at week 30 compared to LETO. Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) is a pleiotropic cytokine that can regulate fibrogenesis and is released from pro-inflammatory cells (e.g. macrophages). The authors showed that mRNA levels of 14-, 30-, and 54-week-old OLETF rats were 1.5-, 1.6-, and 1.3-fold greater, respectively, than in the LETO rats

examined at those same time points. Thus, according to this study, markers of fibrosis are evident at as early as 14 weeks in the OLETF model when animals are overweight and insulin resistant. We expected that markers of fibrosis would be lower in EX and CR groups compared with SED in our study in line with existing myocardial fibrosis suspected at 20 weeks (Mizushige et al., 2000). Contrary to our hypothesis, we found no differences between groups in markers of fibrosis. Since we do not have LETO control animals as a comparison, it is unknown whether the fibrosis occurs in the OLETF heart independent of CR and EX interventions.

Caloric restriction and exercise are known to induce pleiotropic forms of cardiovascular protection (Dolinsky and Dyck, 2011; Shinmura et al., 2010). CR is able to increase longevity and reduce death due to chronic diseases such as cancer, T2DM, obesity, and cardiovascular disease. Indeed, research has shown that CR is able to attenuate the production of ROS and oxidative damage by activation of SIRT1 and transcription factor NF-E2-related factor (Nrf2) (Csiszar et al., 2009). In addition, CR is able to attenuate age-related vascular inflammation by improving the NF- $\kappa$ B inhibition of pro-inflammatory gene transcription, thereby causing NF- $\kappa$ B to become active in anti-inflammatory gene transcription and reducing a pro-inflammatory state (Weiss & Fontana, 2011). Lastly, like exercise, CR is able to reduce adiposity and inflammatory markers, especially in the cardiovascular system, leading to healthy cardiovascular function and can even decrease the adverse effects of cardiomyopathy (Weiss & Fontana, 2011; Kemi et al., 2000).

We hypothesized that the inhibitory effect of CR and EX on overall systemic inflammation in OLETF rats would be reflected in the heart and mediated through

inhibition of NF- $\kappa$ B's pro-inflammatory factor transcription (Medeiros et al., 2011). Adiposity, especially in obesity and diabetes, exhibits paracrine and autocrine signaling of pro-inflammatory markers, thus leading to greater NF- $\kappa$ B activity and mRNA expression of pro-inflammatory markers in adipose tissue (Romacho et al., 2014). In the study conducted by Crissey and colleagues (2014), which analyzed the same animals as the present study, WR and CR in 20-week old OLETF rats showed lower gains in body weight over time, lower adipose tissue mass, and lower adipose tissue expression of inflammatory genes and markers of immune cell infiltration compared to the SED group. Furthermore, in periaortic adipose and aortic tissue, they reported lower expression of cytokines in WR and CR compared with SED. Contrary to our second hypothesis our data showed a trend for increased NF- $\kappa$ B activity in CR compared with SED. We also witnessed that there were no significant differences in the gene expression of markers related to immune cell infiltration and NF- $\kappa$ B target genes in the heart of these 20 week old OLETF rats, providing evidence that inflammation may not play a role in the heart at this time point.

The literature has focused mainly on the pro-inflammatory side of NF- $\kappa$ B and there is still a lack of information on the role of NF- $\kappa$ B on cardioprotection and anti-inflammation in the heart. It is possible that our results could reflect the anti-inflammatory and anti-apoptotic activity of NF- $\kappa$ B, though more research is needed. It has been shown that the anti-apoptotic activity of NF- $\kappa$ B depends on certain gene induction (Lui et al., 1996). NF- $\kappa$ B can induce the expression of cellular inhibitors of apoptosis (c-IAPs), caspase-8-c-FLIP (FLICE inhibitory protein), Bfl1, TNFR-associated factor (TRAF1) and TRAF2. TRAF1 and TRAF2 are adaptor proteins required in

optimizing NF- $\kappa$ B activity and it is postulated that their anti-apoptotic activity is most likely due to their ability in augmenting the activation of NF- $\kappa$ B (Karin & Lin, 2001). The c-IAPs proteins can directly bind and inhibit effector caspases, such as caspase-3 and caspase-7, and prevent activation of pro-caspase-6 and pro-caspase-9 (Deveraux et al., 1998). These c-IAPs can thus inhibit apoptosis induced by both death receptors and mitochondria-dependent pathways. In particular, c-IAP1 and c-IAP2 expression by NF- $\kappa$ B suppresses TNF- $\alpha$  mediated killing through inhibition of caspase-8 (Wang et al., 1998).

There is data to suggest that NF- $\kappa$ B also inhibits apoptosis by DNA damaging agent, which act *via* the mitochondria-dependent pathway (Baldwin, A.S., 2001). This activity could be mediated through members of the Bcl-2 family, a family of pro- and anti-apoptotic proteins situated in the mitochondria, such as Bcl- $\chi$ L and Bfl1. It appears that Bfl1 is able to induce its anti-apoptotic by preventing mitochondrial depolarization, the release of cytochrome c and caspase-9 activation (Wang et al., 1999). In addition, it is possible that Bcl-2 itself may be involved in the anti-apoptotic activity of NF- $\kappa$ B, as it is defective in cells that lack both c-Rel and RelA (p65), the major activating subunits of NF- $\kappa$ B (Grossman et al., 2000). However, more work is required to elucidate these proteins and their pathways in relations to the heart as these pathways have been studied most extensively in immune cells and oncogenesis.

Lastly, this study aimed to determine the comparative effectiveness of exercise and caloric restriction on inflammation and cardiac NF- $\kappa$ B signaling in 20 week old OLETF rats. From our results, it is difficult to state which of these two treatments is more effective in reducing inflammation and cardiac NF- $\kappa$ B activity. Data from Crissey et al.,



shows that exercise is more effective in reducing fasting plasma levels of MCP-1, TNF- $\alpha$ , and other markers of obesity and diabetes (Crissey et al., 2014). In this regard, exercise is more effective than caloric restriction in attenuating the effects of inflammation and cardiometabolic syndrome parameters. Both caloric restriction and exercise have important roles in reducing inflammation, improving longevity, and reducing the risk for a multitude of diseases. In order to guide public health recommendations, more research is needed to determine which intervention is more effective in early progression of diseases such as diabetes and obesity. Further, there is much debate on the effects of NF- $\kappa$ B activity in relation to cardiovascular disease and function that warrants further investigation (Gordon et al., 2011).

### 5.1 Limitations and Future Studies:

A major limitation of this current study was the absence of tissue from LETO control animals for analysis. Since the OLETF rat is a genetic model of T2DM and mild obesity, a proper genetic control is needed to investigate and properly compare the effects of interventions such as caloric restriction, sedentary behavior, and exercise. Future studies require incorporation of LETO animals as a control group. Second, investigating the changes in the heart at different time points, 14 weeks and 30 weeks, can help provide a more complete picture of the effects of pre-diabetes on the hearts of OLETF rats. The literature has witnessed differences at 14 weeks and 30 weeks though there exists limited knowledge on WR and CR at these time periods. Lastly, our analysis of NF- $\kappa$ B activity only provides information on the activation of NF- $\kappa$ B but warrants no information on what regions of DNA it is binding to. In future studies, NF- $\kappa$ B binding should be assessed using chromatin immunoprecipitation (chIP) to determine what consensus

binding regions are being transcribed by NF- $\kappa$ B. Furthermore, microarray analysis on anti-inflammatory genes in cardiac tissues under the different interventions are warranted to see if there is a connection between NF- $\kappa$ B activity and these anti-inflammatory markers.

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