## Cardiac effects of acute hyperinsulinemia and chronic fat feeding

Tadinada, Satya Murthy

# CARDIAC EFFECTS OF ACUTE HYPERINSULINEMIA AND CHRONIC FAT FEEDING

by

Satya Murthy Tadinada

A thesis submitted in partial fulfillment
of the requirements for the Doctor of Philosophy
degree in Pharmacology in the
Graduate College of
The University of Iowa

August 2019

Thesis Supervisor: Professor Evan Dale Abel

To my family and friends

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my mentor Dr. Evan Dale Abel for the training opportunities and the time he's vested in helping me develop as an individual and as a researcher. Dr. Abel's insights and feedback have been invaluable in developing my project and to improve my thinking abilities. Many thanks to my committee members who have been constantly supportive of me throughout my degree.

I would like to also thank all my colleagues in the Abel lab for the conducive environment and their constant feedback on my work. Their insights and involvement in my project have helped me improve my productivity and develop strategies to become efficient at the bench. A special shout-out to Dr. Renata Pereira, Dr. Eric Weatherford and Dr. Yuan Zhang for their constructive criticism and moral support in times of difficulties. They have ensured my growth as an individual and as a researcher.

The amount of data collected for the projects that encompass this thesis would not be possible without cooperation from research cores (Metabolic phenotyping core, Cardiovascular imaging core and the Central Microscopy Research Facility) at the University of Iowa. I would like to thank them for all their time and help with data collection, analysis and for helping me understand the details of the various experimental procedures.

It wasn't without constant support from friends and family that I could endure the rigors of graduate school and successfully graduate with my PhD degree. I would like to express my deep sense of gratitude to my family and friends around the world who were there for me in times of difficulties and happiness. I will cherish all the memories I have made with all my friends here in Iowa. Their support for me was unconditional throughout this phase of my life.

# ABSTRACT

Diabetic cardiomyopathy characterized by left ventricular hypertrophy predisposes diabetic and obese individuals to development of cardiac dysfunction and subsequently to heart failure. Whether hyperinsulinemia has an underlying role in development and or progression of diabetic heart disease is not well understood. We therefore studied the effects of acute hyperinsulinemia on cardiac function in euglycemic states. Acute hyperinsulinemia neither affected baseline nor inotropic response to β-adrenergic stimulation. Previous studies from our laboratory have indicated a potential role for GRK2, a serine threonine kinase in development of cardiac dysfunction in diabetic states in humans as well as in mice. To assess whether GRK2 mediates the detrimental effects of chronic hyperinsulinemia on cardiac dysfunction in mouse model of diet induced obesity, we utilized cardiomyocyte knockout of GRK2. Our results suggested lack of cardiac functional impairments in high fat fed wildtype mice, which hindered our attempts to ascertain the role of GRK2 in diabetic cardiomyopathy. Mouse models of diet induced obesity have been routinely used to study the effects of obesity and diabetes on cardiac dysfunction but recent evidence from multiple research groups has emphasized the need for evaluation of the utility and relevance of the murine diet induced obesity model for studying cardiovascular abnormalities associated with hyperinsulinemic states, including T2DM and obesity. We therefore studied the effect of chronic fat feeding (>20 weeks) alone or in combination with concomitant hypertension on cardiac function in C57BL/6J mice. Different diets were formulated with either lard (32% saturated fat, 68% unsaturated fat) or hydrogenated coconut oil (95% saturated fat) as the source of fat and fatty acids, which contributed 60% of total calories. Insulin resistance and glucose intolerance were readily observed in mice fed a high fat diet in each of the studies. HFD resulted in the development of cardiac hypertrophy; however

cardiac function as measured by B-mode echocardiography and LV catheterization was unaffected in high fat diet groups compared to their respective control diet groups. Further, dietary fat feeding regardless of the source of fat modestly altered the gene expression of a few pathological hypertrophic markers or of fibrosis related genes. However, there was an increase in expression of PPARa target genes such as Pdk4 and fatty acid metabolism genes including CD36, AcadL and Cpt1b. Cardiac mitochondrial function as assessed by oxygen consumption rates, ATP synthesis rates and reactive oxygen species production rates were unaltered in high fat diet fed mice. These results suggest that while chronic fat feeding in mice causes cardiac hypertrophy and potentially cardiometabolic remodeling, it might not be sufficient to activate pathological hypertrophic mechanisms that impair cardiac function and cause cardiac fibrosis.

## PUBLIC ABSTRACT

The incidence and prevalence of type 2 diabetes and obesity has been on the rise in the United States over the last few decades. Heart disease is the leading cause of death in the United States and is an important contributor to morbidity and mortality in individuals with type 2 diabetes and obesity. Of the many aspects of type 2 diabetes and obesity, hyperinsulinemia is most commonly overlooked for its effects on cardiac function. However, recent evidence from epidemiological studies suggest that hyperinsulinemia may indeed be an important contributor to development of diabetic cardiomyopathy- a state of myocardial dysfunction accompanied by cardiac hypertrophy in type 2 diabetes and obesity. The goal of this dissertation was to address specifically the effects of acute and chronic hyperinsulinemia on cardiac function using mouse as a model system. We found that acute hyperinsulinemia does not cause cardiac depression independent of the duration of exposure to insulin and the inotropic response elicited by isoproterenol induced stimulation of cardiac β-adrenergic receptors was not altered by acute hyperinsulinemia. Upon investigation of the long-term effects of hyperinsulinemia on cardiac function in a model of diet induced obesity, we found that cardiac function was unaltered in animals fed a high fat diet for as long as 40 weeks. Further experiments suggested that fatty acid saturation, age at time of induction and concomitant hypertension do not alter the cardiac outcomes of chronic fat feeding. These studies highlight the resilience of the mouse myocardium and its resistance to changes in function upon exposure to states of hyperinsulinemia.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# CHAPTER 1: INTRODUCTION

## 1.1 Type 2 Diabetes Mellitus (T2DM) and Obesity- Statistics and Economic Costs

T2DM has reached the status of a pandemic in the recent years according to the International Diabetic Federation (IDF) with a prevalence of about 425 million individuals in 2017. By 2040 the incidence of T2DM is estimated to rise to 642 million (IDF 2017). In the United States, the incidence of T2DM is rising at an alarming rate. Overall, 30.3 million Americans (~9.4% population) were diabetic in 2017 (Center for Disease Control, CDC 2017) and 84.1 million adults of adult US population (33.9% of adult US population) were prediabetic (CDC 2017). In North America and Europe, obesity, defined when the body mass index (BMI) exceeds 30 is an important risk factor for T2DM. The prevalence of obesity in 2015 was 39.8% of the US adult population affecting 93.3 million adults. In children, the prevalence was 18.5% of the total population younger than 19 years of age affecting approximately 13.7 million individuals (CDC 2015).

These two dysmetabolic states contribute to a major economic burden in the US with T2DM responsible for $237 billion in healthcare costs and $90 billion in reduced productivity in 2017 ("Economic Costs of Diabetes in the U.S. in 2017," 2018) The economic burden of obesity according to the latest available data from CDC suggests that a total of $147 billion in healthcare costs was attributable to obesity in 2008. These remarkably high healthcare costs may be primarily due to complications that develop due to these disease states. A majority of patients afflicted with these progressively deteriorating metabolic disorders develop cardiovascular

complications, which are the major causes of mortality in T2DM and obesity (American Diabetes Association).

### 1.2 Heart Disease in T2DM and Obesity- Diabetic Cardiomyopathy

Heart disease is the leading cause of death in both men and women in the United States (CDC, 2015). According to CDC 2015 statistics, 635,000 individuals die of heart disease every year. T2DM and Obesity are important risk factors for heart disease (Kannel, Hjortland, & Castelli, 1974; Kannel & McGee, 1979; Kenchaiah et al., 2002; Lauer, Anderson, Kannel, & Levy, 1991). A number of epidemiological studies have suggested increased incidence of heart disease in diabetic individuals (de Simone et al., 2010; M. Galderisi, K. M. Anderson, P. Wilson, & D. Levy, 1991; Kannel et al., 1974; Kannel & McGee, 1979; Kenchaiah et al., 2002; Lauer et al., 1991; G. A. Nichols, Gullion, Koro, & Diabetes …, 2004). One of the earliest studies was published in 1974 using population data from the Framingham Heart Study cohort and the findings from these studies have emphasized the association between diabetes and HF risk. T2DM increased the risk of congestive heart failure by more than 2 times in men and 5 times in women. A 4 to 5-fold increased risk of heart failure existed despite adjustment for coronary artery disease and rheumatic heart disease (Kannel et al., 1974). These findings were later corroborated by additional studies from the Framingham Study Cohort (Maurizio Galderisi et al., 1991; Kannel & McGee, 1979) and the Strong Heart Study cohort (de Simone et al., 2010; Devereux et al., 2000). An increased risk of heart failure was found in obese individuals within the Framingham Heart Study cohort (Kannel, LeBauer, Dawber, & McNamara, 1967; Kenchaiah et al., 2002).

In addition to the increased mortality and risk of heart failure in diabetes suggested by the epidemiological studies, a number of other cross-section studies have suggested alterations in cardiac structure and function (Devereux et al., 2000; Ernande et al., 2011; Z. Y. Fang, Schull-Meade, Downey, Prins, & Marwick, 2005; M. Lee et al., 1997; Regan et al., 1977; Rijzewijk, van der Meer, & of the …, 2009). The first identification of a distinct cardiomyopathy associated with T2DM was suggested in 1972. An increased left ventricular mass and interstitial fibrosis was apparent upon histological investigation of autopsy samples from diabetic individuals without a history of coronary artery disease and hypertension compared to samples from non-diabetic individuals (Rubler et al., 1972). More evidence was provided in later studies where increased collagen deposition was detected around the perimysium and in the perivascular spaces (Kawaguchi et al., 1997; M. Shimizu et al., 1993) along with increased thickening of basement membrane and altered endothelial structure and myocyte morphology (Kawaguchi et al., 1997). Given the difficulty with acquiring human tissue for histological analysis and also considering the possible vascular abnormalities that accompany T2DM at later stages of disease progression, investigators have focused on studying gross morphological and functional abnormalities in diabetics primarily by echocardiography to ascertain whether T2DM led to development of cardiac abnormalities. Functional deficits and cardiac hypertrophy were detectable by echocardiography in individuals with T2DM. Cardiac hypertrophy and impaired systolic and diastolic function were observed in individuals with T2DM (Devereux et al., 2000; M. Lee et al., 1997). Strain analysis revealed abnormal systolic function in 27% of individuals with T2DM and without coronary artery disease or hypertension (Z. Y. Fang et al., 2005). In a small sample of individuals with T2DM, elevated end diastolic pressure accompanied by decreased end diastolic

volume and stroke volume resulted in impaired systolic performance. These individuals also failed to respond to experimental increases in afterload where EDP increased rapidly and thereby affecting EDV and SV (Regan et al., 1977).

At least 40% of the individuals with T2DM had diastolic dysfunction in a study sample with normal systolic function (Boyer, Thanigaraj, Schechtman, & Perez, 2004). A decline in early diastolic transmitral flow rate (E) and increased isovolumetric relaxation time (IVRT) was apparent in a small study of individuals with T2DM (Astorri et al., 1997). In a Dutch cohort comprised of individuals with or without T2DM, decreased ratio of early diastolic transmitral flow velocity (E) to late diastolic transmitral flow velocity (A) was observed by magnetic resonance imaging in T2DM men compared to normoglycemic individuals (Rijzewijk et al., 2009). Impaired relaxation and pseudo-normal filling patterns were observed upon Valsalva maneuver in individuals with T2DM and normal systolic function (Poirier, Bogaty, Garneau, Marois, & Dumesnil, 2001). In a Swedish cohort, structural abnormalities that relate to increased LV wall thickness and concentric remodeling correlated well with insulin resistance (Sundström, Lind, Nyström, & Circulation, 2000). In a longitudinal study conducted over 16 years within the Framingham Heart Study cohort, age associated decline in diastolic dimensions were significantly higher in women and in individuals with T2DM (Cheng, Xanthakis, Sullivan, & Circulation, 2010). Increased LV mass was much more pronounced in women, but increased left atrial dimensions were abnormal in both men and women with glucose intolerance suggesting a gender dependent divergence in etiology and presentation of diastolic abnormalities in individuals with glucose intolerance (Rutter et al., 2003). An increased prevalence of diastolic dysfunction in individuals with insulin resistance also correlated positively with the degree of

glucose intolerance (Dinh et al., 2010). A more recent study reported abnormal systolic function by strain analysis in T2DM individuals with normal diastolic function (Ernande et al., 2011) raising questions over the more widely accepted notion that diastolic dysfunction precedes systolic dysfunction in T2DM and obesity. Taken together, the functional deficits described in these various study cohorts identified predominant abnormalities in diastolic function while fewer studies have indicated significant decreases in systolic parameters.

### 1.3 Cardiac Metabolism in T2DM and Obesity

T2DM and obesity are metabolic disorders that impair fuel utilization and mobilization processes. Ectopic lipid accumulation is a cause and consequence of insulin resistance where adipose tissue insulin resistance increases lipolysis resulting in increased circulating fatty acids increased hepatic triglyceride synthesis and accumulation of triglycerides and other lipid intermediates in organs that primarily utilize fatty acids for generating ATP. Altered substrate metabolism was apparent in hearts of obese premenopausal women who were glucose intolerant and had fasting hyperinsulinemia. Increased fatty acid oxidation and oxygen consumption that did not match the cardiac work indicated inefficiency of the heart muscle (Peterson et al., 2004). Positron Emission Tomography revealed decreased glucose uptake and increased fatty acid uptake and oxidation but not high energy phosphates in the hearts of T2DM individuals compared to controls. The decline in diastolic function was however was not correlated with altered substrate metabolism (Rijzewijk et al., 2009). Increased fatty acid uptake was observed in individuals with impaired glucose tolerance which was associated with decreased stroke volume and diastolic function as measured by PET ventriculography (Labbe et al., 2012). In a small cohort of individuals without hypertension and T2DM, there was a significant decline in diastolic

function measured by transmitral flow doppler echocardiography. These individuals also had significant decreases in high energy phosphates measured as ratio between Phosphocreatine/ATP as measured by MRS (Diamant et al., 2003). Elevated triglycerides were found in hearts of overweight and obese individuals compared to control subjects and a positive correlation was found between triglyceride content and concentricity index, which is LV mass indexed to LV volume (Szczepaniak et al., 2003). A more recent study found significant increases in myocardial triglyceride content in individuals with T2DM associated with impaired myocardial energetics and diastolic dysfunction (Levelt et al., 2016). These findings suggest that T2DM and obesity are associated with altered substrate uptake and metabolism. These changes in cardiac metabolism may in part contribute to the observed decline in cardiac function.

**1.4 Animal Models of T2DM and Obesity and Altered Cardiac Metabolism**

Observations in humans from cross-sectional studies and longitudinal studies, indicate that T2DM is associated with progressive deterioration of cardiac function and alterations in substrate metabolism. An understanding of the mechanistic basis for these changes in cardiac metabolism, structure and function could lead to the development of novel therapeutics for improving outcomes of heart disease in T2DM and obesity. To achieve this, it is imperative to conduct longitudinal analysis of cardiac function to detect early changes and perform molecular analysis of pathways that regulate cardiac function. Use of animal models allows for understanding the molecular changes underlying the development of cardiac dysfunction. Coupled with the ease of genetic manipulation in rodents, this strategy may allow for ascertaining the necessity and sufficiency of diverse signaling molecules in mediating the cardiac functional deficits that may result from T2DM.

Impaired contractile function has been reported in the leptin receptor mutant rodent models such as db/db mice (Belke, Larsen, Gibbs, & Severson, 2000; Jonathan Buchanan et al., 2005; Semeniuk, Kryski, & Severson, 2002) as well as the leptin deficient ob/ob mouse model (Christoffersen et al., 2003; Dong et al., 2006) which also exhibits hyperphagia and obesity. Specifically, these animals develop cardiac hypertrophy and compromised systolic function that is accompanied by changes in myocardial substrate utilization (Belke et al., 2000; Jonathan Buchanan et al., 2005; Mazumder et al., 2004). Reduced glucose oxidation and increased fatty acid utilization (uptake and oxidation) was apparent in addition to triglyceride accumulation in the hearts of these leptin signaling deficient animals (Belke et al., 2000; Jonathan Buchanan et al., 2005; Mazumder et al., 2004).

Since the functional deficits and metabolic alterations observed in human diabetic individuals were readily observed in mouse models of T2DM and obesity, many investigators have sought to determine whether altered cardiac metabolism could drive pathological cardiac remodeling in the absence of the humoral milieu of diabetes and obesity. Similar associations between lipid accumulation and impaired cardiac function were also evident in transgenic overexpression models of genes involved in fatty acid oxidation such as Acyl co-A synthase (Chiu et al., 2001), or PPARα. Impaired contractile function and increased expression of fatty acid oxidation related genes were apparent upon transgenic overexpression of PPARα (Finck et al., 2002) or PPARγ (Son et al., 2007) key transcriptional regulators of cardiac fatty acid metabolism and glucose metabolism respectively. Triglyceride accumulation in the random fed state was apparent in PPARγ transgenic but not in PPARα transgenic mice unless after fasting suggesting that altered fatty acid metabolism correlated with contractile dysfunction (Finck et al.,

2002; Son et al., 2007). Increased myocardial triglyceride accumulation was also evident following transgenic overexpression of Plin5, a lipid droplet protein important for formation of lipid droplets (Pollak et al., 2013; H. Wang et al., 2013). The increased lipid droplet formation in Plin5-Tg mice was associated with cardiac hypertrophy, decreased fatty acid oxidation, increased ER stress and transcriptional evidence of oxidative stress. Cardiac function as measured by echocardiography and left ventricular catheterization was not impaired despite impaired fatty acid oxidation (Pollak et al., 2013; H. Wang et al., 2013). On the other hand, knockout of Plin5 resulted in excessive fatty acid oxidation and an age dependent systolic dysfunction that was rescued by neutralization of excessive ROS production in Plin5 knockout mice by treatment with N-acetyl cysteine (Kuramoto et al., 2012). In a similar vein, AcadL knockout hearts exhibited elevated triglyceride content and decreased fatty acid oxidation but develop cardiac dysfunction only upon fasting (Bakersman et al., 2011). Other models with increased TG formation such as the cardiac overexpression of DGAT1, however, challenge the notion that triglyceride accumulation is sufficient to cause lipotoxic cardiomyopathy (Liu et al., 2009). The lack of functional deficits in some of these models despite lipid accumulation suggests that complex mechanisms may link altered myocardial lipid accumulation, altered cardiac metabolism and contractile dysfunction. In addition, whether or not these models truly reflect the cardiac dysfunction associated with diabetes and obesity is unclear given that many of these models overexpress or are deprived of the corresponding gene products from early in development.

**1.5 Hyperinsulinemia as a Potential Mediator of Increased HF risk in T2DM and Obesity:**

Hyperinsulinemic states such as T2DM and obesity are associated with an increased risk of heart disease, but the role of hyperinsulinemia in the etiology of cardiomyopathy associated

with these dysmetabolic states is not well understood. A strong association between hyperinsulinemia and risk of heart disease has been suggested (Despres et al., 1996; Perry et al., 1996). Insulin use was associated with increased risk of congestive heart failure (Bertoni et al., 2004; Kannel et al., 1974; Gregory A. Nichols, Hillier, Erbey, & Brown, 2001). Use of the dipeptidyl peptidase-4 (DPP4) inhibitor Saxagliptin, which indirectly increases insulin secretion is associated with increased risk of heart disease (Benjamin M. Scirica et al., 2014). A more recent study in asymptomatic T2DM individuals revealed that hyperinsulinemia and sulfonylurea (drugs which triggers insulin release by inhibiting the $K^+$ATP channels in pancreatic beta cells) use are associated with diastolic dysfunction (Inoue et al., 2016). The mechanistic basis for these aforementioned findings is currently unknown but suggest that hyperinsulinemia could represent a mechanism linking T2DM and development of cardiac dysfunction. This is a mechanism that is relatively unexplored.

The effects of insulin on the heart are difficult to investigate in isolation due to its effects on blood glucose and other metabolic changes that ensue from hyperinsulinemia. Additionally, the detrimental effects of hyperinsulinemia on cardiac function may only become apparent over long-term exposure since cardiac functional deficits in insulin resistant states may develop slowly in response to the compensatory hyperinsulinemia that develops to counter insulin resistance. Although hyperinsulinemic states such as obesity and T2DM are associated with increased risk of heart disease, not all affected individuals with these dysmetabolic states exhibit cardiac functional deficits. Thus additional studies are necessary to understand the effects of insulin on cardiac function under conditions of acute and chronic exposure.

### 1.6 Metabolic and Signaling Consequences of Insulin on the Heart

Under fasting conditions, the major substrate for ATP generation in the heart is long chain fatty acids (LCFA). Following a rise in blood glucose and insulin levels, insulin increases GLUT4 localization to the plasma membrane, indirectly activates phosphofructokinase 2 and enhances glycolytic flux. Thus, glucose contribution to ATP generation in the heart increases following feeding (Bertrand, Horman, Beauloye, & Vanoverschelde, 2008). The effects of insulin on cardiomyocyte metabolism are associated with increased mitochondrial fusion resulting from upregulated Opa1 protein levels and are mediated by AKT, mTOR and NFkB. Inhibition of the activities of these downstream proteins blunted the effects of insulin on cardiomyocyte metabolism and mitochondrial morphology (Parra et al., 2014). Besides, insulin has growth related effects in the heart. Relevant to its physiological effects, insulin signaling in the heart is required for maintenance of heart size, metabolism and contractile protein isoforms as illustrated by a decreased myocyte size, fetal gene expression patterns of metabolism and contractile function related proteins in the cardiac specific insulin receptor knock out (CIRKO) mice (Belke et al., 2002). Signaling downstream of IRS is critical for myocyte survival and prevention of heart failure as failure to suppress autophagy in the neonatal hearts resulted in premature death from heart failure in the IRS1/2 double knock out mice (Riehle et al., 2013). Excessive insulin signaling in the heart can be detrimental. Systolic dysfunction resulting from activation of insulin signaling following pressure overload and myocyte stretch can be prevented by ablation of insulin secreting pancreatic beta cells or by decreasing cardiac insulin receptor expression (I. Shimizu et al., 2010). In addition, insulin also exhibits pleiotropic effects within cardiomyocytes. For example, insulin signaling regulates the expression of transient outward $K^+$ channels and

contributes to ventricular repolarization (Lopez-Izquierdo et al., 2014). Together, these studies exemplify some non-metabolic roles of insulin signaling in the heart.

### 1.7 Effects of Insulin on Cardiac Function

The acute effects of hyperinsulinemia on the heart have been studied in humans and in different animal models. Results of studies in humans have had varying results. Euglycemic hyperinsulinemia increased heart rate, mean arterial pressure (Rowe et al., 1981) and contractile performance (Klein, van Campen, Sieswerda, Kamp, & Visser, 2010). However, other studies revealed no increase in inotropy (Sasso et al., 2000) or chronotropy (Airaksinen, Lahtela, Ikaheimo, Sotaniemi, & Takkunen, 1985; Sasso et al., 2000) during and after euglycemic hyperinsulinemia.

Autonomic regulation during euglycemic hyperinsulinemia has been investigated by multiple groups given the clinical observations supporting autonomic dysregulation in diabetes (Vinik & Ziegler, 2007). A reduction in low and high frequencies in the R-R power spectrum (Muscelli et al., 1998) (Muscelli et al., 1998; Van De Borne, Hausberg, Hoffman, Mark, & Anderson, 1999) were evident with euglycemic hyperinsulinemia suggesting effects on both sympathetic and parasympathetic arms of autonomic regulation. Euglycemic hyperinsulinemia also increased muscle sympathetic nerve activity (E. A. Anderson, Balon, Hoffman, Sinkey, & Mark, 1992; E. A. Anderson, Hoffman, Balon, Sinkey, & Mark, 1991; Berne, Fagius, Pollare, & Hjemdahl, 1992) and plasma catecholamines, but not blood pressure (E. A. Anderson et al., 1991; Vollenweider et al., 1994; Vollenweider et al., 1993). These data suggest that insulin exerts a range of cardiovascular effects in the absence of hypoglycemia and emphasizing that these effects of insulin are not secondary to counter regulatory responses to hypoglycemia.

In model systems, acute exposure to insulin has been reported to have direct effects on myocardial contractility and indirect effects in modulating the response to catecholamines. Insulin acutely increased force generation in pig moderator bands (Klinge & Wafin, 1971) and chronotropy in isolated dog atria (Chi 1974). These observations are also consistent in vivo in dogs (Liang et al., 1982), although only upon blockade of beta-adrenergic receptors prior to application of insulin (Lucchesi, Medina, & Kniffen, 1972). Substantially high doses of insulin were required to observe increased cardiac contractility in dogs (Reikeras & Gunnes, 1986; Reikeras et al., 1985) and in neonatal pigs (Rieker, Lee, & Downing, 1975). The incessant regulation of cardiac function by the autonomic nervous system has emphasized the necessity for understanding the effect of inotropic agents in insulin treated preparations. Insulin pretreatment attenuated (a) the chronotropic effect of epinephrine in insulin pretreated dogs (Hiatt & Katz, 1969) (b) norepinephrine induced contractility in piglet hearts (Nudel, Lee, & Downing, 1977) and (c) inotropic effect of norepinephrine in pig moderator bands and feline papillary muscle (J. C. Lee & Downing, 1976). These observations are of clinical importance given the impaired cardiac function and cardiac reserve in diabetic individuals (Baldi, Wilson, Wilson, Wilkins, & Lamberts, 2016; Pinto et al., 2014).

A recent study shed additional insight on mechanistic aspects of the acute effects of insulin on cardiac contractility. Decreased inotropic response to isoproterenol in insulin pre-perfused hearts was mediated by insulin induced G-protein coupled Receptor Kinase 2 (GRK2) dependent phosphorylation of the $\beta_2$-adrenergic receptor ($\beta_2$AR). $\beta_2$AR phosphorylation following exposure to insulin resulted in switch in coupling of the receptor from $G\alpha_s$ to $G\alpha_i$. Subsequent activation of the $\beta_2$AR by isoproterenol induced activation of $G\alpha_i$ resulting in

increased inhibitory tone on adenylate cyclase and βarrestin-2 (βarr2) dependent internalization of the receptor (Fu et al., 2014). These findings were primarily from *ex vivo* heart perfusion and *in vitro* cell culture experiments and therefore need validation *in vivo* under conditions of afterload and variable hemodynamics.

G-protein coupled Receptor Kinase 2 (GPCR kinase 2 or GRK2) is a Ser/Thr kinase that phosphorylates GPCRs in a homologous desensitization process. GRK2 expression and activity is increased in the failing hearts and increased expression of this kinase has been suggested to be pathological (Ungerer et al., 1994). More recently, GRK2 has been identified to mediate additional signaling processes in cardiomyocytes that are independent of GPCR activation (Brinks et al., 2010; M. Chen et al., 2013; Ciccarelli et al., 2011; Fan et al., 2013). In this context, cultured cardiomyocytes exposed to insulin for longer durations (24-48h) caused increased ERK1/2 activation and phosphodiesterase 4D (PDE4D) that was sensitive to inhibition of GRK2 (Q. Wang et al., 2017). ERK1/2 is an important kinase with critical role in promoting pathological hypertrophy (Bueno et al., 2000) and PDE4D decreases the tone of PKA signaling indirectly by increasing cAMP degradation. Further role for GRK2 in diabetic cardiomyopathy was apparent when wildtype mice subject to HFD feeding developed cardiac dysfunction and these functional deficits were rescued upon treatment with Paroxetine (Q. Wang et al., 2017), a selective serotonin reuptake inhibitor which also inhibits GRK2 (Thal et al., 2012). These changes also correlated with attenuation of HFD induced PDE4D expression and ERK1/2 activation in mice treated with paroxetine suggesting that PDE4D might be a downstream target of ERK1/2, which is activated by HFD feeding (Q. Wang et al., 2017).

Cardiomyopathy associated with diabetes and obesity has been established in mouse models of T2DM and obesity including the db/db and ob/ob mice (Belke et al., 2000; Jonathan Buchanan et al., 2005; Dong et al., 2006; Mazumder et al., 2004; Semeniuk et al., 2002). These mutant models however suffer the disadvantage of altered cardiac substrate metabolism from as early as 4 weeks (J. Buchanan et al., 2005) and it is possible that compensatory changes in metabolic pathways and gene expression exist in these models that may interfere with understanding the role of GRK2 in diabetic cardiomyopathy. Therefore, in order to study the effect of diabetes and obesity on cardiac function in the absence of genetic manipulations that render mice obese or diabetic, a previously described long-term high fat diet feeding model has been proposed, given the reliability and reproducibility of functional deficits within the myocardium (Battiprolu et al., 2012; S. Y. Park et al., 2005).

**1.8 Project Goals**

From the summary of literature provided thus far, it is apparent that the acute effects of insulin on the heart *ex vivo* and the chronic effects of high fat feeding on myocardial structure and function may be mediated by GRK2, although via different mechanisms. We therefore aimed at understanding the effects of hyperinsulinemia and importance of cardiomyocyte GRK2 in diabetic cardiomyopathy. Since the effects of acute euglycemic hyperinsulinemia on myocardial function have not been validated thus far in mice, the primary goal of this project was to identify the signaling and functional consequences of:

1) Acute euglycemic hyperinsulinemia on endogenous cardiac contractility in vivo
2) Acute euglycemic hyperinsulinemia on myocardial β-adrenergic responsiveness

Contingent on the differences in outcome of acute hyperinsulinemia on the myocardial β-adrenergic responsiveness, our goal was to validate the role of GRK2 in mediating the effects of acute hyperinsulinemia *in vivo*.

The secondary goal is to determine if GRK2 mediates chronic fat feeding induced pathological remodeling and decline in cardiac function.

REFERENCES


Airaksinen, J., Lahtela, J. T., Ikaheimo, M. J., Sotaniemi, E. A., & Takkunen, J. T. (1985). Intravenous insulin has no effect on myocardial contractility or heart rate in healthy subjects. *Diabetologia, 28*(9), 649-652.

Anderson, E. A., Balon, T. W., Hoffman, R. P., Sinkey, C. A., & Mark, A. L. (1992). Insulin increases sympathetic activity but not blood pressure in borderline hypertensive humans. *Hypertension, 19*(6 Pt 2), 621-627.

Anderson, E. A., Hoffman, R. P., Balon, T. W., Sinkey, C. A., & Mark, A. L. (1991). Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans. *J Clin Invest, 87*(6), 2246-2252. doi:10.1172/jci115260

Astorri, E., Fiorina, P., Contini, G. A., Albertini, D., Magnati, G., Astorri, A., & Lanfredini, M. (1997). Isolated and preclinical impairment of left ventricular filling in insulin-dependent and non-insulin-dependent diabetic patients. *Clinical cardiology, 20*(6), 536-540.

Baldi, J. C., Wilson, G. A., Wilson, L. C., Wilkins, G. T., & Lamberts, R. R. (2016). The Type 2 Diabetic Heart: Its Role in Exercise Intolerance and the Challenge to Find Effective Exercise Interventions. *Sports Med, 46*(11), 1605-1617. doi:10.1007/s40279-016-0542-9

Battiprolu, P. K., Hojayev, B., Jiang, N., Wang, Z. V., Luo, X., Iglewski, M., . . . Hill, J. A. (2012). Metabolic stress-induced activation of FoxO1 triggers diabetic cardiomyopathy in mice. *J Clin Invest, 122*(3), 1109-1118. doi:10.1172/jci60329

Belke, D. D., Betuing, S., Tuttle, M. J., Graveleau, C., Young, M. E., Pham, M., . . . Abel, E. D. (2002). Insulin signaling coordinately regulates cardiac size, metabolism, and contractile protein isoform expression. *J Clin Invest, 109*(5), 629-639. doi:10.1172/jci13946

Belke, D. D., Larsen, T. S., Gibbs, E. M., & Severson, D. L. (2000). Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (db/db) mice. *Am J Physiol Endocrinol Metab, 279*(5), E1104-1113. doi:10.1152/ajpendo.2000.279.5.E1104

Berne, C., Fagius, J., Pollare, T., & Hjemdahl, P. (1992). The sympathetic response to euglycaemic hyperinsulinaemia. Evidence from microelectrode nerve recordings in healthy subjects. *Diabetologia, 35*(9), 873-879.

Bertoni, A. G., Hundley, G. W., Massing, M. W., Bonds, D. E., Burke, G. L., & Goff, D. C. (2004). Heart Failure Prevalence, Incidence, and Mortality in the Elderly With Diabetes. *Diabetes Care, 27*(3), 699-703. doi:10.2337/diacare.27.3.699

Bertrand, L., Horman, S., Beauloye, C., & Vanoverschelde, J. L. (2008). Insulin signalling in the heart. *Cardiovasc Res, 79*(2), 238-248. doi:10.1093/cvr/cvn093

Boyer, J. K., Thanigaraj, S., Schechtman, K. B., & Perez, J. E. (2004). Prevalence of ventricular diastolic dysfunction in asymptomatic, normotensive patients with diabetes mellitus. *Am J Cardiol, 93*(7), 870-875. doi:10.1016/j.amjcard.2003.12.026

Brinks, H., Boucher, M., Gao, E., Chuprun, J. K., Pesant, S., Raake, P. W., . . . Koch, W. J. (2010). Level of G protein-coupled receptor kinase-2 determines myocardial ischemia/reperfusion injury via pro- and anti-apoptotic mechanisms. *Circ Res, 107*(9), 1140-1149. doi:10.1161/circresaha.110.221010

Buchanan, J., Mazumder, P. K., Hu, P., Chakrabarti, G., Roberts, M. W., Yun, U., . . . Abel, D. E. (2005). Reduced cardiac efficiency and altered substrate metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity. *Endocrinology, 146*(12), 5341-5349. doi:10.1210/en.2005-0938

Buchanan, J., Mazumder, P. K., Hu, P., Chakrabarti, G., Roberts, M. W., Yun, U. J., . . . Abel, E. D. (2005). Reduced cardiac efficiency and altered substrate metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity. *Endocrinology, 146*(12), 5341-5349. doi:10.1210/en.2005-0938

Bueno, O. F., De Windt, L. J., Tymitz, K. M., Witt, S. A., Kimball, T. R., Klevitsky, R., . . . Molkentin, J. D. (2000). The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. *Embo j, 19*(23), 6341-6350. doi:10.1093/emboj/19.23.6341

Chen, M., Sato, P. Y., Chuprun, J. K., Peroutka, R. J., Otis, N. J., Ibetti, J., . . . Koch, W. J. (2013). Prodeath signaling of G protein-coupled receptor kinase 2 in cardiac myocytes after ischemic stress occurs via extracellular signal-regulated kinase-dependent heat shock protein 90-mediated mitochondrial targeting. *Circ Res, 112*(8), 1121-1134. doi:10.1161/circresaha.112.300754

Cheng, S., Xanthakis, V., Sullivan, L. M., & Circulation, L. W. (2010). Correlates of echocardiographic indices of cardiac remodeling over the adult life course: longitudinal observations from the Framingham Heart Study. *Correlates of echocardiographic indices of cardiac remodeling over the adult life course: longitudinal observations from the Framingham Heart Study*.

Chiu, H.-C., Kovacs, A., Ford, D. A., Hsu, F.-F., Garcia, R., Herrero, P., . . . Schaffer, J. E. (2001). A novel mouse model of lipotoxic cardiomyopathy. *Journal of Clinical Investigation, 107*(7), 813-822. doi:10.1172/JCI10947

Christoffersen, C., Bollano, E., Lindegaard, M. L., Bartels, E. D., Goetze, J. P., Andersen, C. B., & Nielsen, L. B. (2003). Cardiac lipid accumulation associated with diastolic dysfunction in obese mice. *Endocrinology, 144*(8), 3483-3490. doi:10.1210/en.2003-0242

Ciccarelli, M., Chuprun, J. K., Rengo, G., Gao, E., Wei, Z., Peroutka, R. J., . . . Koch, W. J. (2011). G protein-coupled receptor kinase 2 activity impairs cardiac glucose uptake and promotes insulin resistance after myocardial ischemia. *Circulation, 123*(18), 1953-1962. doi:10.1161/circulationaha.110.988642

de Simone, G., Devereux, R. B., Chinali, M., Lee, E. T., Galloway, J. M., Barac, A., . . . Howard, B. V. (2010). Diabetes and incident heart failure in hypertensive and normotensive participants of the Strong Heart Study. *J Hypertens, 28*(2), 353-360. doi:10.1097/HJH.0b013e3283331169

Despres, J. P., Lamarche, B., Mauriege, P., Cantin, B., Dagenais, G. R., Moorjani, S., & Lupien, P. J. (1996). Hyperinsulinemia as an independent risk factor for ischemic heart disease. *N Engl J Med, 334*(15), 952-957. doi:10.1056/nejm199604113341504

Devereux, R. B., Roman, M. J., Paranicas, M., O'Grady, M. J., Lee, E. T., Welty, T. K., . . . Howard, B. V. (2000). Impact of diabetes on cardiac structure and function: the strong heart study. *Circulation, 101*(19), 2271-2276.

Diamant, M., Lamb, H. J., Groeneveld, Y., Endert, E. L., Smit, J. W., Bax, J. J., . . . Radder, J. K. (2003). Diastolic dysfunction is associated with altered myocardial metabolism in asymptomatic normotensive patients with well-controlled type 2 diabetes mellitus. *J Am Coll Cardiol, 42*(2), 328-335.

Dinh, W., Lankisch, M., Nickl, W., Scheyer, D., Scheffold, T., Kramer, F., . . . Futh, R. (2010). Insulin resistance and glycemic abnormalities are associated with deterioration of left ventricular diastolic function: a cross-sectional study. *Cardiovasc Diabetol, 9*, 63. doi:10.1186/1475-2840-9-63

Dong, F., Zhang, X., Yang, X., Esberg, L. B., Yang, H., Zhang, Z., . . . Ren, J. (2006). Impaired cardiac contractile function in ventricular myocytes from leptin-deficient ob/ob obese mice. *J Endocrinol, 188*(1), 25-36. doi:10.1677/joe.1.06241

Economic Costs of Diabetes in the U.S. in 2017. (2018). *Diabetes Care, 41*(5), 917-928. doi:10.2337/dci18-0007

Ernande, L., Bergerot, C., Rietzschel, E. R., De Buyzere, M. L., Thibault, H., Pignonblanc, P. G., . . . Derumeaux, G. (2011). Diastolic dysfunction in patients with type 2 diabetes mellitus: is it really the first marker of diabetic cardiomyopathy? *J Am Soc Echocardiogr, 24*(11), 1268-1275.e1261. doi:10.1016/j.echo.2011.07.017

Fan, Q., Chen, M., Zuo, L., Shang, X., Huang, M. Z., Ciccarelli, M., . . . Gao, E. (2013). Myocardial Ablation of G Protein-Coupled Receptor Kinase 2 (GRK2) Decreases Ischemia/Reperfusion Injury through an Anti-Intrinsic Apoptotic Pathway. *PLoS One, 8*(6), e66234. doi:10.1371/journal.pone.0066234

Fang, Z. Y., Schull-Meade, R., Downey, M., Prins, J., & Marwick, T. H. (2005). Determinants of subclinical diabetic heart disease. *Diabetologia, 48*(2), 394-402. doi:10.1007/s00125-004-1632-z

Finck, B. N., Lehman, J. J., Leone, T. C., Welch, M. J., Bennett, M. J., Kovacs, A., . . . Kelly, D. P. (2002). The cardiac phenotype induced by PPARα overexpression mimics that caused by diabetes mellitus. *Journal of Clinical Investigation, 109*(1), 121. doi:10.1172/JCI14080

Fu, Q., Xu, B., Liu, Y., Parikh, D., Li, J., Li, Y., . . . Xiang, Y. K. (2014). Insulin inhibits cardiac contractility by inducing a Gi-biased beta2-adrenergic signaling in hearts. *Diabetes, 63*(8), 2676-2689. doi:10.2337/db13-1763

Galderisi, M., Anderson, K. M., Wilson, P., & Levy, D. (1991). Echocardiographic evidence for the existence of a distinct diabetic cardiomyopathy (The Framingham Heart Study). *Am J Cardiol, 68*(1), 85-89. doi:10.1016/0002-9149(91)90716-X

Hiatt, N., & Katz, J. (1969). Modification of cardiac and hyperglycemic effects of epinephrine by iulin. *Life Sci, 8*(9), 551-558.

Inoue, T., Maeda, Y., Sonoda, N., Sasaki, S., Kabemura, T., Kobayashi, K., & Inoguchi, T. (2016). Hyperinsulinemia and sulfonylurea use are independently associated with left ventricular diastolic dysfunction in patients with type 2 diabetes mellitus with suboptimal blood glucose control. *BMJ Open Diabetes Res Care, 4*(1), e000223. doi:10.1136/bmjdrc-2016-000223

Kannel, W. B., Hjortland, M., & Castelli, W. P. (1974). Role of diabetes in congestive heart failure: the Framingham study. *Am J Cardiol, 34*(1), 29-34.

Kannel, W. B., LeBauer, E. J., Dawber, T. R., & McNamara, P. M. (1967). Relation of body weight to development of coronary heart disease. The Framingham study. *Circulation, 35*(4), 734-744.

Kannel, W. B., & McGee, D. L. (1979). Diabetes and cardiovascular disease. The Framingham study. *Jama, 241*(19), 2035-2038.

Kawaguchi, M., Techigawara, M., Ishihata, T., Asakura, T., Saito, F., Maehara, K., & Maruyama, Y. (1997). A comparison of ultrastructural changes on endomyocardial biopsy specimens obtained from patients with diabetes mellitus with and without hypertension. *Heart Vessels, 12*(6), 267-274.

Kenchaiah, S., Evans, J. C., Levy, D., Wilson, P. W., Benjamin, E. J., Larson, M. G., . . . Vasan, R. S. (2002). Obesity and the risk of heart failure. *N Engl J Med, 347*(5), 305-313. doi:10.1056/NEJMoa020245

Klein, L. J., van Campen, C. M., Sieswerda, G. T., Kamp, O., & Visser, F. C. (2010). Effects of high-dose insulin infusion on left ventricular function in normal subjects. *Neth Heart J, 18*(4), 183-189.

Klinge, E., & Wafin, F. (1971). Increase in cardiac contractile force caused by pork insulin. *Ann Med Exp Biol Fenn, 49*(3), 138-142.

Kuramoto, K., Okamura, T., Yamaguchi, T., Nakamura, T. Y., Wakabayashi, S., Morinaga, H., . . . Osumi, T. (2012). Perilipin 5, a lipid droplet-binding protein, protects heart from oxidative burden by sequestering fatty acid from excessive oxidation. *J Biol Chem, 287*(28), 23852-23863. doi:10.1074/jbc.M111.328708

Labbe, S. M., Grenier-Larouche, T., Noll, C., Phoenix, S., Guerin, B., Turcotte, E. E., & Carpentier, A. C. (2012). Increased myocardial uptake of dietary fatty acids linked to cardiac dysfunction in glucose-intolerant humans. *Diabetes, 61*(11), 2701-2710. doi:10.2337/db11-1805

Lauer, M. S., Anderson, K. M., Kannel, W. B., & Levy, D. (1991). The impact of obesity on left ventricular mass and geometry. The Framingham Heart Study. *Jama, 266*(2), 231-236.

Lee, J. C., & Downing, S. E. (1976). Effects of insulin on cardiac muscle contraction and responsiveness to norepinephrine. *Am J Physiol, 230*(5), 1360-1365. doi:10.1152/ajplegacy.1976.230.5.1360

Lee, M., Gardin, J. M., Lynch, J. C., Smith, V. E., Tracy, R. P., Savage, P. J., . . . Ward, B. J. (1997). Diabetes mellitus and echocardiographic left ventricular function in free-living elderly men and women: The Cardiovascular Health Study. *Am Heart J, 133*(1), 36-43.

Levelt, E., Mahmod, M., Piechnik, S. K., Ariga, R., Francis, J. M., Rodgers, C. T., . . . Neubauer, S. (2016). Relationship Between Left Ventricular Structural and Metabolic Remodeling in Type 2 Diabetes. *Diabetes, 65*(1), 44-52. doi:10.2337/db15-0627

Liang, C., Doherty, J. U., Faillace, R., Maekawa, K., Arnold, S., Gavras, H., & Hood, W. B., Jr. (1982). Insulin infusion in conscious dogs. Effects on systemic and coronary hemodynamics, regional blood flows, and plasma catecholamines. *J Clin Invest, 69*(6), 1321-1336.

Liu, L., Shi, X., Bharadwaj, K. G., Ikeda, S., Yamashita, H., Yagyu, H., . . . Goldberg, I. J. (2009). DGAT1 Expression Increases Heart Triglyceride Content but Ameliorates Lipotoxicity. *Journal of Biological Chemistry, 284*(52), 36312-36323. doi:10.1074/jbc.M109.049817

Lopez-Izquierdo, A., Pereira, R. O., Wende, A. R., Punske, B. B., Abel, E. D., & Tristani-Firouzi, M. (2014). The absence of insulin signaling in the heart induces changes in potassium channel expression and ventricular repolarization. *Am J Physiol Heart Circ Physiol, 306*(5), H747-754. doi:10.1152/ajpheart.00849.2013

Lucchesi, B. R., Medina, M., & Kniffen, F. J. (1972). The positive inotropic action of insulin in the canine heart. *Eur J Pharmacol, 18*(1), 107-115.

Mazumder, P. K., O'Neill, B. T., Roberts, M. W., Buchanan, J., Yun, U., Cooksey, R. C., . . . Abel, D. E. (2004). Impaired Cardiac Efficiency and Increased Fatty Acid Oxidation in Insulin-Resistant ob/ob Mouse Hearts. *Diabetes, 53*(9), 2366-2374. doi:10.2337/diabetes.53.9.2366

Muscelli, E., Emdin, M., Natali, A., Pratali, L., Camastra, S., Gastaldelli, A., . . . Ferrannini, E. (1998). Autonomic and hemodynamic responses to insulin in lean and obese humans. *J Clin Endocrinol Metab, 83*(6), 2084-2090. doi:10.1210/jcem.83.6.4878

Nichols, G. A., Gullion, C. M., Koro, C. E., & Diabetes …, E. S. A. (2004). The incidence of congestive heart failure in type 2 diabetes: an update. *The incidence of congestive heart failure in type 2 diabetes: an update*. doi:10.2337/diacare.27.8.1879

Nichols, G. A., Hillier, T. A., Erbey, J. R., & Brown, J. B. (2001). Congestive Heart Failure in Type 2 Diabetes. *Diabetes Care, 24*(9), 1614-1619. doi:10.2337/diacare.24.9.1614

Nudel, D. B., Lee, J. C., & Downing, S. E. (1977). Reciprocal inhibition of cardiac responses to norepinephrine and insulin. *Am J Physiol, 233*(6), H665-669. doi:10.1152/ajpheart.1977.233.6.H665

Park, S. Y., Cho, Y. R., Kim, H. J., Higashimori, T., Danton, C., Lee, M. K., . . . Kim, J. K. (2005). Unraveling the temporal pattern of diet-induced insulin resistance in individual organs and cardiac dysfunction in C57BL/6 mice. *Diabetes, 54*(12), 3530-3540.

Parra, V., Verdejo, H. E., Iglewski, M., Del Campo, A., Troncoso, R., Jones, D., . . . Lavandero, S. (2014). Insulin stimulates mitochondrial fusion and function in cardiomyocytes via the Akt-mTOR-NFkappaB-Opa-1 signaling pathway. *Diabetes, 63*(1), 75-88. doi:10.2337/db13-0340

Perry, I. J., Wannamethee, S. G., Whincup, P. H., Shaper, A. G., Walker, M. K., & Alberti, K. G. (1996). Serum insulin and incident coronary heart disease in middle-aged British men. *Am J Epidemiol, 144*(3), 224-234.

Peterson, L. R., Herrero, P., Schechtman, K. B., Racette, S. B., Waggoner, A. D., Kisrieva-Ware, Z., . . . Gropler, R. J. (2004). Effect of Obesity and Insulin Resistance on Myocardial Substrate Metabolism and Efficiency in Young Women. *Circulation, 109*(18), 2191-2196. doi:10.1161/01.cir.0000127959.28627.f8

Pinto, T. E., Gusso, S., Hofman, P. L., Derraik, J. G., Hornung, T. S., Cutfield, W. S., & Baldi, J. C. (2014). Systolic and diastolic abnormalities reduce the cardiac response to exercise in adolescents with type 2 diabetes. *Diabetes Care, 37*(5), 1439-1446. doi:10.2337/dc13-2031

Poirier, P., Bogaty, P., Garneau, C., Marois, L., & Dumesnil, J.-G. (2001). Diastolic Dysfunction in Normotensive Men with Well-Controlled Type 2 Diabetes. *Diabetes Care, 24*(1), 5-10. doi:10.2337/diacare.24.1.5

Pollak, N. M., Schweiger, M., Jaeger, D., Kolb, D., Kumari, M., Schreiber, R., . . . Haemmerle, G. (2013). Cardiac-specific overexpression of perilipin 5 provokes severe cardiac steatosis via the formation of a lipolytic barrier. *J Lipid Res, 54*(4), 1092-1102. doi:10.1194/jlr.M034710

Regan, T. J., Lyons, M. M., Ahmed, S. S., Levinson, G. E., Oldewurtel, H. A., Ahmad, M. R., & Haider, B. (1977). Evidence for cardiomyopathy in familial diabetes mellitus. *J Clin Invest, 60*(4), 884-899. doi:10.1172/jci108843

Reikeras, O., & Gunnes, P. (1986). Effects of high doses of insulin on systemic haemodynamics and regional blood flows in dogs. *Clin Physiol, 6*(2), 129-138.

Reikeras, O., Gunnes, P., Sorlie, D., Ekroth, R., Jorde, R., & Mjos, O. D. (1985). Haemodynamic effects of low and high doses of insulin during beta receptor blockade in dogs. *Clin Physiol, 5*(5), 455-467.

Riehle, C., Wende, A. R., Sena, S., Pires, K. M., Pereira, R. O., Zhu, Y., . . . Abel, E. D. (2013). Insulin receptor substrate signaling suppresses neonatal autophagy in the heart. *J Clin Invest, 123*(12), 5319-5333. doi:10.1172/jci71171

Rieker, R. P., Lee, J. C., & Downing, S. E. (1975). Positive inotropic action of insulin on piglet heart. *Yale J Biol Med, 48*(5), 353-360.

Rijzewijk, L. J., van der Meer, R. W., & of the …, L. H. J. (2009). Altered myocardial substrate metabolism and decreased diastolic function in nonischemic human diabetic cardiomyopathy: studies with cardiac positron emission …. *Altered myocardial substrate metabolism and decreased diastolic function in nonischemic human diabetic cardiomyopathy: studies with cardiac positron emission ….*

Rowe, J. W., Young, J. B., Minaker, K. L., Stevens, A. L., Pallotta, J., & Landsberg, L. (1981). Effect of insulin and glucose infusions on sympathetic nervous system activity in normal man. *Diabetes, 30*(3), 219-225.

Rubler, S., Dlugash, J., Yuceoglu, Y. Z., Kumral, T., Branwood, A. W., & Grishman, A. (1972). New type of cardiomyopathy associated with diabetic glomerulosclerosis. *Am J Cardiol, 30*(6), 595-602.

Rutter, M. K., Parise, H., Benjamin, E. J., Levy, D., Larson, M. G., Meigs, J. B., . . . Vasan, R. S. (2003). Impact of glucose intolerance and insulin resistance on cardiac structure and function: sex-related differences in the Framingham Heart Study. *Circulation*, 448-454.

Sasso, F. C., Carbonara, O., Cozzolino, D., Rambaldi, P., Mansi, L., Torella, D., . . . Salvatore, T. (2000). Effects of insulin-glucose infusion on left ventricular function at rest and during dynamic exercise in healthy subjects and noninsulin dependent diabetic patients: a radionuclide ventriculographic study. *J Am Coll Cardiol, 36*(1), 219-226.

Scirica, B. M., Braunwald, E., Raz, I., Cavender, M. A., Morrow, D. A., Jarolim, P., . . . and Investigators*, S.-T. (2014). Heart Failure, Saxagliptin, and Diabetes Mellitus: Observations from the SAVOR-TIMI 53 Randomized Trial. *Circulation, 130*(18), 1579-1588. doi:10.1161/CIRCULATIONAHA.114.010389

Semeniuk, L. M., Kryski, A. J., & Severson, D. L. (2002). Echocardiographic assessment of cardiac function in diabetic db/db and transgenic db/db-hGLUT4 mice. *Am J Physiol Heart Circ Physiol, 283*(3), H976-982. doi:10.1152/ajpheart.00088.2002

Shimizu, I., Minamino, T., Toko, H., Okada, S., Ikeda, H., Yasuda, N., . . . Komuro, I. (2010). Excessive cardiac insulin signaling exacerbates systolic dysfunction induced by pressure overload in rodents. *J Clin Invest, 120*(5), 1506-1514. doi:10.1172/jci40096

Shimizu, M., Umeda, K., Sugihara, N., Yoshio, H., Ino, H., Takeda, R., . . . Nakanishi, I. (1993). Collagen remodelling in myocardia of patients with diabetes. *J Clin Pathol, 46*(1), 32-36.

Son, N. H., Park, T. S., Yamashita, H., Yokoyama, M., Huggins, L. A., Okajima, K., . . . Goldberg, I. J. (2007). Cardiomyocyte expression of PPARgamma leads to cardiac dysfunction in mice. *J Clin Invest, 117*(10), 2791-2801. doi:10.1172/jci30335

Sundström, J., Lind, L., Nyström, N., & Circulation, Z. B. (2000). Left ventricular concentric remodeling rather than left ventricular hypertrophy is related to the insulin resistance syndrome in elderly men. *Left ventricular concentric remodeling rather than left ventricular hypertrophy is related to the insulin resistance syndrome in elderly men*.

Szczepaniak, L. S., Dobbins, R. L., Metzger, G. J., Sartoni-D'Ambrosia, G., Arbique, D., Vongpatanasin, W., . . . Victor, R. G. (2003). Myocardial triglycerides and systolic function in humans: in vivo evaluation by localized proton spectroscopy and cardiac imaging. *Magn Reson Med, 49*(3), 417-423. doi:10.1002/mrm.10372

Thal, D. M., Homan, K. T., Chen, J., Wu, E. K., Hinkle, P. M., Huang, Z. M., . . . Tesmer, J. J. (2012). Paroxetine is a direct inhibitor of g protein-coupled receptor kinase 2 and increases myocardial contractility. *ACS Chem Biol, 7*(11), 1830-1839. doi:10.1021/cb3003013

Ungerer, M., Parruti, G., Bohm, M., Puzicha, M., DeBlasi, A., Erdmann, E., & Lohse, M. J. (1994). Expression of beta-arrestins and beta-adrenergic receptor kinases in the failing human heart. *Circ Res, 74*(2), 206-213.

Van De Borne, P., Hausberg, M., Hoffman, R. P., Mark, A. L., & Anderson, E. A. (1999). Hyperinsulinemia produces cardiac vagal withdrawal and nonuniform sympathetic activation in normal subjects. *Am J Physiol, 276*(1), R178-183. doi:10.1152/ajpregu.1999.276.1.R178

Vinik, A. I., & Ziegler, D. (2007). Diabetic cardiovascular autonomic neuropathy. *Circulation, 115*(3), 387-397. doi:10.1161/circulationaha.106.634949

Vollenweider, P., Randin, D., Tappy, L., Jequier, E., Nicod, P., & Scherrer, U. (1994). Impaired insulin-induced sympathetic neural activation and vasodilation in skeletal muscle in obese humans. *J Clin Invest, 93*(6), 2365-2371. doi:10.1172/jci117242

Vollenweider, P., Tappy, L., Randin, D., Schneiter, P., Jequier, E., Nicod, P., & Scherrer, U. (1993). Differential effects of hyperinsulinemia and carbohydrate metabolism on sympathetic nerve activity and muscle blood flow in humans. *J Clin Invest, 92*(1), 147-154. doi:10.1172/jci116542

Wang, H., Sreenivasan, U., Gong, D. W., O'Connell, K. A., Dabkowski, E. R., Hecker, P. A., . . . Sztalryd, C. (2013). Cardiomyocyte-specific perilipin 5 overexpression leads to myocardial steatosis and modest cardiac dysfunction. *J Lipid Res, 54*(4), 953-965. doi:10.1194/jlr.M032466

Wang, Q., Liu, Y., Fu, Q., Xu, B., Zhang, Y., Kim, S., . . . Xiang, Y. K. (2017). Inhibiting Insulin-Mediated beta2-Adrenergic Receptor Activation Prevents Diabetes-Associated Cardiac Dysfunction. *Circulation, 135*(1), 73-88. doi:10.1161/circulationaha.116.022281

# CHAPTER 2: ACUTE EFFECTS OF EUGLYCEMIC-HYPERINSULINEMIA ON CARDIAC CONTRACTILITY INDUCED BY β-ADRENERGIC RECEPTOR STIMULATION

## 2.1 Introduction:

Hyperinsulinemia is an established risk factor for ischemic heart disease (Despres et al., 1996; Perry et al., 1996). Even after adjusting for hypertension and coronary artery disease, hyperinsulinemic states such as Type 2 diabetes and obesity are associated with increased risk of heart disease, suggesting that hyperinsulinemia could independently contribute to the development of cardiac dysfunction in these dysmetabolic states (M. Galderisi, K. M. Anderson, P. W. Wilson, & D. Levy, 1991). Whether hyperinsulinemia affects cardiac function is not well understood. In humans, euglycemic hyperinsulinemia increased heart rate, mean arterial pressure (Rowe et al., 1981) and contractile performance (Klein et al., 2010). However, these findings are challenged by other studies where no difference in inotropic state (Sasso et al., 2000) or a chronotropic effect (Airaksinen et al., 1985; Sasso et al., 2000) was observed during and after euglycemic hyperinsulinemia.

Studies using model systems for investigating the acute effects of hyperinsulinemia have suggested a direct effect of insulin on cardiac function. Acute exposure to insulin resulted in increased force generation in cardiac muscle strips from pigs (Klinge & Wafin, 1971). These observations are also consistent *in vivo* in dogs (Liang et al., 1982), although only upon blockade of β-adrenergic receptors prior to application of insulin (Lucchesi et al., 1972). Substantially high doses of insulin were required to elicit chronotropy in isolated dog atria (Chiba, 1974), to increase cardiac contractility in dogs (Reikeras & Gunnes, 1986; Reikeras et al., 1985) and in neonatal pigs (Rieker et al., 1975). However, the sensitivity of the direct cardiac effect of insulin

to beta-blockade was also not consistent between different studies (Chiba, 1974; Rieker et al., 1975) highlighting the need for more rigorous analysis.

The incessant regulation of cardiac function by the autonomic nervous system has emphasized the necessity for understanding the effect of inotropic stimuli in insulin treated preparations. In a recent report (Fu Q et al 2014), insulin pretreatment attenuated the inotropic response to isoproterenol in Langendorff perfused mouse hearts. Mechanistically, this effect of insulin was mediated by GRK2 dependent phosphorylation of the $\beta_2$-adrenergic receptor, which primed the $\beta_2$-adrenergic receptor for coupling with $G\alpha_i$ upon subsequent stimulation with β-adrenergic receptor agonists. The switch in coupling of $\beta_2$-adrenergic receptor was sufficient to attenuate PKA activation following stimulation of β-adrenergic receptors by isoproterenol. A similar attenuated response to catecholamines was also demonstrated previously in insulin pretreated preparations, although in different species (Hiatt & Katz, 1969; Nudel et al., 1977) (J. C. Lee & Downing, 1976). These observations could be of clinical importance given the impaired cardiac function and cardiac reserve in diabetic individuals (Baldi et al., 2016; Pinto et al., 2014). Whether insulin inhibits cardiac contractility induced by β-adrenergic stimulation in an intact animal under euglycemic state has not been thoroughly assessed. Therefore, the goal of this study was to understand the *in vivo* effects of supraphysiological levels of insulin on contractility induced by β-adrenergic receptor stimulation.

## 2.2 Methods:

### 2.2.1. Animals:

C57BL6/J mice aged 6-8 weeks were purchased from Jackson Laboratories (Bar Harbor, Maine) and were housed in the animal facility maintained with a 12h dark:light cycle. Mice had adlib access to water and food and were fasted for 4-6h on the day of experiments.

### 2.2.2. Reagents:

Humulin R-100 was purchased from Eli Lilly (Indianpolis, IN). Isoproterenol (Catalog# I6504) was purchased from Sigma (St. Louis, MO). pPLN S16 antibody (Catalog# A010-12) was purchased from Badrilla (Leeds, UK), total PLN antibody (Catalog# ab2865) was purchased from Abcam (Cambridge, UK). pAKT S473 antibody (Catalog# 4060S) and Pan AKT (Catalog# 2920S) were purchased from Cell Signaling.

### 2.2.3. Intravenous Injections:

Insulin was injected at a dose of 1U/kg into the right jugular vein of mice under isoflurane anesthesia (induction dose - 5%v/v, maintenance dose - 1.5% v/v). The injection volume was fixed at 20uL and the control group received an equivalent volume of saline. Cardiac catheterization was performed to measure changes in contractile function from baseline following i.v. insulin injection and in response to intraperitoneal injections of isoproterenol.

### 2.2.4. Hyperinsulinemic Euglycemic Clamps:

The procedure for euglycemic hyperinsulinemic clamps has been previously described (Ayala et al., 2011). Briefly, mice were surgically implanted with a PE-50 tubing catheter in the right external jugular vein and allowed to recover for 7-10 days. On the day of experiment mice were fasted for 5-6h and anesthetized using 5% (v/v) isoflurane anesthesia to test the patency of

catheters and animals with patent catheters were used for the experiments. Blood glucose measurements were made on each mouse, one measurement every 10 minutes beginning at 20 minutes prior to the start of infusions until 120 minutes after the infusion. At time T0, mice were infused with a bolus dose of 160mU/kg insulin followed by 16mU/kg/min insulin for 2h concomitantly with a variable infusion rate of 50% dextrose solution to maintain euglycemia.

**2.2.5. Invasive Hemodynamics:**

Cardiac function was assessed by cardiac catheterization in mice anesthetized using isoflurane anesthesia. A 1.4Fr catheter equipped with a pressure transducer (SPR-671, Millar Instruments, Houston, TX) was guided retrogradely into the left ventricle and traces recorded using Powerlab 8/30 series (AD Instruments, Colorado Springs, CO). For mice subjected to euglycemic hyperinsulinemic clamps, after recording the baseline left ventricular (LV) pressures for 2 minutes, isoproterenol was injected intraperitoneally at increasing doses of 1ug/kg, 10ug/kg and 100ug/kg at 3-minute intervals to assess changes in cardiac contractility.

**2.2.6. Western Blots:**

Tris-tricine gels (15%) were used for PLN immunoblots. Proteins were transferred on to PVDF membranes overnight at constant voltage (25V) at 4°C. Primary antibodies were used at 1:2000 dilution and blots were incubated overnight. Fluorophore conjugated secondary antibodies were used for detection using a LiCOR Odyssey scanner.

**2.2.7. Statistical Analysis:**

Data were analyzed using Graphpad Prism v7 software. Hyperinsulinemic-euglycemic clamp data were analyzed by unpaired t-test. Cardiac functional data was analyzed using

repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's or Sidak's test to determine significant differences. Significance is reported at $p<0.05$.

**2.3 Results:**

**2.3.1. Effect of insulin bolus on baseline cardiac function and responses to isoproterenol:**

To determine whether insulin influences baseline cardiac function of mice, we measured real-time cardiac contractile function in stably anesthetized mice by LV catheterization. We first established that there was no significant difference in blood glucose (Figure 1A) or in blood pressure (as measured from the carotid artery, Figure 1B) between baseline and 5 minutes after intravenous insulin injection suggesting that insulin treatment did not cause hypoglycemia or alter systemic hemodynamics while changes to baseline cardiac function were assessed. Intravenous insulin injection did not alter baseline cardiac function measured over 5 minutes (Figure 2). To further assess the effect of insulin on β-adrenergic receptor induced cardiac inotropy, we assessed myocardial contractility in response to increasing doses of isoproterenol given intraperitoneally starting at 5-7 minutes following intravenous injection of saline or insulin. Relative to baseline, intraperitoneal injections of isoproterenol in doses of 0.01ug/kg to 10ug/kg resulted in a significant increase in first derivatives of LV pressure and heart rates after isoproterenol injection in both saline and insulin groups (Figure 3). However, there was no significant difference between insulin and saline groups in terms of β-adrenergic responsiveness. These results suggest that acute exposure of the myocardium to a bolus injection of insulin does not modulate the overall responsiveness to β-adrenergic stimulation *in vivo*. Myocardial exposure to insulin in this study was confirmed by increased phosphorylation of Akt on Ser 473 (Fig 4A). While there were no differences in functional responses to isoproterenol between the

saline and insulin treated mice, previous studies (Fu et al., 2014; Steinhorn et al., 2017) reported an attenuated phosphorylation of Ser 16 residue of phospholamban following isoproterenol exposure in insulin pretreated cardiomyocytes. We therefore determined if acute exposure to insulin altered the response to isoproterenol-induced induction of Ser 16 phosphorylation of phospholamban by western blot. As shown in figure 4B, phosphorylation status of the Ser 16 residue on PLN was not different in the hearts of saline and insulin treated mice.

**2.3.2. Effect of euglycemic-hyperinsulinemia on baseline cardiac function and responses to isoproterenol:**

Whether a longer duration exposure of the myocardium to insulin *in vivo* influences response to β-adrenergic stimulation is not known. Additionally, hypoglycemia was evident at the end of isoproterenol dose-response (~15 minutes after insulin injection) and could have potentially activated counter regulatory responses mediated by sympathetic activation, which can be prevented by constant glucose monitoring coupled with maintenance of euglycemia. We therefore performed euglycemic hyperinsulinemic clamps in mice to study the effects on cardiac function and the myocardial inotropic response to β-adrenergic stimulation. Euglycemic hyperinsulinemia was confirmed by stable blood glucose levels in mice subjected to a variable infusion of glucose and a constant infusion of insulin for 120 minutes (Figure 5). Blood glucose was measured every 10 minutes and glucose infusion rate was adjusted to maintain euglycemia. A steady state for glucose was achieved as the 120 minutes time point approached. This is reflected by a relatively constant glucose infusion rate (Figure 5A) that maintains euglycemia (Figure 5B) during insulin infusion. Hyperinsulinemia was confirmed by substantial elevation in serum insulin levels after 120 minutes in mice infused with insulin compared to the saline group

(Figure 5C). Invasive hemodynamics revealed a significant difference in LV pressures between the saline and insulin groups at the end of euglycemic hyperinsulinemia (Figure 6). Further, when mice were injected with increasing doses of isoproterenol, there was a significant increase in heart rate and first derivatives of LV pressure indicative of increased contractile performance (Figure 6). However, there were no significant differences in cardiac contractile performance between the saline and insulin groups. These results suggest that inotropic response following stimulation of the cardiac β-adrenergic receptors is unaltered by steady state hyperinsulinemia.

We further assessed euglycemic hyperinsulinemia in mice altered the response to isoproterenol-induced induction of Ser 16 phosphorylation of phospholamban by western blot. As shown in figure 7, phosphorylation status of the Ser 16 residue on PLN was not different between saline and insulin treated mice. Taken together, these findings suggest that insulin might not attenuate isoproterenol-induced contractile and signaling changes in a physiological setting *in vivo*.

**2.4 Discussion:**

Epidemiological studies suggest that hyperinsulinemic states such as type 2 diabetes and obesity are associated with an increased risk of heart disease (M. Galderisi et al., 1991; Kannel & McGee, 1979). Exogenous insulin use, to maintain glycemia has also been strongly associated with a higher risk of heart failure (Kannel et al., 1974; G. A. Nichols, Gullion, Koro, Ephross, & Brown, 2004). These studies suggest that hyperinsulinemia may be an important contributor to development of cardiac dysfunction in dysmetabolic states such as type 2 diabetes and obesity. However, the direct short-term effects of hyperinsulinemia on cardiac function are relatively less well understood. To address this gap in knowledge, we performed cardiac catheterization in

anesthetized mice following a bolus dose of insulin. Relative to baseline, a transient but statistically insignificant decrease in contractile performance was evident after insulin bolus. Since there was an apparent decline in cardiac function following insulin bolus, we studied whether exposure to insulin under euglycemic conditions for a longer duration would impact cardiac function. We therefore assessed LV pressures in mice subject to euglycemic-hyperinsulinemic clamps and found no significant differences in baseline cardiac function compared to control group, although the baseline LV developed pressures were significantly lower in the insulin group compared to the saline group. To our knowledge, no studies have previously investigated the effect of hyperinsulinemia under euglycemic states in mice and our results suggest that under isoflurane anesthesia, euglycemic hyperinsulinemia does not significantly affect cardiac function over the course of 5 minutes or after 2h. Our results disagree with some previous reports on cardiac effects of insulin in other species. In healthy individuals, euglycemic hyperinsulinemia resulted in increases in heart rate (Siani, Strazzullo, Giorgione, De Leo, & Mancini, 1990) and contractile function (Klein et al., 2010) but these findings have been challenged by other studies (Airaksinen et al., 1985; Lager, Attvall, Eriksson, von Schenk, & Smith, 1986; Sasso et al., 2000). In organ bath studies using papillary muscles from cats (J. C. Lee & Downing, 1976) and dogs (Lucchesi et al., 1972), and moderator bands (muscle bundles that link papillary muscles to the septum) from pigs (Bella et al., 1998), insulin treatment resulted in an increased contractile performance. In dogs, intracoronary administration of insulin resulted in increased contractile function (Lucchesi et al., 1972) but the underlying molecular mechanisms mediating these responses are not understood. Lack of chronotropy or inotropy following insulin in our studies and the apparent differences in the effects reported in previous

studies may be due to species related differences the source of insulin or differences in innervation of the preparations and the consequences of autonomic reflexes.

Hyperinsulinemic states such as diabetes and obesity are associated with decreased cardiac reserve (Baldi et al., 2016; Pinto et al., 2014). In this context, the effect of acute hyperinsulinemia in modulating myocardial β-adrenergic responsiveness has been previously assessed. The responses to increasing doses of isoproterenol were used to measure myocardial β-adrenergic responsiveness in our studies. We found that acute hyperinsulinemia for durations of 5 minutes and 2h does not attenuate cardiac contractile response to β-adrenergic stimulation. These findings are in contrast with some previously reported literature and the reasons may include the species used for the study, altered milieu following abrogation of cephalic circulation (Nudel et al., 1977), anesthetic agent used and differences that may relate to the nature of preparation itself (*in vivo* vs. *ex vivo*).

Recent studies using isolated cardiomyocytes from adult mice showed altered cAMP dynamics and PKA activity when insulin pretreated myocytes were exposed to isoproterenol compared to myocytes treated with isoproterenol alone (Fu et al., 2014). Attenuation in isoproterenol induced PKA activity in insulin pretreated myocytes also correlated with reduced phosphorylation of PKA substrates such as Ser 16 residue of phospholamban, which is important for regulating SERCA activity (Fu et al., 2014; Steinhorn et al., 2017). When we examined signaling changes following *in vivo* administration of isoproterenol in insulin treated mice, we did not observe a significant difference in Ser 16 phosphorylation on phospholamban suggesting that insulin does not influence the biochemical or functional consequences of cardiac β-adrenergic receptor activation under physiological settings.

**2.5 Limitations:**

Anesthesia effects cardiac function and therefore, the observed effects of insulin on the myocardial function and myocardial β-adrenergic responsiveness may or may not be relevant in conscious state. Moreover, the studies were performed in animals with intact myocardial innervation. Thus, the possibility exists that the autonomic nervous system could override the potential direct effects on myocardial contractility which have been observed in *ex vivo* preparations.

In summary, results from our studies suggest that in mice subject to euglycemic hyperinsulinemia for 5 minutes or 2h, insulin does not exert a significant effect on cardiac function. In contrast to *in vitro* and *ex vivo* preparations, acute hyperinsulinemia up to 2h does not attenuate myocardial contractility induced by β-adrenergic stimulation.

REFERENCES


Airaksinen, J., Lahtela, J. T., Ikaheimo, M. J., Sotaniemi, E. A., & Takkunen, J. T. (1985). Intravenous insulin has no effect on myocardial contractility or heart rate in healthy subjects. *Diabetologia, 28*(9), 649-652.

Ayala, J. E., Bracy, D. P., Malabanan, C., James, F. D., Ansari, T., Fueger, P. T., . . . Wasserman, D. H. (2011). Hyperinsulinemic-euglycemic clamps in conscious, unrestrained mice. *J Vis Exp*(57). doi:10.3791/3188

Baldi, J. C., Wilson, G. A., Wilson, L. C., Wilkins, G. T., & Lamberts, R. R. (2016). The Type 2 Diabetic Heart: Its Role in Exercise Intolerance and the Challenge to Find Effective Exercise Interventions. *Sports Med, 46*(11), 1605-1617. doi:10.1007/s40279-016-0542-9

Bella, J. N., Devereux, R. B., Roman, M. J., O'Grady, M. J., Welty, T. K., Lee, E. T., . . . Howard, B. V. (1998). Relations of Left Ventricular Mass to Fat-Free and Adipose Body Mass. *Circulation, 98*(23), 2538-2544. doi:10.1161/01.cir.98.23.2538

Chiba, S. (1974). Positive chronotropic response of the canine SA node to insulin. *Tohoku J Exp Med, 114*(2), 193-194.

Despres, J. P., Lamarche, B., Mauriege, P., Cantin, B., Dagenais, G. R., Moorjani, S., & Lupien, P. J. (1996). Hyperinsulinemia as an independent risk factor for ischemic heart disease. *N Engl J Med, 334*(15), 952-957. doi:10.1056/nejm199604113341504

Fu, Q., Xu, B., Liu, Y., Parikh, D., Li, J., Li, Y., . . . Xiang, Y. K. (2014). Insulin inhibits cardiac contractility by inducing a Gi-biased beta2-adrenergic signaling in hearts. *Diabetes, 63*(8), 2676-2689. doi:10.2337/db13-1763

Galderisi, M., Anderson, K. M., Wilson, P. W., & Levy, D. (1991). Echocardiographic evidence for the existence of a distinct diabetic cardiomyopathy (the Framingham Heart Study). *Am J Cardiol, 68*(1), 85-89.

Hiatt, N., & Katz, J. (1969). Modification of cardiac and hyperglycemic effects of epinephrine by iulin. *Life Sci, 8*(9), 551-558.

Kannel, W. B., Hjortland, M., & Castelli, W. P. (1974). Role of diabetes in congestive heart failure: the Framingham study. *Am J Cardiol, 34*(1), 29-34.

Kannel, W. B., & McGee, D. L. (1979). Diabetes and cardiovascular disease. The Framingham study. *Jama, 241*(19), 2035-2038.

Klein, L. J., van Campen, C. M., Sieswerda, G. T., Kamp, O., & Visser, F. C. (2010). Effects of high-dose insulin infusion on left ventricular function in normal subjects. *Neth Heart J, 18*(4), 183-189.

Klinge, E., & Wafin, F. (1971). Increase in cardiac contractile force caused by pork insulin. *Ann Med Exp Biol Fenn, 49*(3), 138-142.

Lager, I., Attvall, S., Eriksson, B. M., von Schenk, H., & Smith, U. (1986). Studies on the insulin-antagonistic effect of catecholamines in normal man. Evidence for the importance of beta 2-receptors. *Diabetologia, 29*(7), 409-416.

Lee, J. C., & Downing, S. E. (1976). Effects of insulin on cardiac muscle contraction and responsiveness to norepinephrine. *Am J Physiol, 230*(5), 1360-1365. doi:10.1152/ajplegacy.1976.230.5.1360

Liang, C., Doherty, J. U., Faillace, R., Maekawa, K., Arnold, S., Gavras, H., & Hood, W. B., Jr. (1982). Insulin infusion in conscious dogs. Effects on systemic and coronary hemodynamics, regional blood flows, and plasma catecholamines. *J Clin Invest, 69*(6), 1321-1336.

Lucchesi, B. R., Medina, M., & Kniffen, F. J. (1972). The positive inotropic action of insulin in the canine heart. *Eur J Pharmacol, 18*(1), 107-115.

Nichols, G. A., Gullion, C. M., Koro, C. E., Ephross, S. A., & Brown, J. B. (2004). The incidence of congestive heart failure in type 2 diabetes: an update. *Diabetes Care, 27*(8), 1879-1884.

Nudel, D. B., Lee, J. C., & Downing, S. E. (1977). Reciprocal inhibition of cardiac responses to norepinephrine and insulin. *Am J Physiol, 233*(6), H665-669. doi:10.1152/ajpheart.1977.233.6.H665

Perry, I. J., Wannamethee, S. G., Whincup, P. H., Shaper, A. G., Walker, M. K., & Alberti, K. G. (1996). Serum insulin and incident coronary heart disease in middle-aged British men. *Am J Epidemiol, 144*(3), 224-234.

Pinto, T. E., Gusso, S., Hofman, P. L., Derraik, J. G., Hornung, T. S., Cutfield, W. S., & Baldi, J. C. (2014). Systolic and diastolic abnormalities reduce the cardiac response to exercise in adolescents with type 2 diabetes. *Diabetes Care, 37*(5), 1439-1446. doi:10.2337/dc13-2031

Reikeras, O., & Gunnes, P. (1986). Effects of high doses of insulin on systemic haemodynamics and regional blood flows in dogs. *Clin Physiol, 6*(2), 129-138.

Reikeras, O., Gunnes, P., Sorlie, D., Ekroth, R., Jorde, R., & Mjos, O. D. (1985). Haemodynamic effects of low and high doses of insulin during beta receptor blockade in dogs. *Clin Physiol, 5*(5), 455-467.

Rieker, R. P., Lee, J. C., & Downing, S. E. (1975). Positive inotropic action of insulin on piglet heart. *Yale J Biol Med, 48*(5), 353-360.

Rowe, J. W., Young, J. B., Minaker, K. L., Stevens, A. L., Pallotta, J., & Landsberg, L. (1981). Effect of insulin and glucose infusions on sympathetic nervous system activity in normal man. *Diabetes, 30*(3), 219-225.

Sasso, F. C., Carbonara, O., Cozzolino, D., Rambaldi, P., Mansi, L., Torella, D., . . . Salvatore, T. (2000). Effects of insulin-glucose infusion on left ventricular function at rest and during dynamic exercise in healthy subjects and noninsulin dependent diabetic patients: a radionuclide ventriculographic study. *J Am Coll Cardiol, 36*(1), 219-226.

Siani, A., Strazzullo, P., Giorgione, N., De Leo, A., & Mancini, M. (1990). Insulin-induced increase in heart rate and its prevention by propranolol. *Eur J Clin Pharmacol, 38*(4), 393-395.

Steinhorn, B., Sartoretto, J. L., Sorrentino, A., Romero, N., Kalwa, H., Abel, E. D., & Michel, T. (2017). Insulin-dependent metabolic and inotropic responses in the heart are modulated by hydrogen peroxide from NADPH-oxidase isoforms NOX2 and NOX4. *Free Radic Biol Med, 113*, 16-25. doi:10.1016/j.freeradbiomed.2017.09.006

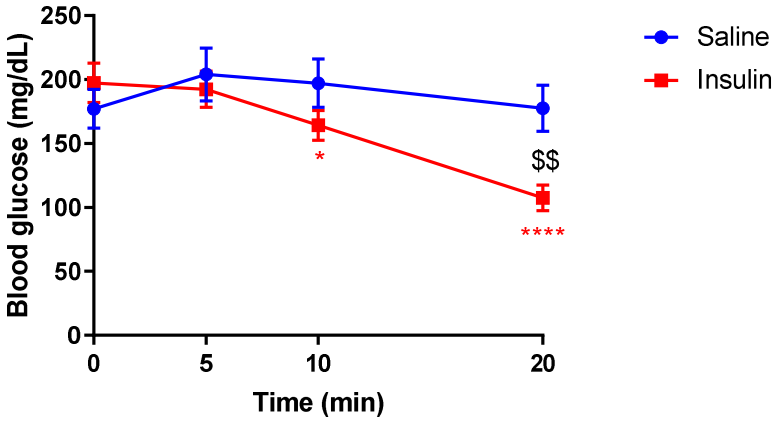
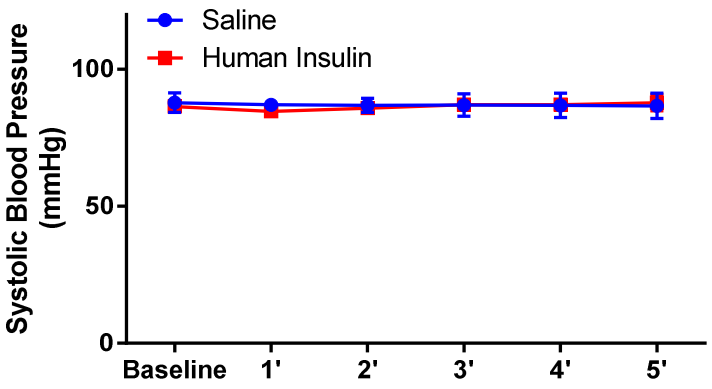
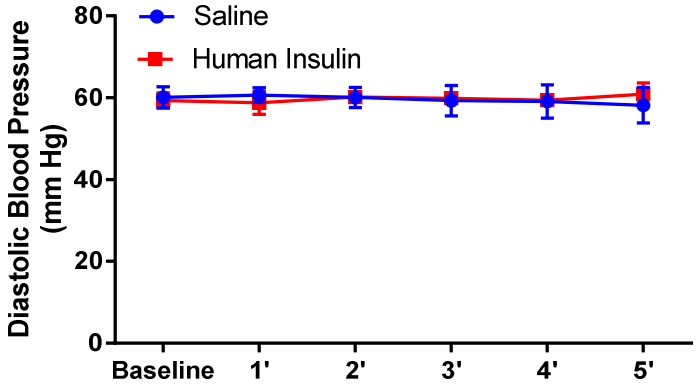
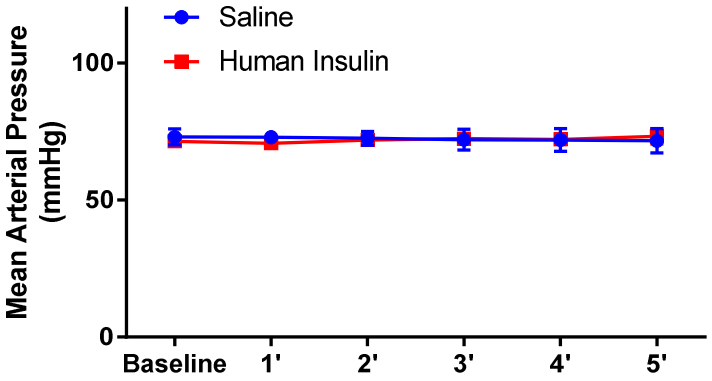



**Figure 1. Effect of i.v. insulin bolus on blood glucose in anesthetized mice.**

Data are presented as Mean±SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis by Tukey's test (within group comparisons) or Sidak's test (between group comparisons). Statistical significance was set at $p<0.05$ (N>7/group) (*-$p<0.05$, ****- $p<0.0001$ vs baseline within group; $$- $p<0.01$ vs saline).


Saline
Human Insulin
Systolic Blood Pressure (mmHg)
100
50
0
Baseline
1'
2'
3'
4'
5'


Saline
Human Insulin
Diastolic Blood Pressure (mm Hg)
80
60
40
20
0
Baseline
1'
2'
3'
4'
5'


Saline
Human Insulin
Mean Arterial Pressure (mmHg)
100
50
0
Baseline
1'
2'
3'
4'
5'

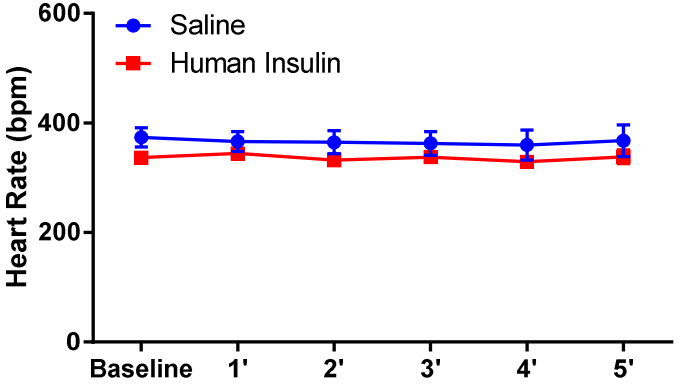
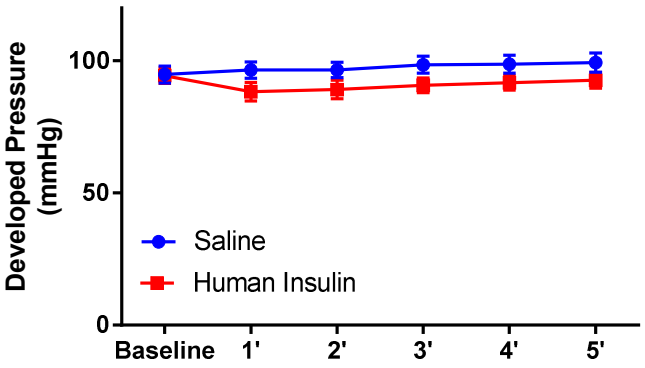
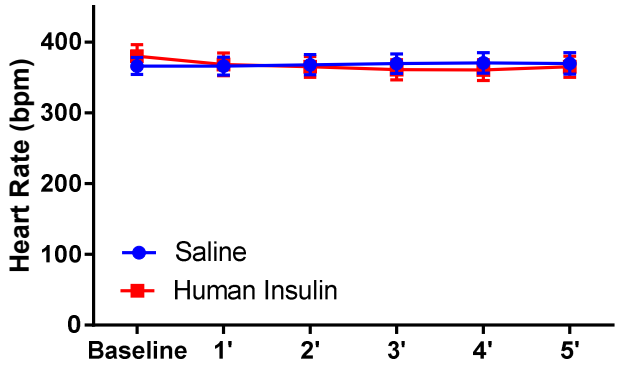
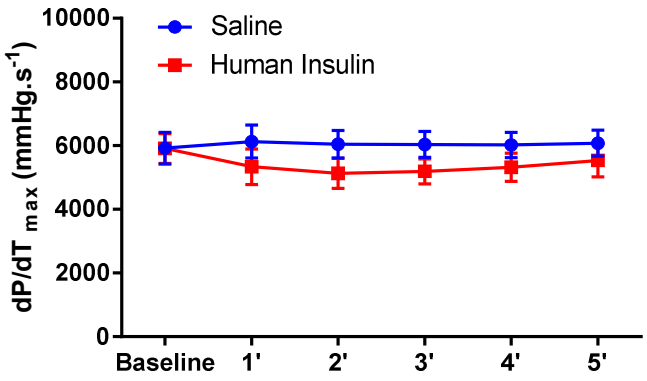

Saline
Human Insulin
Heart Rate (bpm)
600
400
200
0
Baseline
1'
2'
3'
4'
5'

**Figure 2. Effect of i.v. insulin on blood pressure measured in the carotid artery.**

Baseline values refer to measurements immediately before the i.v. bolus injections. Data are presented as Mean±SEM and were analyzed by repeated measures two-way ANOVA. Statistical significance was set at $p<0.05$ ($N>3$/group).


Developed Pressure (mmHg)
100
50
0
Saline
Human Insulin
Baseline
1'
2'
3'
4'
5'


Heart Rate (bpm)
400
300
200
100
0
Saline
Human Insulin
Baseline
1'
2'
3'
4'
5'


dP/dT max (mmHg.s-1)
10000
8000
6000
4000
2000
0
Saline
Human Insulin
Baseline
1'
2'
3'
4'
5'

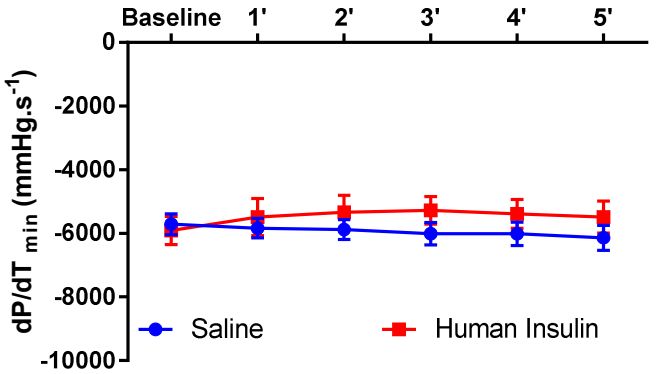
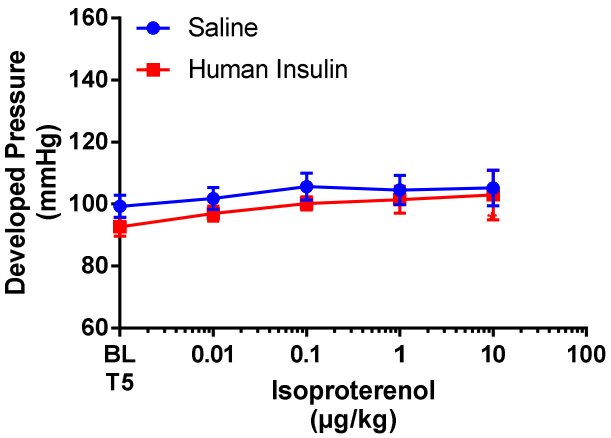
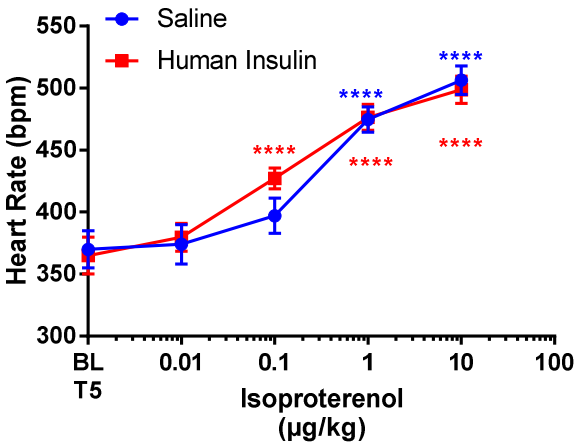
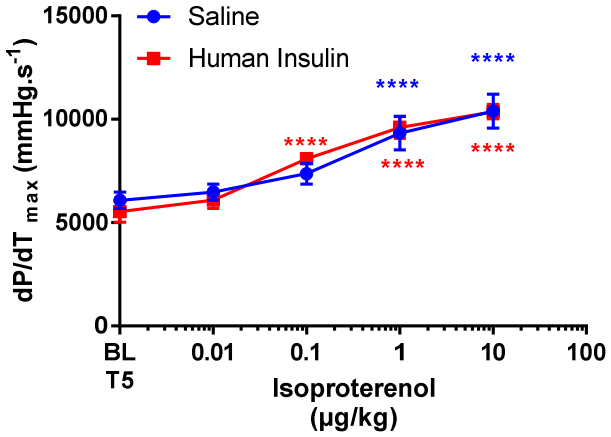

Baseline
1'
2'
3'
4'
5'
0
-2000
-4000
-6000
-8000
-10000
dP/dT min (mmHg.s-1)
Saline
Human Insulin

**Figure 3. Effect of i.v. insulin on cardiac function measured by LV catheterization.**

Baseline values refer to measurements immediately before the i.v. bolus injections. Data are presented as Mean+SEM and were analyzed by multiple t-tests to compare saline and insulin groups at corresponding time points. Statistical significance was set at $p<0.05$ ($N\geq10$/group). Repeated measures two-way ANOVA revealed no significant differences between the groups.


Saline
Human Insulin
Developed Pressure (mmHg)
160
140
120
100
80
60
BL
T5
0.01
0.1
1
10
100
Isoproterenol (µg/kg)


Saline
Human Insulin
Heart Rate (bpm)
550
500
450
400
350
300
****
BL
T5
0.01
0.1
1
10
100
Isoproterenol (µg/kg)


Saline
Human Insulin
dP/dT max (mmHg.s-1)
15000
10000
5000
0
****
BL
T5
0.01
0.1
1
10
100
Isoproterenol (µg/kg)

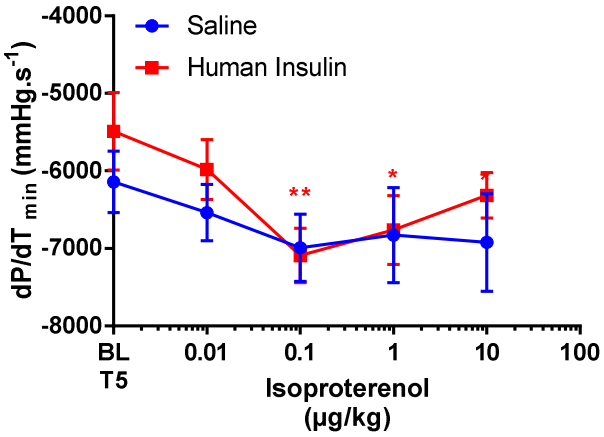

Saline
Human Insulin
dP/dT min (mmHg.s-1)
-4000
-5000
-6000
-7000
-8000
**
*
BL
T5
0.01
0.1
1
10
100
Isoproterenol (µg/kg)

**Figure 4. Cardiac inotropy induced by β-adrenergic stimulation as measured by LV cathetization in mice subject to i.v. saline or i.v. insulin.**

BL refers to values 5 minutes after i.v. bolus injection of saline or insulin. Data are presented as Mean+SEM and were analyzed by repeated measures two-way ANOVA to determine difference in inotropic response within and betweengroups. Statistical significance was set at $p<0.05$ ($N>10$/group) revealed no significant differences between the groups by Sidak's test. (*-$p<0.05$, **-$p<0.01$, ***-$p<0.001$, ****-$p<0.0001$ between baseline and response to isoproterenol within same group by Tukey's multiple comparison test

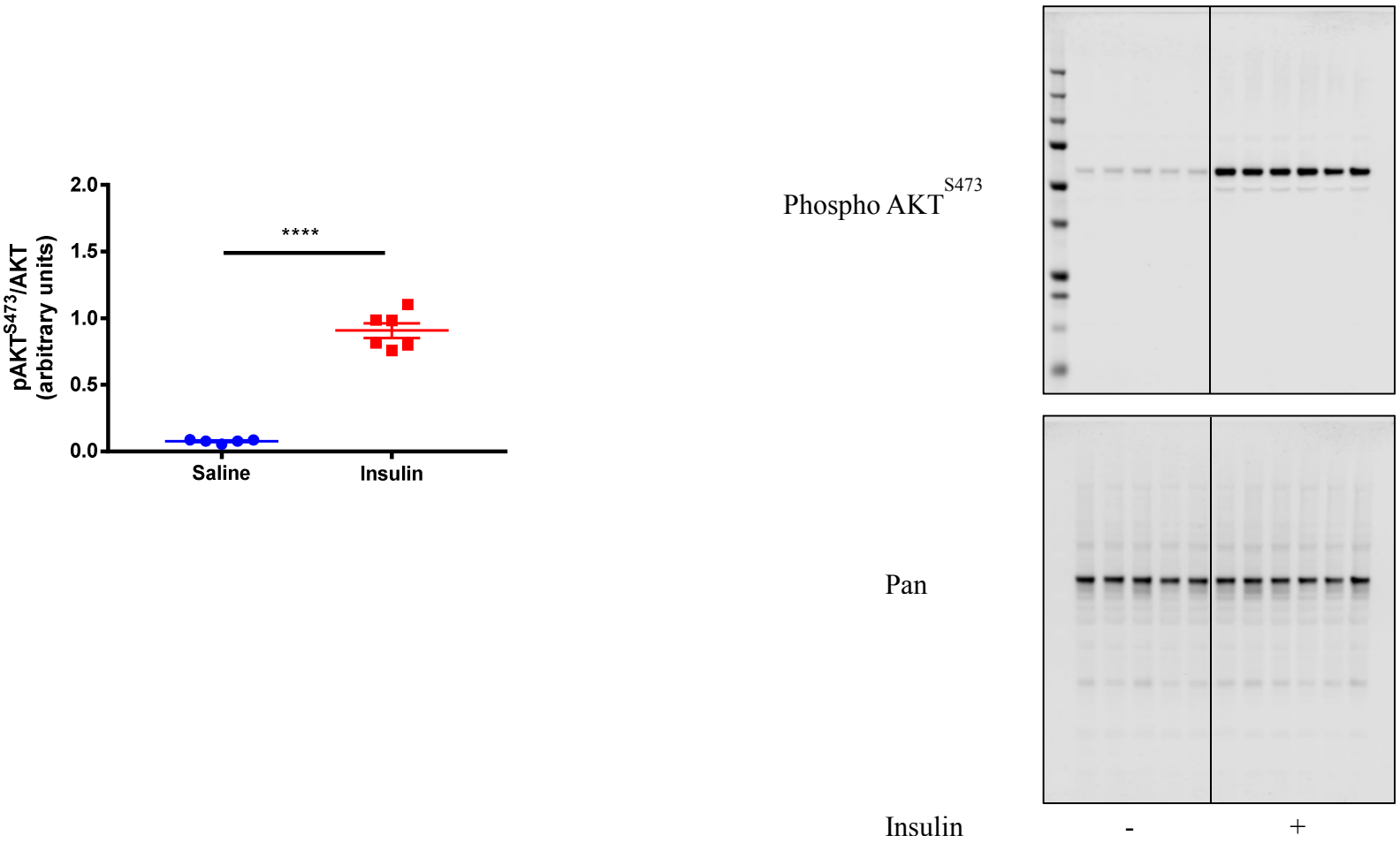



**Figure 5. Immunoblot showing increased AKT$^{S473}$ phosphorylation in mouse hearts acutely exposed to i.v. saline or insulin (1U/kg) ahead of isoproterenol.**

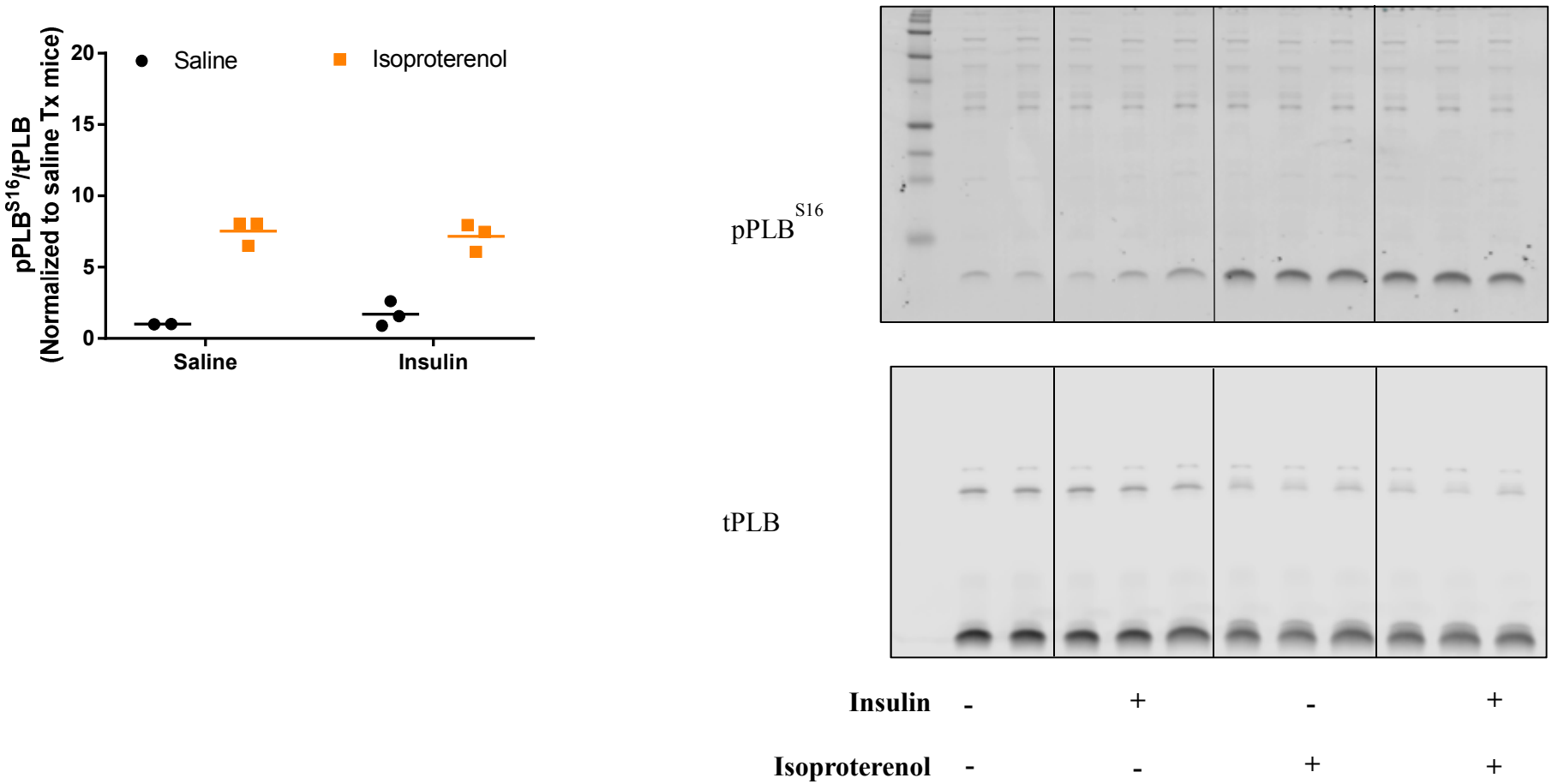
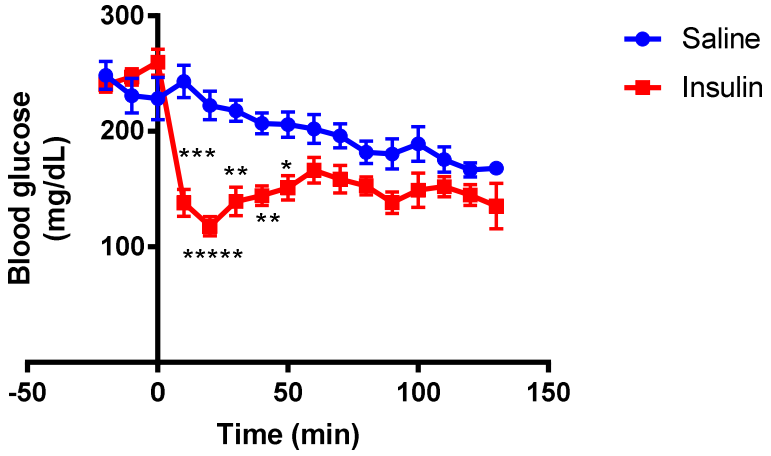
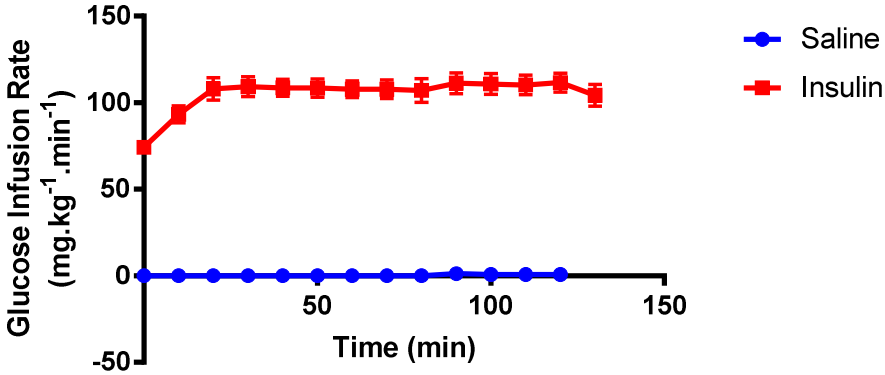



**Figure 6. Immunoblot showing increased phospholamban$^{S16}$ phosphorylation in mouse hearts acutely exposed to intravenous saline or insulin (1U/kg) and intraperitoneal isoproterenol.**

A)


300
200
100
Blood glucose (mg/dL)
***
**
*
**
*****
-50
0
50
100
150
Time (min)
Saline
Insulin

B)


150
100
50
0
-50
Glucose Infusion Rate (mg.kg-1.min-1)
50
100
150
Time (min)
Saline
Insulin

C)

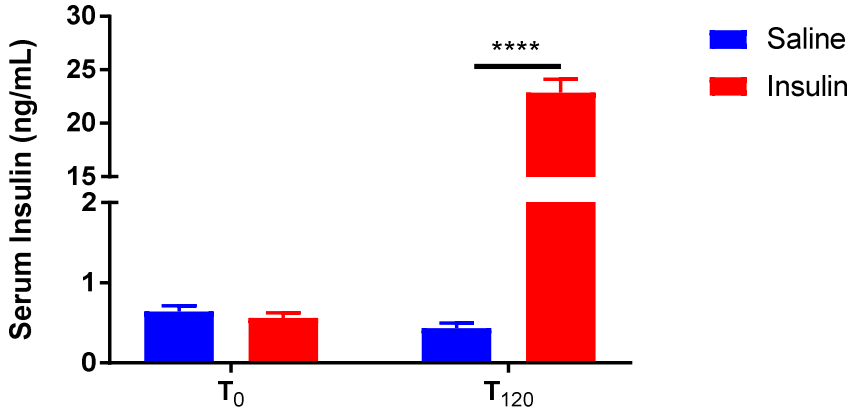
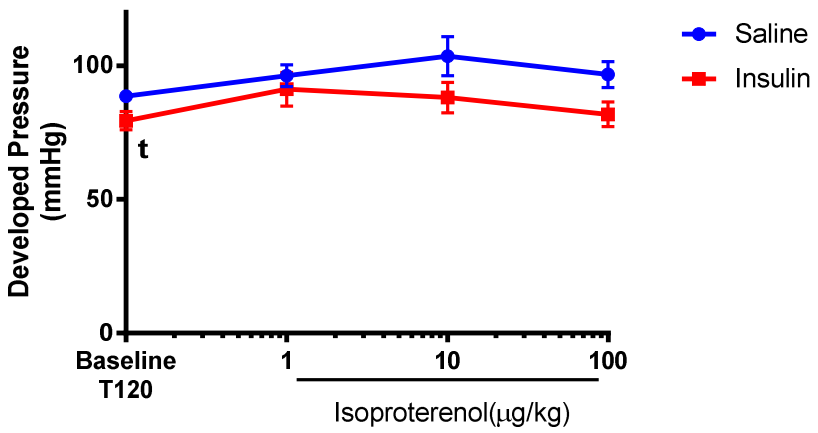
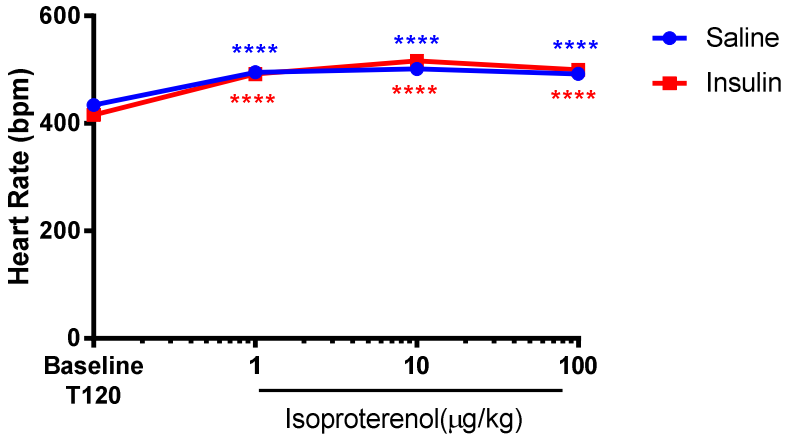
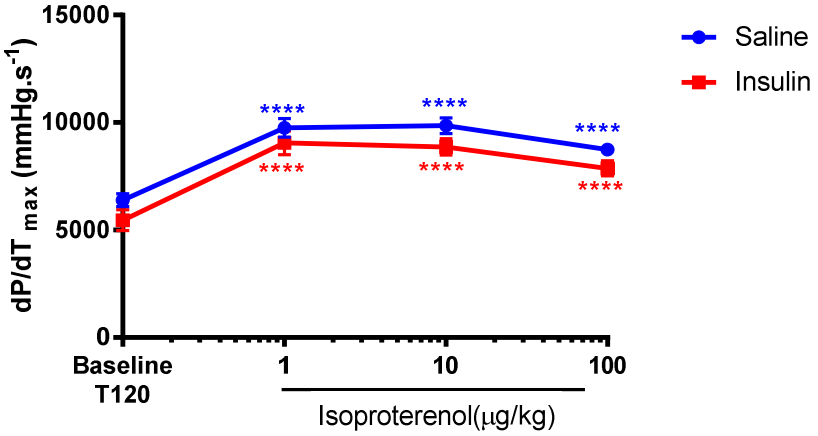

30
25
20
15
2
1
0
Serum Insulin (ng/mL)
****
T0
T120
Saline
Insulin

**Figure 7. Euglycemic hyperinsulinemic clamp elevated serum insulin levels at the end of 120 minutes.**

A) Blood glucose B) Glucose infusion rates during euglycemic hyperinsulinemic clamps. C) Serum insulin levels as measured by insulin ELISA. Data are presented as Mean+SEM and were analyzed by student's t-test in (A) and by repeated measures two-way ANOVA in (C). Statistical significance was set at $p<0.05$ ($N>9$/group).


Developed Pressure (mmHg)
100
50
0
t
Baseline
T120
1
10
100
Isoproterenol(μg/kg)
Saline
Insulin


Heart Rate (bpm)
600
400
200
0
****
Baseline
T120
1
10
100
Isoproterenol(μg/kg)
Saline
Insulin


dP/dT max (mmHg.s-1)
15000
10000
5000
0
****
Baseline
T120
1
10
100
Isoproterenol(μg/kg)
Saline
Insulin

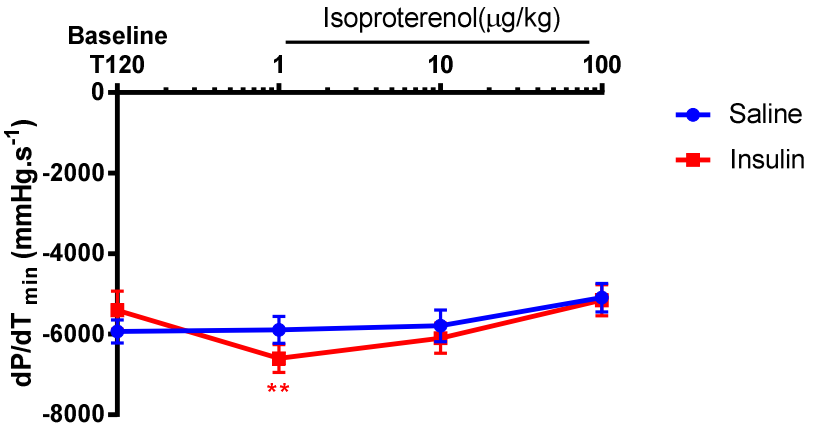

dP/dT min (mmHg.s-1)
Baseline
T120
Isoproterenol(μg/kg)
1
10
100
0
-2000
-4000
-6000
-8000
**
Saline
Insulin

**Figure 8. Cardiac inotropy induced by β-adrenergic stimulation as measured by LV cathetization in mice subject to euglycemic-hyperinsulinemic clamp.**

BL refers to values measured at 120 minutes after the start of euglycemic hyperinsulinemic clamp. Data are presented as Mean+SEM and were analyzed by repeated measures two-way ANOVA to determine difference in inotropic response within and between groups. Statistical significance was set at $p<0.05$ ($N>8$/group; **-$p<0.01$, ****-$p<0.0001$ between baseline and response to isoproterenol within same group by Tukey's multiple comparison test) (t- $p<0.05$ between saline and insulin groups at baseline as analyzed by student's t-test).

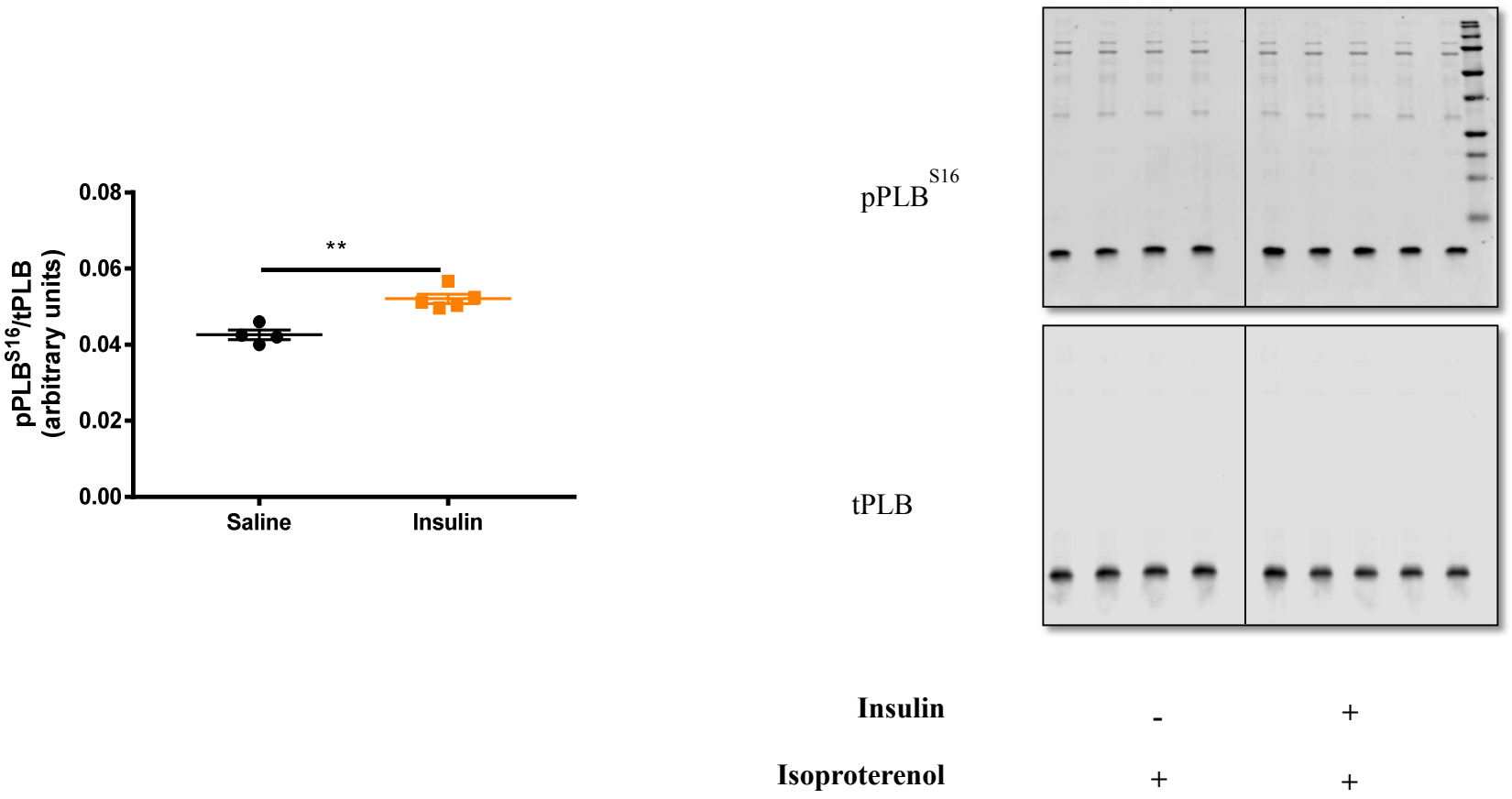



**Figure 9. Immunoblot showing increased phospholamban$^{S16}$ phosphorylation in heart lysates from mice subject to euglycemic hyperinsulinemia and injected with isoproterenol intraperitoneally.**

(**-$p<0.01$ as assessed by student's t-test)

# CHAPTER 3: CHRONIC FAT FEEDING DOES NOT IMPAIR SYSTOLIC FUNCTION IN GRK2 DEFICIENT MICE

## 3.1 Introduction:

Heart disease is the leading cause of morbidity and mortality in the US (CDC, 2015). According to data from the Center for Disease Control (CDC), increased mortality due to heart disease overlaps with the increased incidence and prevalence of diabetes and obesity. While diabetes (de Simone et al., 2010; M. Galderisi et al., 1991; Kannel & McGee, 1979) and obesity (Kenchaiah et al., 2002; Lauer et al., 1991) have been established as risk factors for heart disease, the molecular mechanisms that predispose this hyperinsulinemic and dyslipidemic patient population to cardiac dysfunction are not well understood.

Hyperinsulinemia is an independent risk factor for ischemic heart disease (Despres et al., 1996). Multiple strategies are available for achieving metabolic control in individuals with type 2 diabetes, some of which result in hyperinsulinemia such as insulin and insulin secretagogues. Exogenous insulin use (Kannel et al., 1974; G. A. Nichols, Gullion, Koro, Ephross, et al., 2004) or increasing endogenous insulin levels indirectly using saxagliptin (B. M. Scirica et al., 2015) is associated with an increased risk of heart failure. These studies suggest a correlation between hyperinsulinemia and the development of cardiac dysfunction associated with diabetes and obesity.

Effects of insulin on cardiac muscle have been previously characterized. Acute exposure of isolated papillary muscle preparation or isolated hearts to insulin resulted in increased cardiac contractility (Downing, Lee, & Rieker, 1977; Klinge & Wafin, 1971; J. C. Lee & Downing, 1976; Rieker et al., 1975) but these findings were not supported by other studies (Lucchesi et al.,

1972; Regan, Frank, Lehan, & Hellems, 1963). Subsequent studies documented attenuated β-adrenergic responsiveness upon insulin pretreatment but the mechanistic basis for these important findings remained elusive (Hiatt & Katz, 1969; J. C. Lee & Downing, 1976; Nudel et al., 1977).

A recent study (Fu et al., 2014) has shed some light on mechanistic aspects of the acute effects of insulin on contractility of the heart. Decreased inotropic response to isoproterenol in insulin pre-perfused hearts was mediated by insulin induced GRK2 dependent phosphorylation of the $\beta_2$-adrenergic receptor ($\beta_2$AR). $\beta_2$AR phosphorylation following exposure to insulin resulted in switch in coupling of the receptor from $G\alpha_s$ to $G\alpha_i$. Subsequent activation of the $\beta_2$AR by isoproterenol induced activation of $G\alpha_i$ resulting in increased inhibitory tone on adenylate cyclase and β arrestin-2 (βarr2) dependent internalization of the receptor.

G-protein coupled Receptor Kinase 2 (GRK2) is a Ser/Thr kinase that phosphorylates GPCRs in a homologous desensitization process. GRK2 is a cytosolic kinase that translocates to the plasma membrane upon activation of the heterotrimeric G-proteins and requires free Gβγ subunits to phosphorylate the activated GPCRs. Subsequent desensitization and internalization of the receptor are mediated by β arrestin1/2 binding (Evron, Daigle, & Caron, 2012; Huang, Gao, Chuprun, & Koch, 2014).

GRK2 expression and activity is increased in the failing hearts and the increased expression of this kinase has been suggested to be pathological (Ungerer et al., 1994). More recently, GRK2 has been identified to mediate additional signaling processes in cardiomyocytes that are independent of GPCR activation (Brinks et al., 2010; M. Chen et al., 2013; Ciccarelli et

al., 2011; Fan et al., 2013). In this context, cultured cardiomyocytes exposed to insulin for longer durations (24-48h) caused increased ERK1/2 activation and phosphodiesterase 4D (PDE4D) that was sensitive to inhibition of GRK2 (Q. Wang et al., 2017). ERK1/2 is an important kinase with critical role in promoting pathological hypertrophy (Bueno et al., 2000) and PDE4D decreases the tone of PKA signaling indirectly by increasing cAMP degradation. Further role for GRK2 in diabetic cardiomyopathy was apparent when wildtype mice subject to HFD feeding developed cardiac dysfunction and these functional deficits were rescued upon treatment with Paroxetine (Q. Wang et al., 2017), a selective serotonin reuptake inhibitor which also inhibits GRK2 (Thal et al., 2012). These changes also correlated with attenuation of HFD induced PDE4D expression and ERK1/2 activation in mice treated with paroxetine suggesting that PDE4D might be a downstream target of ERK1/2, which is activated by HFD feeding (Q. Wang et al., 2017). Given GRK2 is an important downstream kinase of signaling cascades activated following acute and chronic exposure to insulin, we hypothesized that GRK2 potentially mediates cardiac dysfunction associated with long term high fat feeding.

Long term high fat diet feeding in mice has been established as a model to understand the molecular pathways underlying diabetes and obesity related cardiomyopathy (Battiprolu et al., 2012; S. Y. Park et al., 2005). To determine if cardiomyocyte GRK2 mediates the pathological effects of chronic hyperinsulinemia on the myocardium, we hypothesized that cardiomyocyte specific knockout of GRK2 would render mice resistant to cardiac dysfunction induced by long term high fat feeding.

### 3.2 Methods:

#### 3.2.1. Animals:

$GRK2^{fl/fl}$ mice have been previously described (Matkovich et al., 2006). $GRK2^{fl/fl}$ mice were crossed with $GRK2^{fl/fl}$ Myh6-ERT2-Cre mice to generate tamoxifen-inducible GRK2 cardiomyocyte knockout mice. Both male and female mice were used for the studies and the mice were on a mixed background. At 6 weeks of age, tamoxifen was injected for 5 consecutive days at a dose of 10mg/kg/day in all the mice used in the studies to induce recombination of the floxed alleles in mice expressing the Myh6-ERT2-Cre transgene.

#### 3.2.2. Metabolic Parameters:

Mice were fed a low-fat diet or control diet (12451J, Research Diets, New Brunswick, NJ) or a high-fat diet (12492, Research Diets, New Brunswick, NJ) for 36-38 weeks beginning at 10 weeks of age. Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed after 30 weeks of dietary intervention. For GTT, mice were fasted for 6h, baseline blood glucose was measured and 2g/kg body weight of 20% (w/v) glucose in saline was injected intraperitoneally. Blood glucose was measured at various time points until 120 minutes from the time of injection. ITT was performed following baseline blood glucose measurements in mice fasted for 3-4h prior to the experiment. Humulin R-100 (Eli Lilly, Indianapolis, IN) was injected at a dose of 0.75U/kg body weight and blood glucose measured at various time intervals over the next 2h. Serum insulin was measured by using a mouse insulin ELISA kit (Crystal Chem, IL) using samples collected in the fasted state and after glucose load during the GTT.

### 3.2.3. Echocardiography:

Cardiac function was assessed by 2D-transthoracic echocardiography in sedated mice (100uL/mouse of 2mg/mL Midazolam) using Vevo 2100 (Visual Sonics, Canada) equipped with a 30MHz transducer. Images were captured in B-mode both in short axis at mid papillary level and in parasternal long axis. LV function was calculated from measurements made from both axes and heart rate was extrapolated from the duration of 4 consecutive cardiac cycles.

### 3.2.4. High Resolution Respirometry:

High resolution respirometry was performed in saponin permeabilized fibers prepared from the interventricular septum of wild type and cardiomyocyte GRK2 knockout mice. Muscle bundles and fibers were always maintained hydrated in cold buffer during incubation and washes. Briefly, fibers were separated using Dumont #5/45 forceps in BiOPS buffer (7.23mM $K_2EGTA$, 2.77mM $CaK_2EGTA$, 20mM imidazole, 0.5mM DTT, 20mM Taurine, 5.7mM ATP, 14.3mM Phosphocreatine, 6.56mM $MgCl_2.6H_2O$, 50mM MES, pH 7.1 with KOH) by carefully separating apart the fibers perpendicular to the direction of their orientation within the muscle bundle. After an adequate increase in the surface area, fibers were incubated in 50ug/mL saponin in BiOPS buffer at 4ºC for 30min on a rocker followed by 2X10min washes at 4ºC in buffer Z heavy (105mM KMES, 30mM KCl, 10mM $KH_2PO_4$, 5mM $MgCl_2.6H_2O$, 5mg/mL BSA, pH 7.4 with KOH). Fibers were incubated at 4ºC on the rocker in buffer Z heavy until they were assayed. 1-3mg of fiber bundles were weighed out and assayed Oxygraph chambers (Oroboros, Innsbruck, Austria) in buffer Z heavy. After baseline stabilization, ADP stimulated respiration with Pyruvate/Malate (2.5mM/0.5mM) and Palmitoylcarnitine/Malate (0.02mM/0.5mM) were measured (Katunga et al., 2015).

### 3.2.5. RNA Isolation and Quantitative PCR:

RNA was isolated from mouse hearts after 36-38 weeks of dietary intervention using Ambion Purelink RNA isolation kit according to the manufacturer's instructions. On column DNA digestion step was included to eliminate genomic DNA contamination. qPCR primers (IDT, Coralville, IA) were custom designed using primer blast. 1ug of cDNA was prepared by reverse transcription using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's instructions. For qPCR, 5ng cDNA was loaded per well in triplicate for each sample and a house keeping control was run for each plate. Data were analyzed by the ΔΔCt method and represented as fold change relative to wild type mice fed control diet.

### 3.2.6. Histology:

Heart tissue was fixed in 10% Zinc-formalin fixative, dehydrated in ethanol and were subsequently embedded in paraffin. Trichrome staining was performed on 5μm thick sections after deparaffinization. Stained sections were mounted with coverslips using permount. Slides were scanned using an Ariol Slide Scanner (Leica Biosystems) and images were captured at 20X magnification. Images were analyzed using Fiji after conversion to RGB and analysis parameters for all the images were kept constant. Fibrosis was quantified and presented as % area of blue relative to the total area.

### 3.2.7. Statistical Analysis:

Data were analyzed by student t-test where appropriate. When analyzing data from 4 groups, two-way ANOVA was performed followed by post-hoc analysis using Tukey's multiple comparisons to determine statistical significance between groups. Data are presented as Mean$\pm$SEM. Statistical significance is reported at $p<0.05$.

### 3.3 Results:

Decreased levels of GRK2 mRNA and protein content in the hearts were achieved by intraperitoneal delivery of tamoxifen for 5 consecutive days. GRK2 mRNA expression (Fig 10) and protein content (Fig 10) was lowered by 60% in the knockout hearts compared to the wildtype hearts by 4 weeks after the final tamoxifen injection. The residual expression is perhaps due to GRK2 expression in non-cardiomyocytes in the heart since GRK2 is expressed ubiquitously (Matkovich et al., 2006).

#### 3.3.1. High fat feeding resulted in insulin resistance and glucose intolerance:

Wild type and knockout GRK2 mice of both sexes were subject to a high fat diet protocol for 36-38 weeks. Fat feeding resulted in development of insulin resistance (Fig 11), glucose intolerance (Fig 12) and significantly elevated fasting insulin levels (Fig 13). These results confirm that high fat feeding resulted in significant insulin resistance that was largely not modified by the cardiomyocyte knockout of GRK2 either in male or in female mice.

#### 3.3.2. High fat feeding did not induce cardiac dysfunction in wildtype mice:

After 36-38 weeks of dietary intervention, we measured cardiac function using 2D echocardiography but did not observe significant differences in ejection fraction (EF) between the control diet and high fat diet fed groups regardless of the genotype or sex (Table 1). High fat feeding for duration as long as 40 weeks was insufficient to cause cardiac dysfunction measured as EF in wild type mice on pure C57BL/6J background (Table 2) suggesting that strain specific differences between pure C57BL/6J and mixed background do not explain the resistance of these mice to cardiac dysfunction induced by dietary fat feeding. High fat feeding however, induced significant cardiac hypertrophy independent of the genotype or sex after 36-38 weeks after

dietary intervention (Fig 14). These results suggest that long-term high fat feeding in mice on a pure background or a mixed background reproducibly causes cardiac hypertrophy but does not reliably precipitate systolic cardiac dysfunction.

### 3.3.3. High fat feeding did not cause mitochondrial dysfunction in the heart:

Cardiomyocyte overexpression of GRK2 decreased oxygen consumption rate with palmitoyl carnitine as substrate (Sato et al., 2015) and high fat feeding has been previously shown to decrease mitochondrial oxygen consumption and increase uncoupling (S. Boudina et al., 2012). We therefore investigated whether chronic high fat diet feeding in wildtype mice resulted in a decrease in cardiac mitochondrial oxygen consumption and if cardiomyocyte GRK2 knockout prevented the high fat diet-induced impairment of mitochondrial function. Permeabilized fibers from wildtype and GRK2 cardiac knockout mice studied by high resolution respirometry in Oroboros oxygraph chambers had comparable oxygen consumption rates regardless of the dietary fat content, or the substrate used (Fig 15). Oxygen consumption was comparable among the groups even after blocking ATP synthase using oligomycin suggesting that the mitochondrial oxygen consumption in the high fat fed hearts was potentially not uncoupled from ATP synthesis (Figure 15, bottom panel). These results suggest that cardiac mitochondrial function might not be altered by chronic fat feeding as measured in permeabilized fibers and knockout of GRK2 does not alter cardiac mitochondrial oxygen consumption rates regardless of the dietary fat content.

### 3.3.4. High fat feeding did not cause pathological remodeling in the heart:

Since cardiac hypertrophy was apparent in the high fat fed mice regardless of the genotype, we further assessed changes in expression levels of genes related to pathological

hypertrophy and cardiac fibrosis in male mice. High fat feeding, independent of the genotype, did not induce a significant increase in expression levels of hypertrophic markers including ANP and BNP or in the fetal myosin heavy chain Myh7 (Fig 16A). High fat feeding in the wild type mice as well as GRK2 cardiomyocyte knockout mice caused modest but significant elevations in collagen transcript levels- Col1a1 and Col3a1 (Fig 16B). However, the increased collagen isoform expression following long term HFD feeding did not result in increased cardiac fibrosis (Fig 17). Taken together these results suggest that high fat feeding does not result in activation of pathological hypertrophic markers or increased interstitial fibrosis in the heart. Moreover, these changes are not influenced by altered GRK2 expression.

**3.4 Discussion:**

GRK2 is a cytosolic kinase that mediates the agonist-dependent homologous desensitization of G protein-coupled receptors (GPCRs). Activation of GPCRs has been described as the required step for translocation of GRK2 from cytosol to the membrane. The free Gβγ subunits that dissociate from the heterotrimeric Gαβγ following receptor activation serves in anchoring and activating GRK2 in the membrane (Evron et al., 2012; Huang et al., 2014).

Increased expression and activity of GRK2 has been documented in human heart failure (Ungerer et al., 1994). Insulin activation of GRK2 to phosphorylate $\beta_2$AR has been described in acute studies and is a novel mechanism that uses the homologous desensitization machinery to phosphorylate $\beta_2$AR in the absence of a ligand for the $\beta_2$AR (Fu et al., 2014). Insulin resistance is a common feature of dysmetabolic states with chronic hyperinsulinemia being a compensatory change adaptation. Hyperinsulinemia is also an established risk factor for heart disease with little knowledge of underlying mechanisms. Given that GRK2 is critical for mediating the effects of

acute (Fu et al., 2014) and prolonged exposure to insulin *in vitro* (Q. Wang et al., 2017), we hypothesized that cardiomyocyte GRK2 mediates development of cardiac dysfunction associated with chronic hyperinsulinemic states. Our preliminary studies also suggested that HFD-induced cardiac dysfunction was ameliorated in mice injected with paroxetine, an inhibitor of GRK2 (Q. Wang et al., 2017). To test the hypothesis that cardiomyocyte GRK2 mediates chronic fat feeding induced cardiac dysfunction, we rendered $GRK2^{fl/fl}$ and cardiomyocyte GRK2 knockout mice hyperinsulinemic by long term high fat diet feeding. While high fat feeding resulted in significant increases in measures of insulin resistance (glucose intolerance, fasting serum insulin) our observations on cardiac functional parameters suggest that the mouse model of diet-induced obesity utilized in this study might not be a reliable model for studying diabetes and obesity related cardiomyopathy. Our findings are at odds with previously published reports on cardiac dysfunction in diet-induced obesity (Battiprolu et al., 2012; S. Y. Park et al., 2005). The reasons for the differences in observations may include differences in echocardiographic measurements (M-mode versus B-mode), strain of the mice used for the study and differences in housing conditions. High fat feeding however resulted in significant cardiac hypertrophy, but whether the hypertrophic response is driven by increased body mass upon high fat feeding needs further investigation.

Recent studies have described non-canonical roles for GRK2 (M. Chen et al., 2013; Ciccarelli et al., 2011; Ciccarelli, Cipolletta, & Iaccarino, 2012; Pfleger et al., 2018; Sato et al., 2015; Theccanat et al., 2016). GRK2 knockdown in cardiomyocytes was associated with better handling of cardiac triglycerides and GRK2 knockout in the adult mice attenuated development of diet-induced obesity and insulin resistance in high fat fed models (Lucas et al., 2014; Lucas et

al., 2016). A recent report documented decreased CD36 activity and protein content following cardiomyocyte overexpression of GRK2, which in part contributed to decreased palmitate uptake in the heart (Pfleger et al., 2018). These observations place GRK2 at the intersection between cardiac function and cardiac metabolism. Since recent literature suggests a role for GRK2 in mitochondrial fatty acid oxidation and ROS production, we performed high resolution respirometry in permeabilized fibers from heart tissue in wild type and knockout mice after 36-38 weeks of dietary intervention. Oxygen consumption rates were comparable in state 3 regardless of the substrate used. Succinate driven oxygen consumption was comparable in all the groups and so was oxygen consumption after oligomycin suggesting lack of mitochondrial functional deficits upon high fat feeding in wild type mice or an improvement of mitochondria function in knockout mice. Since hypertrophy was consistently observed in high fat fed mice, we further investigated for alterations in expression levels of markers related to cardiac hypertrophy and pathological remodeling. However, we did not observe significant increases in natriuretic peptides in high fat fed mice regardless of the genotype. Significant increases in collagen isoforms- Col1a1 and Col3a1 were noted in both wildtype and knockout mice fed high fat diet. This increase in collagen isoform gene expression however, was not sufficient to cause fibrosis or to impair cardiac function.

In summary, long-term high fat feeding does not impair cardiac systolic function or compromise cardiac mitochondrial function neither in wildtype and GRK2 knockout mice but reproducibly causes cardiac hypertrophy in both wildtype and GRK2 knockout mice. These findings also suggest that cardiomyocyte GRK2 knockout does not predispose murine hearts to high fat diet-induced cardiac dysfunction. The hypertrophic phenotype of high fat fed mice is

however, not accompanied by pathological hypertrophic gene expression patterns or increased interstitial fibrosis. These findings emphasize the need for identification and development of reliable and reproducible models of cardiomyopathy associated with type 2 diabetes and obesity, which would allow for subsequent evaluation of cardiomyocyte GRK2 in development of diabetic cardiomyopathy.

REFERENCES:


Battiprolu, P. K., Hojayev, B., Jiang, N., Wang, Z. V., Luo, X., Iglewski, M., . . . Hill, J. A. (2012). Metabolic stress-induced activation of FoxO1 triggers diabetic cardiomyopathy in mice. *J Clin Invest, 122*(3), 1109-1118. doi:10.1172/jci60329

Boudina, S., Han, Y. H., Pei, S., Tidwell, T. J., Henrie, B., Tuinei, J., . . . Abel, E. D. (2012). UCP3 regulates cardiac efficiency and mitochondrial coupling in high fat-fed mice but not in leptin-deficient mice. *Diabetes, 61*(12), 3260-3269. doi:10.2337/db12-0063

Brinks, H., Boucher, M., Gao, E., Chuprun, J. K., Pesant, S., Raake, P. W., . . . Koch, W. J. (2010). Level of G protein-coupled receptor kinase-2 determines myocardial ischemia/reperfusion injury via pro- and anti-apoptotic mechanisms. *Circ Res, 107*(9), 1140-1149. doi:10.1161/circresaha.110.221010

Chen, M., Sato, P. Y., Chuprun, J. K., Peroutka, R. J., Otis, N. J., Ibetti, J., . . . Koch, W. J. (2013). Prodeath signaling of G protein-coupled receptor kinase 2 in cardiac myocytes after ischemic stress occurs via extracellular signal-regulated kinase-dependent heat shock protein 90-mediated mitochondrial targeting. *Circ Res, 112*(8), 1121-1134. doi:10.1161/circresaha.112.300754

Ciccarelli, M., Chuprun, J. K., Rengo, G., Gao, E., Wei, Z., Peroutka, R. J., . . . Koch, W. J. (2011). G protein-coupled receptor kinase 2 activity impairs cardiac glucose uptake and promotes insulin resistance after myocardial ischemia. *Circulation, 123*(18), 1953-1962. doi:10.1161/circulationaha.110.988642

Ciccarelli, M., Cipolletta, E., & Iaccarino, G. (2012). GRK2 at the control shaft of cellular metabolism. *Curr Pharm Des, 18*(2), 121-127.

de Simone, G., Devereux, R. B., Chinali, M., Lee, E. T., Galloway, J. M., Barac, A., . . . Howard, B. V. (2010). Diabetes and incident heart failure in hypertensive and normotensive participants of the Strong Heart Study. *J Hypertens, 28*(2), 353-360. doi:10.1097/HJH.0b013e3283331169

Despres, J. P., Lamarche, B., Mauriege, P., Cantin, B., Dagenais, G. R., Moorjani, S., & Lupien, P. J. (1996). Hyperinsulinemia as an independent risk factor for ischemic heart disease. *N Engl J Med, 334*(15), 952-957. doi:10.1056/nejm199604113341504

Downing, S. E., Lee, J. C., & Rieker, R. P. (1977). Mechanical and metabolic effects of insulin on newborn lamb myocardium. *Am J Obstet Gynecol, 127*(6), 649-656.

Evron, T., Daigle, T. L., & Caron, M. G. (2012). GRK2: multiple roles beyond G protein-coupled receptor desensitization. *Trends Pharmacol Sci, 33*(3), 154-164. doi:10.1016/j.tips.2011.12.003

Fan, Q., Chen, M., Zuo, L., Shang, X., Huang, M. Z., Ciccarelli, M., . . . Gao, E. (2013). Myocardial Ablation of G Protein-Coupled Receptor Kinase 2 (GRK2) Decreases Ischemia/Reperfusion Injury through an Anti-Intrinsic Apoptotic Pathway. *PLoS One, 8*(6), e66234. doi:10.1371/journal.pone.0066234

Fu, Q., Xu, B., Liu, Y., Parikh, D., Li, J., Li, Y., . . . Xiang, Y. K. (2014). Insulin inhibits cardiac contractility by inducing a Gi-biased beta2-adrenergic signaling in hearts. *Diabetes, 63*(8), 2676-2689. doi:10.2337/db13-1763

Galderisi, M., Anderson, K. M., Wilson, P. W., & Levy, D. (1991). Echocardiographic evidence for the existence of a distinct diabetic cardiomyopathy (the Framingham Heart Study). *Am J Cardiol, 68*(1), 85-89.

Hiatt, N., & Katz, J. (1969). Modification of cardiac and hyperglycemic effects of epinephrine by iulin. *Life Sci, 8*(9), 551-558.

Huang, Z. M., Gao, E., Chuprun, J. K., & Koch, W. J. (2014). GRK2 in the heart: a GPCR kinase and beyond. *Antioxid Redox Signal, 21*(14), 2032-2043. doi:10.1089/ars.2014.5876

Kannel, W. B., Hjortland, M., & Castelli, W. P. (1974). Role of diabetes in congestive heart failure: the Framingham study. *Am J Cardiol, 34*(1), 29-34.

Kannel, W. B., & McGee, D. L. (1979). Diabetes and cardiovascular disease. The Framingham study. *Jama, 241*(19), 2035-2038.

Katunga, L. A., Gudimella, P., Efird, J. T., Abernathy, S., Mattox, T. A., Beatty, C., . . . Anderson, E. J. (2015). Obesity in a model of gpx4 haploinsufficiency uncovers a causal role for lipid-derived aldehydes in human metabolic disease and cardiomyopathy. *Mol Metab, 4*(6), 493-506. doi:10.1016/j.molmet.2015.04.001

Kenchaiah, S., Evans, J. C., Levy, D., Wilson, P. W., Benjamin, E. J., Larson, M. G., . . . Vasan, R. S. (2002). Obesity and the risk of heart failure. *N Engl J Med, 347*(5), 305-313. doi:10.1056/NEJMoa020245

Klinge, E., & Wafin, F. (1971). Increase in cardiac contractile force caused by pork insulin. *Ann Med Exp Biol Fenn, 49*(3), 138-142.

Lauer, M. S., Anderson, K. M., Kannel, W. B., & Levy, D. (1991). The impact of obesity on left ventricular mass and geometry. The Framingham Heart Study. *Jama, 266*(2), 231-236.

Lee, J. C., & Downing, S. E. (1976). Effects of insulin on cardiac muscle contraction and responsiveness to norepinephrine. *Am J Physiol, 230*(5), 1360-1365. doi:10.1152/ajplegacy.1976.230.5.1360

Lucas, E., Jurado-Pueyo, M., Fortuno, M. A., Fernandez-Veledo, S., Vila-Bedmar, R., Jimenez-Borreguero, L. J., . . . Murga, C. (2014). Downregulation of G protein-coupled receptor kinase 2 levels enhances cardiac insulin sensitivity and switches on cardioprotective gene expression patterns. *Biochim Biophys Acta, 1842*(12 Pt A), 2448-2456. doi:10.1016/j.bbadis.2014.09.004

Lucas, E., Vila-Bedmar, R., Arcones, A. C., Cruces-Sande, M., Cachofeiro, V., Mayor, F., Jr., & Murga, C. (2016). Obesity-induced cardiac lipid accumulation in adult mice is modulated by G protein-coupled receptor kinase 2 levels. *Cardiovasc Diabetol, 15*(1), 155. doi:10.1186/s12933-016-0474-6

Lucchesi, B. R., Medina, M., & Kniffen, F. J. (1972). The positive inotropic action of insulin in the canine heart. *Eur J Pharmacol, 18*(1), 107-115.

Matkovich, S. J., Diwan, A., Klanke, J. L., Hammer, D. J., Marreez, Y., Odley, A. M., . . . Dorn, G. W., 2nd. (2006). Cardiac-specific ablation of G-protein receptor kinase 2 redefines its roles in heart development and beta-adrenergic signaling. *Circ Res, 99*(9), 996-1003. doi:10.1161/01.RES.0000247932.71270.2c

Nichols, G. A., Gullion, C. M., Koro, C. E., Ephross, S. A., & Brown, J. B. (2004). The incidence of congestive heart failure in type 2 diabetes: an update. *Diabetes Care, 27*(8), 1879-1884.

Nudel, D. B., Lee, J. C., & Downing, S. E. (1977). Reciprocal inhibition of cardiac responses to norepinephrine and insulin. *Am J Physiol, 233*(6), H665-669. doi:10.1152/ajpheart.1977.233.6.H665

Park, S. Y., Cho, Y. R., Kim, H. J., Higashimori, T., Danton, C., Lee, M. K., . . . Kim, J. K. (2005). Unraveling the temporal pattern of diet-induced insulin resistance in individual organs and cardiac dysfunction in C57BL/6 mice. *Diabetes, 54*(12), 3530-3540.

Pfleger, J., Gross, P., Johnson, J., Carter, R. L., Gao, E., Tilley, D. G., . . . Koch, W. J. (2018). G protein-coupled receptor kinase 2 contributes to impaired fatty acid metabolism in the failing heart. *J Mol Cell Cardiol, 123*, 108-117. doi:10.1016/j.yjmcc.2018.08.025

Regan, T. J., Frank, M. J., Lehan, P. H., & Hellems, H. K. (1963). RELATIONSHIP OF INSULIN AND STROPHANTHIDIN TO MYOCARDIAL METABOLISM AND FUNCTION. *Am J Physiol, 205*, 790-794. doi:10.1152/ajplegacy.1963.205.4.790

Rieker, R. P., Lee, J. C., & Downing, S. E. (1975). Positive inotropic action of insulin on piglet heart. *Yale J Biol Med, 48*(5), 353-360.

Sato, P. Y., Chuprun, J. K., Ibetti, J., Cannavo, A., Drosatos, K., Elrod, J. W., & Koch, W. J. (2015). GRK2 compromises cardiomyocyte mitochondrial function by diminishing fatty acid-mediated oxygen consumption and increasing superoxide levels. *J Mol Cell Cardiol, 89*(Pt B), 360-364. doi:10.1016/j.yjmcc.2015.10.002

Scirica, B. M., Braunwald, E., Raz, I., Cavender, M. A., Morrow, D. A., Jarolim, P., . . . Bhatt, D. L. (2015). Heart Failure, Saxagliptin, and Diabetes Mellitus: Observations from the SAVOR-TIMI 53 Randomized Trial. *Circulation, 132*(15), e198. doi:10.1161/cir.0000000000000330

Theccanat, T., Philip, J. L., Razzaque, A. M., Ludmer, N., Li, J., Xu, X., & Akhter, S. A. (2016). Regulation of cellular oxidative stress and apoptosis by G protein-coupled receptor kinase-2; The role of NADPH oxidase 4. *Cell Signal, 28*(3), 190-203. doi:10.1016/j.cellsig.2015.11.013

Ungerer, M., Parruti, G., Bohm, M., Puzicha, M., DeBlasi, A., Erdmann, E., & Lohse, M. J. (1994). Expression of beta-arrestins and beta-adrenergic receptor kinases in the failing human heart. *Circ Res, 74*(2), 206-213.

Wang, Q., Liu, Y., Fu, Q., Xu, B., Zhang, Y., Kim, S., . . . Xiang, Y. K. (2017). Inhibiting Insulin-Mediated beta2-Adrenergic Receptor Activation Prevents Diabetes-Associated Cardiac Dysfunction. *Circulation, 135*(1), 73-88. doi:10.1161/circulationaha.116.022281

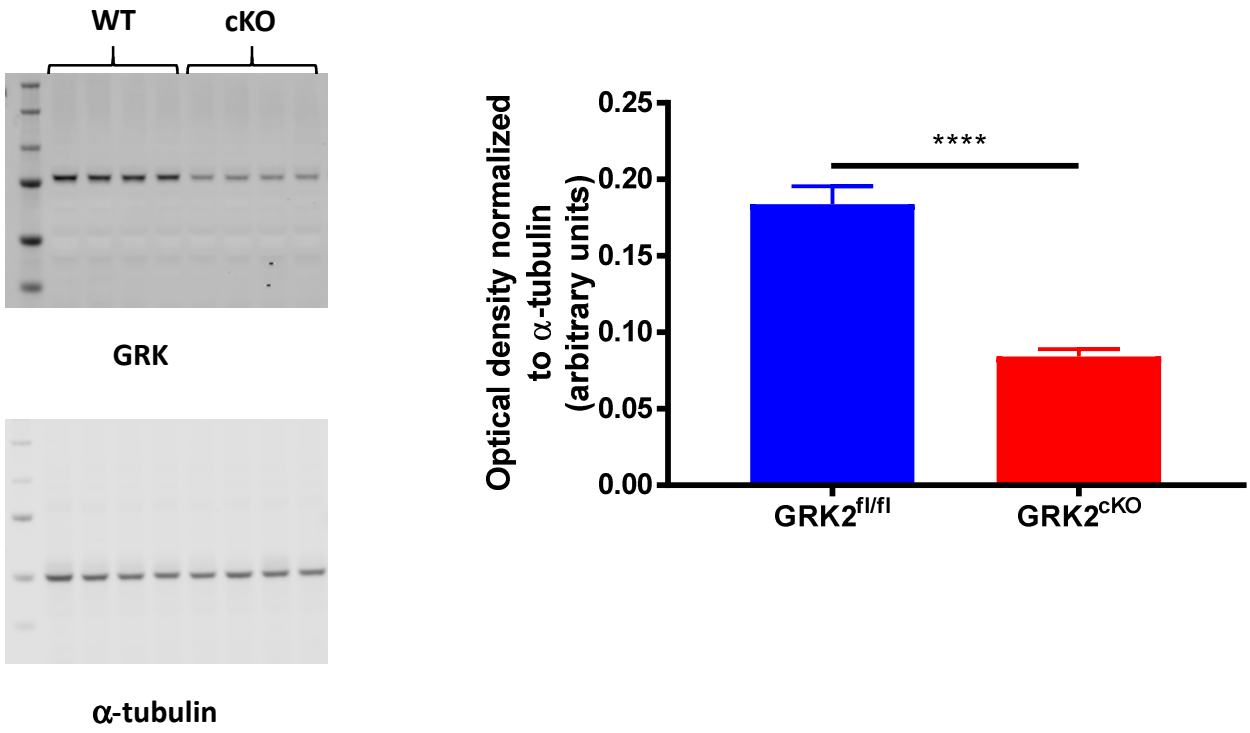



**Figure 10. Validation of GRK2 knockout in the hearts of GRK2$^{cKO}$ mice.**

cKO mice refer to floxed mice carrying a transgene encoding Cre recombinase fused to a mutant estrogen receptor (designated Mer-Cre-Mer). GRK2 protein was measured in heart by western blot 4 weeks after the last tamoxifen injection (N=4/group). Data are presented as Mean±SEM and statistical significance was tested by student's t-test (****-p<0.0001).

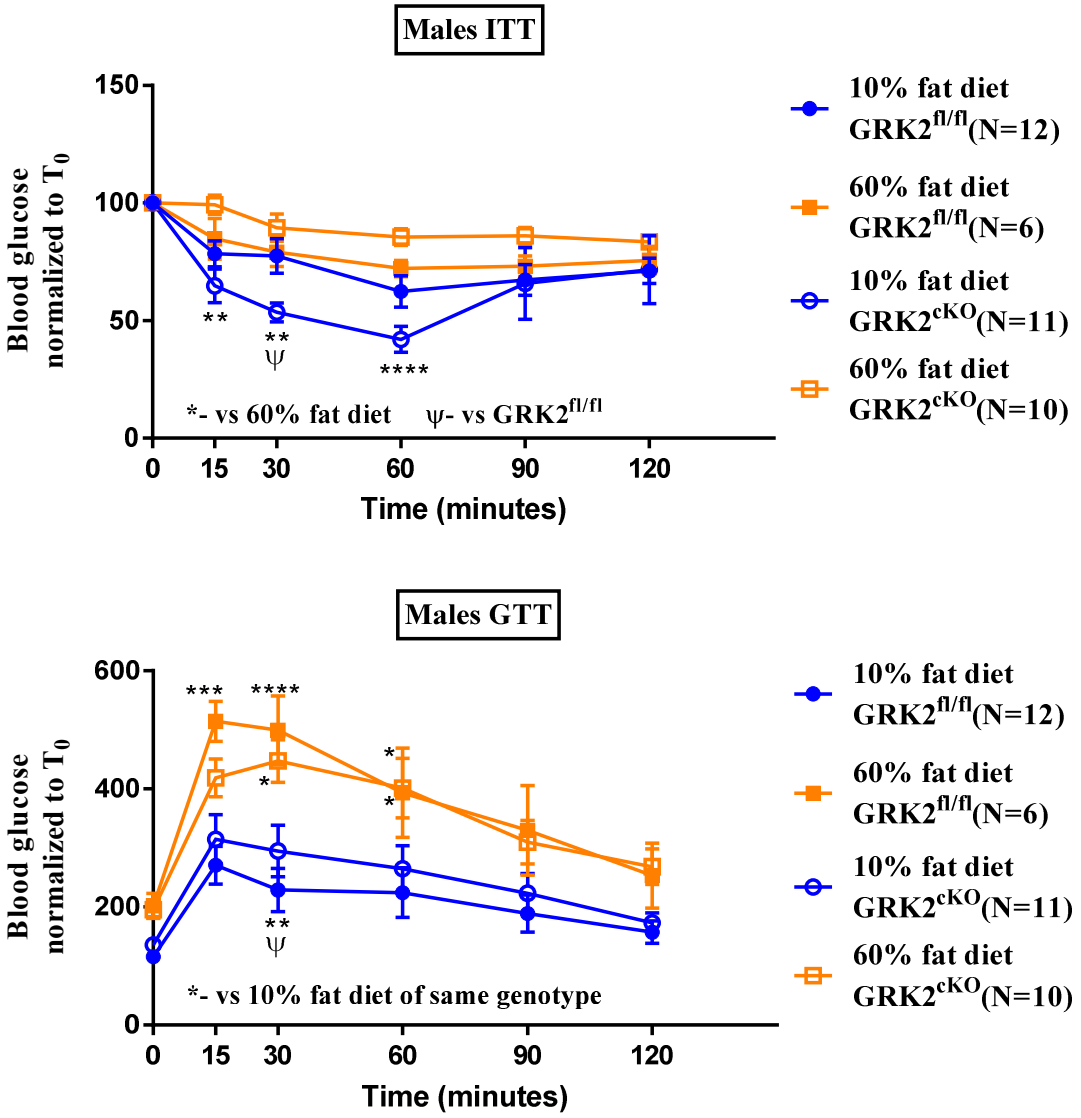



**Figure 11. High fat feeding resulted in insulin resistance and glucose intolerance as measured at 30 weeks after high fat feeding in male mice.**

Data were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's test for within genotype differences. (*-$p<0.05$, **-$p<0.01$, ****-$p<0.0001$)

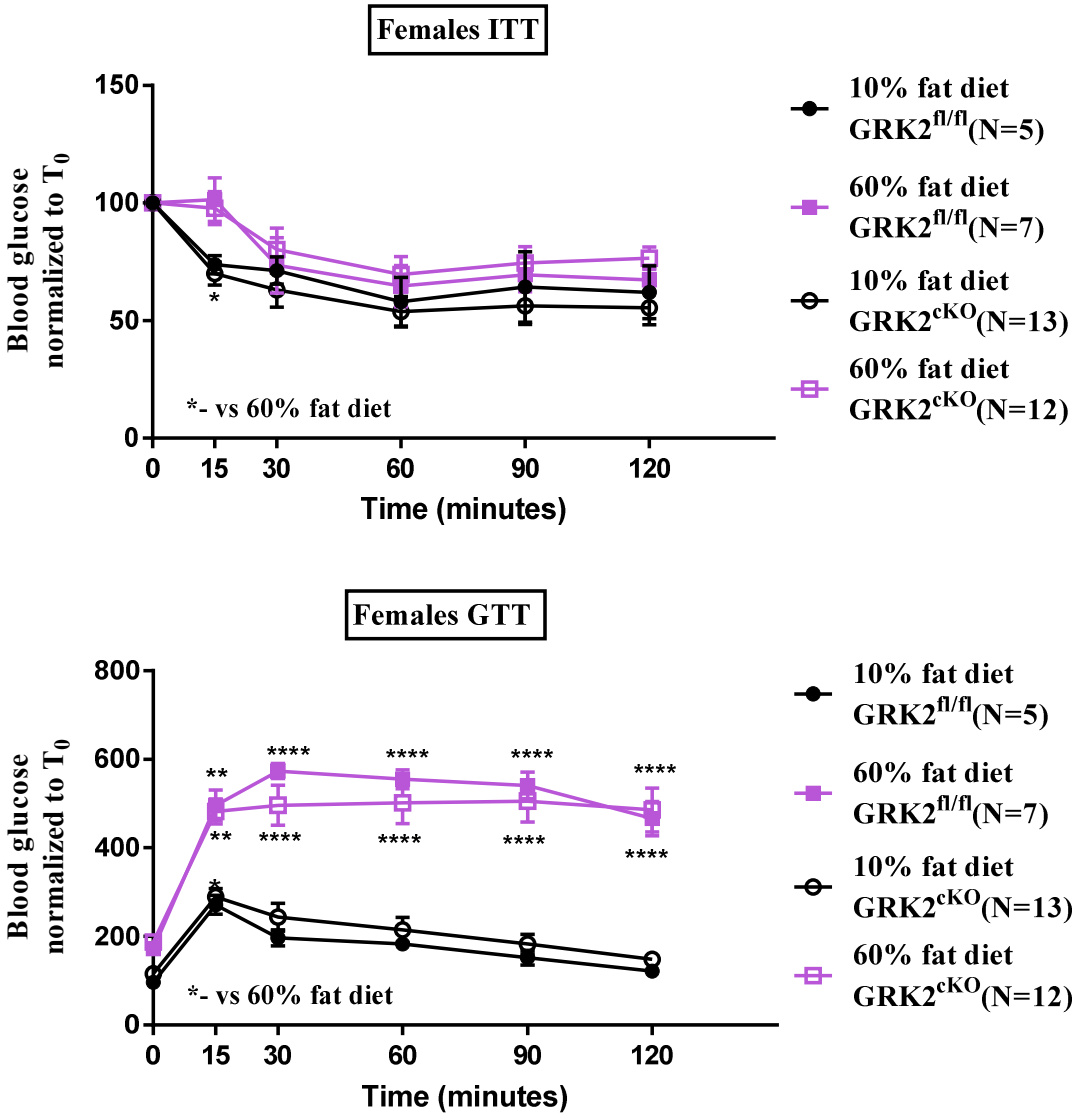
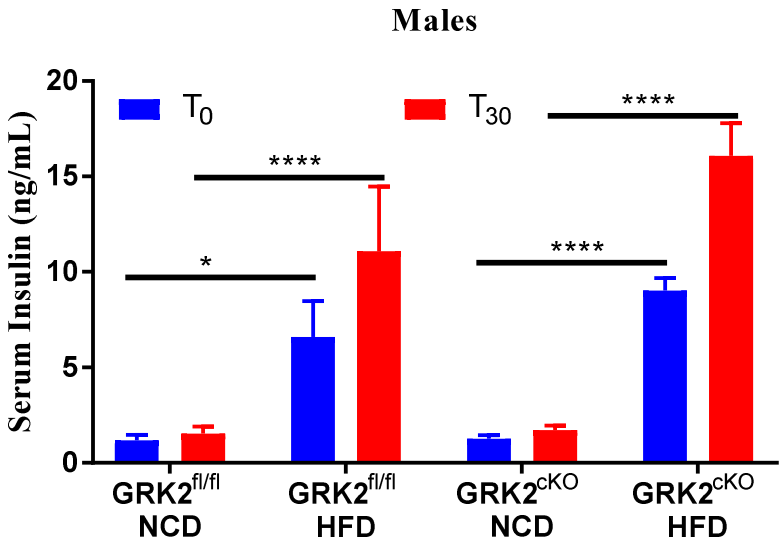



**Figure 12. High fat feeding resulted in insulin resistance and glucose intolerance as measured at 30 weeks after high fat feeding in female mice.**

Data were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's test for within genotype differences. (*-p<0.05, **-p<0.01, ****-p<0.0001)


Males
Serum Insulin (ng/mL)
20
15
10
5
0
T0
T30
****
****
*
****
GRK2fl/fl NCD
GRK2fl/fl HFD
GRK2cKO NCD
GRK2cKO HFD

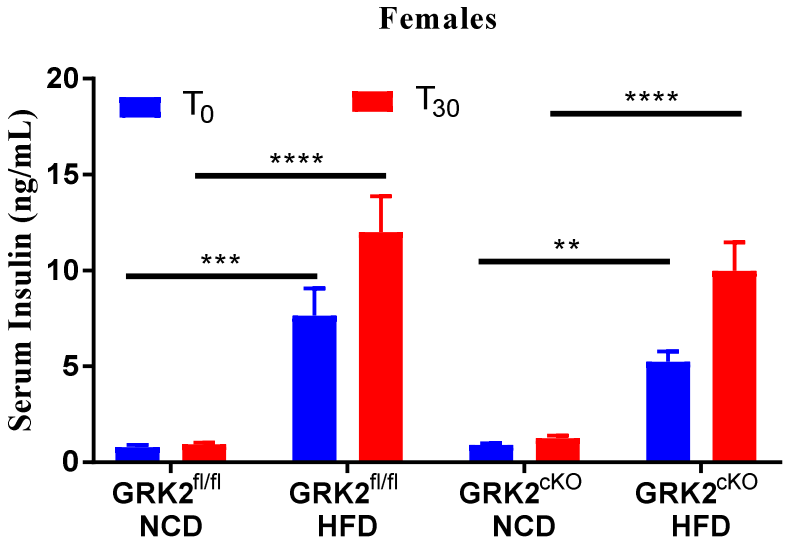

Females
Serum Insulin (ng/mL)
20
15
10
5
0
T0
T30
****
****
***
**
GRK2fl/fl NCD
GRK2fl/fl HFD
GRK2cKO NCD
GRK2cKO HFD

**Figure 13. High fat feeding elevated serum insulin levels in GRK2 fl/fl and GRK2cKO mice.**

Serum insulin was measured by insulin ELISA after 6h fasting and 30minutes after intraperitoneal glucose load during GTT. Data are presented as Mean±SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Sidak's multiple comparison test. Statistical significance was set at $p<0.05$ (*-$p<0.05$, **-$p<0.01$, ***-$p<0.001$, ****-$p<0.0001$)

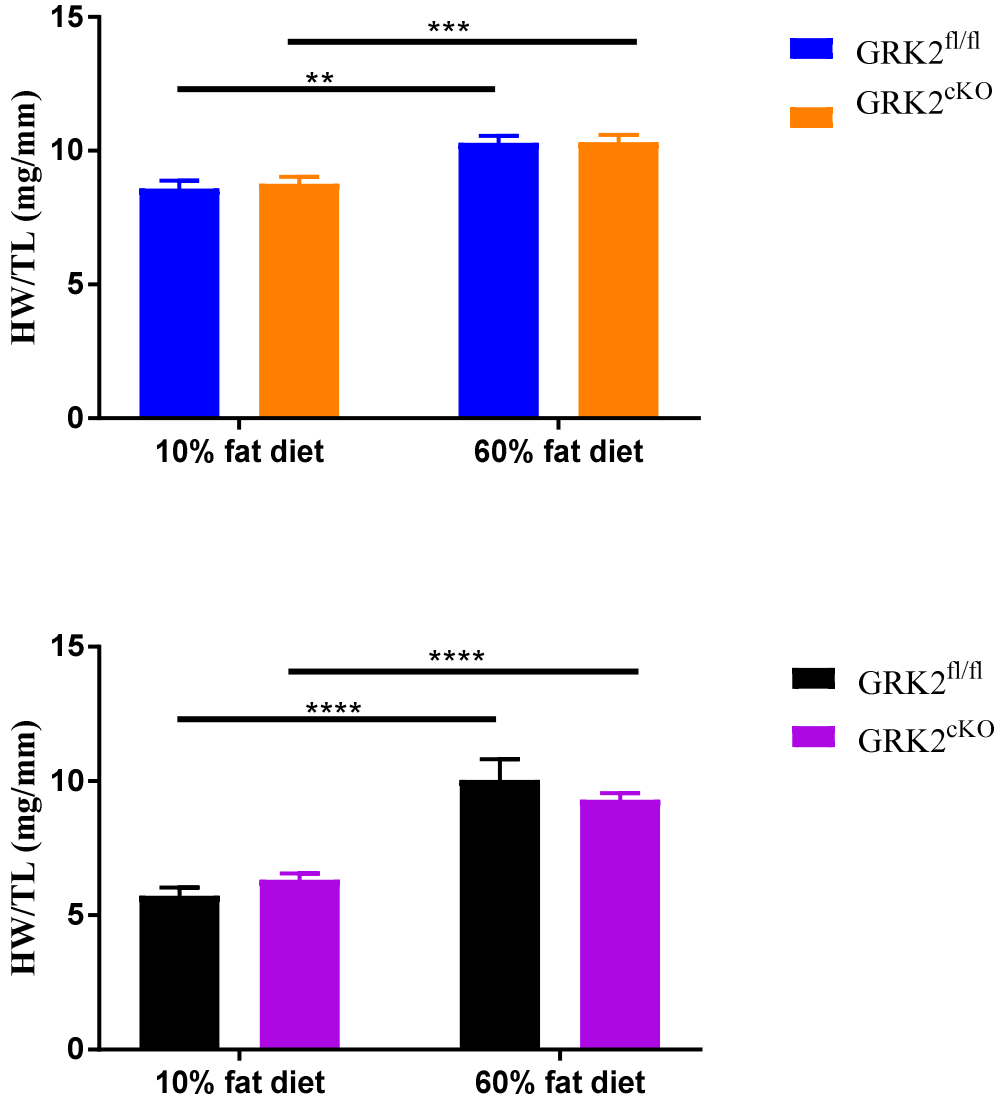



**Figure 14. Cardiac hypertrophy in wildtype and GRK2 cardiomyocyte knockout male and female mice after 36-38 weeks of dietary intervention.**

Statistical analysis was performed by two-way ANOVA followed by significance testing using Tukey's post-hoc analysis. Statistical significance was assessed at $p<0.05$ (**-$p<0.01$, ***-$p<0.001$, ****-$p<0.0001$)

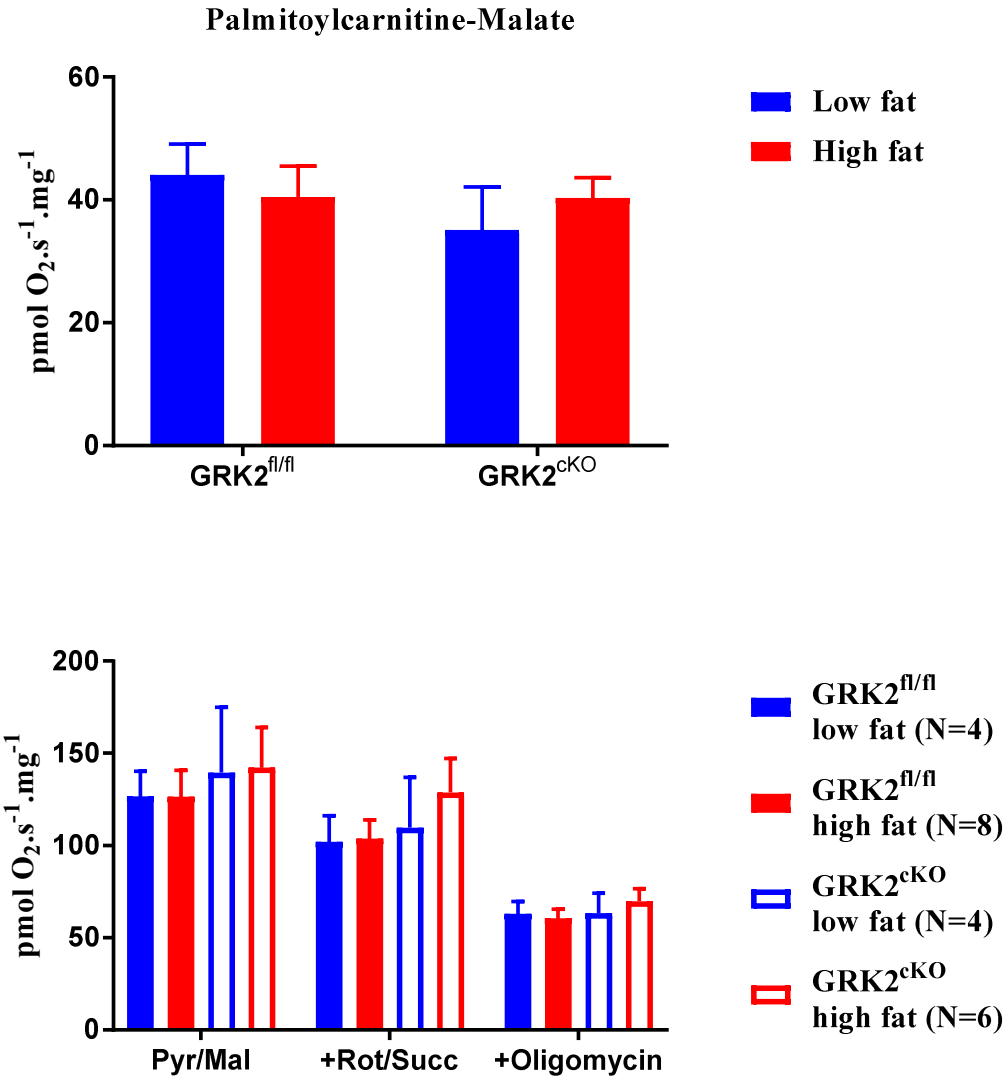
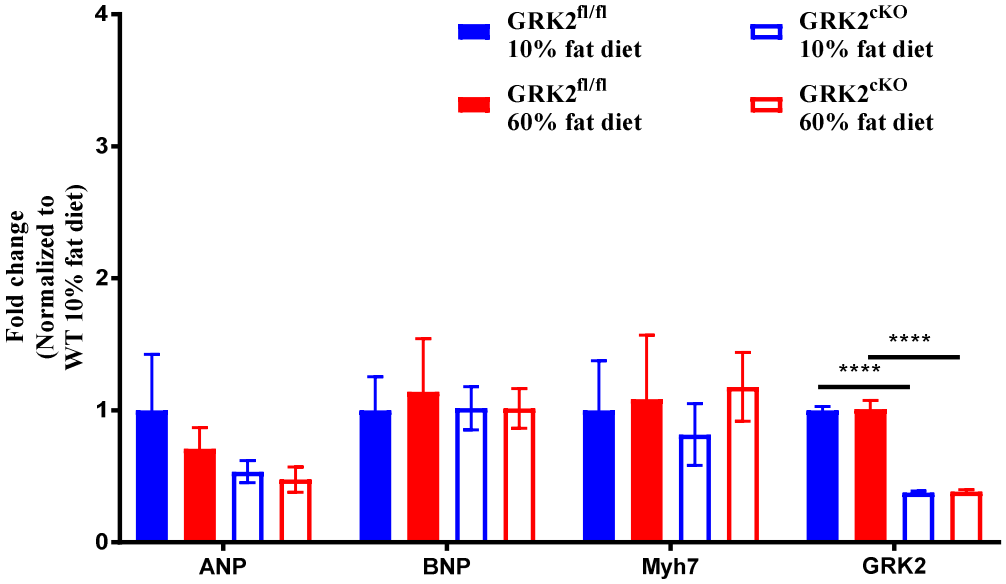

Palmitoylcarnitine-Malate
pmol O2.s-1.mg-1
60
40
20
0
GRK2fl/fl
GRK2cKO
Low fat
High fat
pmol O2.s-1.mg-1
200
150
100
50
0
Pyr/Mal
+Rot/Succ
+Oligomycin
GRK2fl/fl low fat (N=4)
GRK2fl/fl high fat (N=8)
GRK2cKO low fat (N=4)
GRK2cKO high fat (N=6)

**Figure 15. Mitochondrial respiration as measured in permeabilized fibers from hearts of GRK2$^{fl/fl}$ and GRK2$^{cKO}$ mice subject to various dietary conditions.**

Oxygen consumption in State 3 (2mM ADP) using Palmitoylcarnitine/Malate (0.02mM/0.25mM) as substrates (top) or using Pyruvate/Malate (2.5mM/0.5mM) as substrates (bottom) followed by injections with Rotenone (8mM) and Succinate (2mM). Experiment was concluded after addition of Oligomycin (8mg/mL). Each group comprised of fibers prepared from at least 2 male and 2 female mice. Two-way ANOVA revealed no significant differences among the groups.

A


GRK2fl/fl 10% fat diet
GRK2cKO 10% fat diet
GRK2fl/fl 60% fat diet
GRK2cKO 60% fat diet
Fold change (Normalized to WT 10% fat diet)
0
1
2
3
4
****
****
ANP
BNP
Myh7
GRK2

B

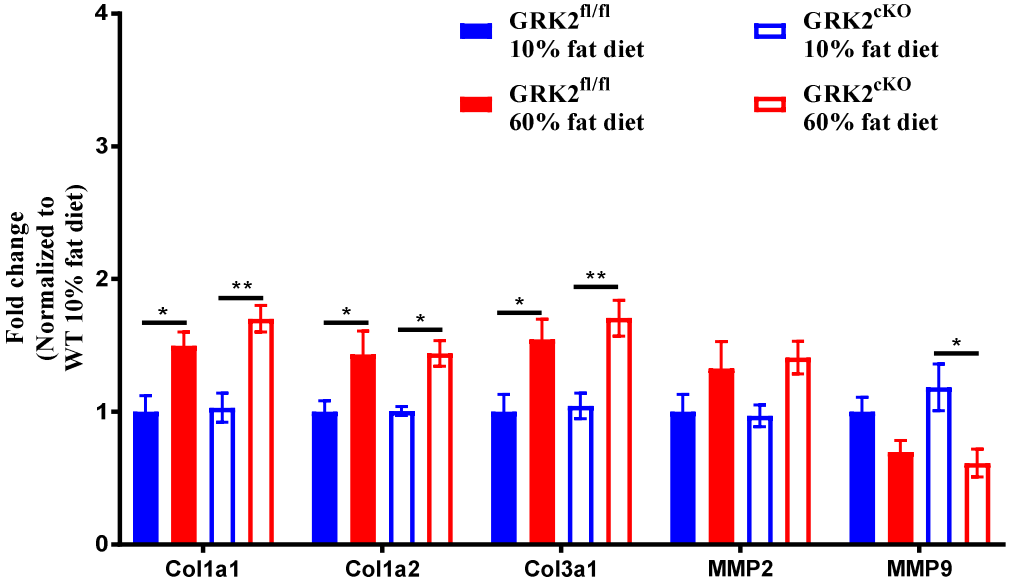

GRK2fl/fl 10% fat diet
GRK2cKO 10% fat diet
GRK2fl/fl 60% fat diet
GRK2cKO 60% fat diet
Fold change (Normalized to WT 10% fat diet)
0
1
2
3
4
*
**
*
*
*
**
*
Col1a1
Col1a2
Col3a1
MMP2
MMP9

**Figure 16. Chronic fat feeding induced alterations in cardiac gene expression of GRK2$^{fl/fl}$ and GRK2$^{cKO}$ mice as determined by qPCR.**

A) no significant differences in pathological hypertrophic markers (ANP, BNP, Myh7). GRK2 mRNA levels in these samples (plotted in A) are shown for reference. B) Modest increases in genes related to extracellular matrix remodeling were observed in high fat fed groups compared to low fat diet independent of the genotype. Data are presented as Mean±SEM and analyzed by two-way ANOVA followed by Tukey's post-hoc analysis (N>5 samples per group, all male mice; *-$p<0.05$, **-$p<0.01$, ****-$p<0.0001$).

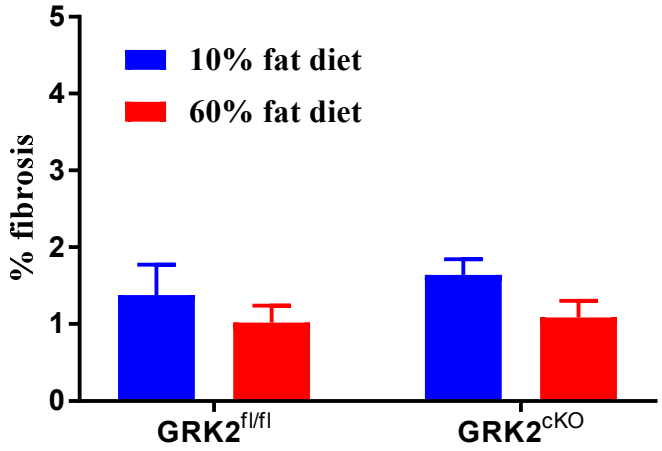



**Figure 17. Quantification of fibrosis in the hearts of GRK2 $^{fl/fl}$ and GRK2$^{cKO}$ mice after 36-38 weeks of dietary intervention.**

Date are presented as Mean±SEM and statistical testing was performed by Two-way ANOVA (N=4/group, only male mice).

| Parameter | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|
| | Wildtype | | Knockout | | Wildtype | | Knockout | |
| | 10% fat diet (N=13) | 60% fat diet (N=9) | 10% fat diet (N=12) | 60% fat diet (N=11) | 10% fat diet (N=6) | 60% fat diet (N=8) | 10% fat diet (N=11) | 60% fat diet (N=13) |
| **Ejection Fraction** | 0.797+0.017 | 0.805+0.014 | 0.822+0.008 | 0.786+0.022 | 0.817+0.017 | 0.83+0.015 | 0.79+0.016 | 0.82+0.0129 |
| **Heart Rate (bpm)** | 616+15.35 | 599.9+31.15 | 647.8+14.79 | 586.8+25.47 | 650+17.88 | 636.6+27.6 | 658.2+18 | 666.2+12.56 |
| **ESV (µL)** | 10.04+0.973 | 11.84+1.716 | 8.051+0.673 | 11.82+1.653 | 4.84+0.407 | 6.757+1.343 | 5.73+0.395 | 8.042+0.68 |
| **EDV (µL)** | 50.11+3.507 | 57.06+4.935 | 45.01+2.75 | 54.94+3.532 | 27.04+2.3 | 40.62+6.01 | 27.92+2.213 | 44.69+2.506 |
| **LV mass (mg)** | 94.06+3.966 | 109.3+5.396 | 93.11+3.676 | 103.1+3.25 | 66.98+3.42 | 83.44+8.999 | 69.83+3.914 | 94.76+5.665 |

**Table 1. Cardiac functional parameters as measured by 2D-echocardiography (B-mode) in GRK2$^{fl/fl}$ and GRK2$^{cKO}$ mice after 36-38 weeks of high fat feeding.**

Data were analyzed by 2-way ANOVA followed by post-hoc analysis to determine differences between groups by Tukey's multiple comparison test. Statistical significance was set at $p<0.05$.

| Parameter | 10% fat diet (N=6) | | 60% fat diet (N=6) | |
|---|---|---|---|---|
| | Baseline | 41 weeks | Baseline | 41 weeks |
| Ejection Fraction | 0.816±0.0156 | 0.819±0.01 | 0.776±0.03 | 0.834±0.03 |
| Heart Rate (bpm) | 705±6.99 | 625.8±27.9 | 687.8±22.9 | 543.8±32.8* |
| ESV (µL) | 5.29±0.49 | 5.51±0.46 | 7.87±1.47 | 8.61±2.14 |
| EDV (µL) | 28.89±2.04 | 30.88±1.95 | 35.52±4.96 | 50.73±5.23* |
| EDV/LV mass | 0.439±0.019 | 0.483±0.031 | 0.43±0.037 | 0.49±0.049 |
| LV mass (mg) | 65.83±3.57 | 82.56±8.83 | 64.36±3.92 | 102.17±5.51* |

**Table 2. Cardiac function measured by transthoracic echocardiography in mice fed a lard-based high fat diet for 41 weeks beginning at 9 weeks of age.**

Data were analyzed by repeated measures two-way ANOVA and are represented as Mean±SEM followed by post-hoc analysis using Sidak's test. Significance is reported at $p<0.05$ (*- $p<0.05$ compared to 10% fat diet at the same time point)

# CHAPTER 4: RESILIENCE OF THE C57BL/6J MOUSE TO CARDIAC DYSFUNCTION INDUCED BY METABOLIC STRESS

## 4.1 Introduction:

Cardiovascular disease (CVD) is the leading cause of death in the United States the incidence of which has been increasing for the last 3 decades (CDC 2015). The obesity and diabetes epidemic has a significant impact on modern day health care and economy especially because- these two dysmetabolic states are established risk factors of cardiovascular disease (CDC 2015). Studies from the Framingham cohort revealed a significant association between type 2 diabetes (Galderisi, Anderson, Wilson, & Levy, 1991; Kannel, Hjortland, & Castelli, 1974; Kannel & McGee, 1979) or obesity (Kenchaiah et al., 2002; Lauer, Anderson, Kannel, & Levy, 1991) and risk of heart failure. Therefore, there is an urgent need to improve our understanding of mechanisms underlying the etiology and pathogenesis of cardiac dysfunction associated with these dysmetabolic states.

Impaired contractile function has been reported in leptin receptor mutant models including db/db mice (Belke, Larsen, Gibbs, & Severson, 2000; Buchanan et al., 2005; Semeniuk, Kryski, & Severson, 2002) and in leptin deficient ob/ob mouse model (Christoffersen et al., 2003; Dong et al., 2006) which also exhibits hyperphagia and obesity. Specifically, these animals develop cardiac hypertrophy and compromised systolic function that is accompanied by changes in myocardial substrate utilization (Belke et al., 2000; Buchanan et al., 2005; Mazumder et al., 2004). Decrease in glucose oxidation and an increased fatty acid preference was apparent in addition to triglyceride accumulation in these hearts (Belke et al., 2000; Buchanan et al., 2005; Mazumder et al., 2004). An association between lipid accumulation and impaired cardiac

function was also evident in transgenic overexpression models of genes involved in fatty acid oxidation such as Acyl co-A synthase (Chiu et al., 2001). These studies suggest that lipotoxicity might be a key contributor to development of cardiac dysfunction when excess fatty acid uptake is not matched with increased fatty acid utilization. Other studies in models with increased TG formation such as the cardiac overexpression of DGAT1, however, challenge the notion that triglyceride accumulation is per se sufficient to cause lipotoxic cardiomyopathy (Liu et al., 2009). Whether or not these models reflect the cardiac dysfunction associated with diabetes and obesity is unclear given that these transgenes and mutants are expressed throughout development.

Diabetes and obesity cause severe systemic metabolic perturbations and an understanding of the molecular pathways that predispose these individuals to heart disease is incomplete. To determine whether diabetes and obesity in the absence of genetic manipulations can result in cardiac dysfunction, many investigators have used long-term high fat diet feeding in rodents to model cardiac dysfunction in dysmetabolic states (Battiprolu et al., 2012; S. Y. Park et al., 2005). Impaired organelle homeostasis has been suggested as one contributing mechanism for cardiac dysfunction in hearts of diet-induced obese mice. However, the reproducibility and reliability of this model for studying cardiac dysfunction associated with diabetes and obesity has been questioned by well-designed studies (Brainard et al., 2013; Gupte et al., 2013). Thus, these differences in observations necessitate further validation of the model. We therefore aimed at understanding whether high fat diet reliably and reproducibly resulted in development of cardiac dysfunction and investigated if age, fatty acid saturation in the diet and concomitant hypertension altered the cardiac functional phenotype in high fat diet fed mice.

## 4.2 Methods:

### 4.2.1. Animals:

All studies were performed using C57BL/6J strain (stock: 00664) purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were purchased at 8 weeks of age or 18 weeks of age and were allowed to acclimatize in the animal facility at University of Iowa for 1-2 weeks. Animals were maintained on 12h light-dark cycle with access to food and water adlib. Animals were sacrificed in random fed state at the end of the study.

### 4.2.2. Diets:

Formulated diets were purchased from Research Diets (New Brunswick, NJ). The composition of diets and caloric values are provided in Table 3.

### 4.2.3. Echocardiography:

Cardiac function was measured at baseline and at different time points by echocardiography in sedated mice (100uL of Midazolam) using Vevo 2100 equipped with a 30MHz probe. Images were acquired in 2D mode from short axis at the level of papillary muscles and in parasternal long axis at a frame rate of 300fps. A trained sonographer blinded to the different groups captured and analyzed the images.

### 4.2.4. Mitochondrial Isolation:

A total mitochondrial fraction was isolated from the interventricular septum of the myocardium. Briefly, after sacrifice, the tissue was maintained in ice-cold BiOPS buffer (7.23mM $K_2$EGTA, 2.77mM Ca$K_2$EGTA, 20mM imidazole, 0.5mM DTT, 20mM Taurine, 5.7mM ATP, 14.3mM Phosphocreatine, 6.56mM $MgCl_2.6H_2O$, 50mM MES, pH 7.1 with KOH) until homogenization.

Mitochondrial isolation was performed as previously described (Makrecka-Kuka, Krumschnabel, & Gnaiger, 2015).Bradford assay was performed to determine the protein concentration.

**4.2.5. Oxygen Consumption:**

Mitochondrial function was assessed using Seahorse XF96 analyzer. Briefly, 2.5ug of cardiac mitochondria were seeded on a Polyethylene Terephthalate (PET) plate and centrifuged for 20 minutes at 2000xg at 4ºC. Substrates were added to the assay buffer at the following final concentrations- Pyruvate at 2.5mM, Malate at 0.5mM and Palmitoylcarnitine at 0.02mM. Injections were made in the following sequential order (final concentrations): ADP (2mM), Succinate (2mM) and Cytochrome C (6.4μM). Substrates were freshly prepared and reagents were purchased from Sigma (St.Louis, MO)

**4.2.6. ROS Production:**

Mitochondrial ROS production was measured in isolated mitochondria using a microplate assay as previously described. Briefly, ROS production in state 3 was assessed in respiration assay buffer containing amplex red, superoxide dismutase, ADP at varying concentrations and succinate(5mM), glutamate(10mM), malate(2mM), 5mM deoxyglucose and 5U/mL hexokinase were used as substrates. 1.5-2ug of mitochondria were added right before kinetic based measurement of the accumulating fluorescent resorufin product. ROS production in state 4 was assessed under identical conditions in buffer supplemented with oligomycin (0.01mg/mL).

**4.2.7. ATP Synthesis**

ATP synthesis rates were assessed in isolated mitochondria using a fluorometer (Horiba Systems). Briefly, buffer Z lite (105mM KMES, 30mM KCl, 10mM $KH_2PO_4$, 5mM $MgCl_2.6H_2O$, 0.5mg/mL BSA, pH 7.4 with KOH) supplemented with glucose, hexokinase/G-6-

PDH (Sigma, St.Louis, MO), Ap5a (an inhibitor of adenylate kinase, Sigma, St.Louis, MO) and NADP (Sigma, St.Louis, MO) was added with 5ug of mitochondria. Basal and ADP stimulated ATP synthesis rate were measured kinetically from the formation of NADPH in a coupled reaction that uses ATP for conversion of glucose to Glucose-6-Phosphate (G6P) by hexokinase and subsequently to 6-phosphogluconolactone by G6P dehydrogenase coupled with reduction of NADP to NADPH. NADPH accumulation is measured by fluorometry using excitation/emission wavelengths of 345nm/460nm respectively (Lark et al., 2016).

#### 4.2.8. Gene Expression

qPCR was performed using cDNA prepared from the hearts of various high fat diet study cohorts. Changes in expression levels of different genes were measured using Power-SYBR master mix (Applied biosystems) and custom designed primer pairs (IDT, Coralville, IA). Data were analyzed using the ΔΔCt method and expressed as fold change normalized to control diet groups.

#### 4.2.9. L-NAME Supplementation and Tail-Cuff Plethysmography

L-NAME (Cayman Chemicals, Ann Arbor, MI) was prepared as a frozen aqueous stock (20g/L) and diluted to 1mg/mL in water. Mice were provided adlib access to L-NAME containing drinking water in water bottles and water was changed on alternate days. 8 weeks after exposure to L-NAME, mice were subjected to tail-cuff plethysmography for 6 consecutive days. The first 4 days was considered acclimatization phase and data were analyzed from recordings made on days 5 and 6. At least 20 measurements were made from each mouse and averaged data from individual mice were pooled based on groups.

### 4.2.10. Histology

At the time of sacrifice, a small portion of the apex was placed in Zinc-formalin buffered saline fixative. Tissues were stored in the fixative until they were processed for paraffin embedding. Sections as thin as 5μm were cut using a microtome and samples were processed for Masson's trichrome staining. Sections were scanned using Ariol Slide scanner (Leica Biosystems) and processed for analysis. Quantification was performed using Fiji software.

### 4.2.11. Metabolic Phenotyping

Body composition was measured by NMR after 16 weeks of dietary intervention. Insulin sensitivity was measured by insulin tolerance test (ITT) following a 3-4h fast. Briefly, 0.75U/kg of Humulin-R100 (Eli Lilly, Indianapolis, IN) was injected intraperitoneally (i.p.) after baseline blood glucose was measured. Blood glucose measurements were made at various time points over the next 120min. Data were normalized to blood glucose levels at baseline. Glucose tolerance tests (GTTs) were performed following a 6h fast. Glucose was injected i.p. at 2g/kg body weight and blood glucose was measured at baseline and at various time points over the next 2h. ITTs and GTTs were performed between 16-18 weeks after dietary intervention with a week day recovery period between the two tests.

### 4.2.12. Statistics

Data were analyzed depending on the number of groups in the experiment. Student's t-test was used when comparing 2 groups and one-way ANOVA was used when comparing more than 2 groups to determine simple effects followed by post-hoc analysis. Repeated measures ANOVA was used where appropriate. Statistical significance was set at $p<0.05$ and date are presented as Mean$\pm$SEM.

## 4.3 Results:

### 4.3.1. Lard-based HFD feeding induced cardiac hypertrophy but not cardiac dysfunction:

High fat diet-induced cardiac dysfunction has been established as a model of diabetes and obesity related cardiomyopathy (Battiprolu et al., 2012; Dirkx, Schwenk, Glatz, Luiken, & van Eys, 2011; C. X. Fang et al., 2008; Ouwens et al., 2005; Ouwens et al., 2007; S.-Y. Park et al., 2005). To understand the molecular mechanisms that lead to cardiac dysfunction in a model of diet-induced obesity, we fed C57BL/6J mice with a lard-based high fat diet and performed serial echocardiographic measurements to assess changes in cardiac function. Although some studies revealed an apparent cardiac dysfunction as early as 15-20 weeks after feeding with HFD (Battiprolu et al., 2012; S. Y. Park et al., 2005), we did not observe any effect of high fat feeding on cardiac function in wild type C57BL/6J mice even after 40 weeks of fat feeding (Supplementary Table 1). However, cardiac hypertrophy and increased LV end diastolic volume (Supplementary Figure 1) was a consistent effect.

### 4.3.2. Older C57BL/6J mice are not susceptible to cardiac dysfunction following metabolic stress:

We tested the hypothesis of whether age could modulate the cardiac adaptation to HFD. To test this possibility, we subjected mice that were either 10 weeks of age or 20 weeks of age to high fat feeding for 20 weeks and examined cardiac function by echocardiography. HFD feeding resulted in significant increase in fat mass by 16 weeks (Supplementary Figure 2A) and glucose intolerance and insulin resistance by 18 weeks (Supplementary Figure 3A). After 20 weeks of feeding with high fat diet, transthoracic echocardiography revealed no significant alterations in cardiac function regardless of the age at which HFD feeding was initiated (Figure 18). These

results were corroborated by LV catheterization studies. No differences were observed in first derivatives of LV pressure between control and high fat fed groups in younger mice, but older mice fed a high fat diet had higher $dP/dT_{max}$ compared to control diet (Figure 19). Taken together, these data indicate that high fat feeding for up to 40 weeks in younger mice did not impair cardiac function. Moreover, mice at 20 weeks of age do not respond differently to a 20-week HFD regimen in terms of overall cardiac function as measured by TTE and LV catheterization relative to mice that are 10 weeks of age at the time of initiation with HFD.

**4.3.3. Saturated fat rich diet causes cardiac hypertrophy but not cardiac dysfunction in younger C57BL/6J mice:**

Previous studies in cell culture models using neonatal rat ventricular myocytes (NRVMs) showed that treatment with palmitate, a 16-carbon chain saturated fatty acid induced a robust apoptotic effect that was rescued by addition of unsaturated fatty acids such as oleic acid. The lard-based diet used in our studies is a combination of saturated and unsaturated fat (Supplementary Table 3). Therefore, to assess whether feeding a diet rich in saturated fat impairs cardiac function in mice and whether this response may be affected by age, we subjected 10-week old and 20-week old mice to dietary intervention using a diet rich in saturated fat (58% calories) and sucrose (19% calories). The study was performed for 20 weeks and cardiac function was assessed at 10 and 20 weeks after initiation of HFD feeding. Younger and older mice fed with high fat high sucrose diet had significantly higher fat mass by 16 weeks (Supplementary Figure 2B) after dietary intervention and older mice developed glucose intolerance and insulin resistance (Supplementary Figure 3B) relative to mice fed a low-fat high-sucrose or low-fat low-sucrose diet. Cardiac function as assessed using transthoracic

echocardiography revealed no impairment in function in the high fat group compared to the low-fat groups at 10 weeks as well as 20 weeks after dietary intervention (Figure 20). These observations were not affected by the age of mice at the time of initiation of dietary intervention. Further analysis of cardiac function using LV catheterization in younger mice (10-week old at the time of dietary intervention) revealed no significant changes in first derivatives of LV pressures consistent with preserved systolic function (Figure 21).

**4.3.4. HFD feeding caused cardiac hypertrophy in younger mice regardless of saturation fat content:**

Cardiac dysfunction in many disease models is preceded by cardiac hypertrophy which might be a compensatory response to alleviate the increased LV wall stress. We therefore assessed whether high fat feeding with lard-based diet or saturated fat rich diet resulted in cardiac hypertrophy. A significant increase in heart weight normalized to tibia length was apparent with high fat feeding regardless of the saturated fat content (Figure 22). Further, we assessed whether there was an increase in interstitial fibrosis in high fat fed hearts in light of observations that diabetes and obesity related cardiomyopathy is often accompanied by increased interstitial fibrosis. Trichrome staining revealed no difference in fibrosis in the high fat fed hearts compared to the control hearts regardless of the saturated fat content of the diets (Supplementary Figure 4).

**4.3.5. Younger mice but not older mice exhibit triglyceride accumulation upon HFD feeding:**

One of the proposed mechanisms implicated in cardiac dysfunction in diabetes and obesity is lipotoxicity characterized by accumulation of lipid species such as TAG, DAG and

ceramides in the heart (Bugger & Abel, 2014; Wende & Abel, 2010). We therefore tested whether high fat feeding in mice resulted in lipid accumulation. A small but significant increase in cardiac triglycerides was apparent in younger mice fed with high fat diet regardless of the content of saturated fat in the diet, but this increase in cardiac triglycerides was not evident in older mice fed the high fat diets (Figure 23).

**4.3.6. HFD feeding did not cause mitochondrial dysfunction regardless of differences in saturated fat content:**

Mitochondrial dysfunction has been described in cardiomyopathy associated with diabetes and obesity (S. Boudina et al., 2012; D. Chen, Li, Zhang, Zhu, & Gao, 2018; Marciniak, Marechal, Montaigne, Neviere, & Lancel, 2014; Sverdlov et al., 2015). Since cardiac hypertrophy and lipid accumulation were apparent only in younger mice subjected to dietary intervention with high fat diets, we assessed mitochondrial oxygen consumption in isolated mitochondria from mice fed HFD (both saturated fat rich and lard-based diets) starting at 10 weeks of age using the Seahorse bioanalyzer. HFD feeding did not cause significant alterations in ADP stimulated oxygen consumption rates in isolated mitochondria with pyruvate-malate as substrates or palmitoylcarnitine-malate as substrates (Figure 24, 25). Additionally, we did not observe difference in ADP stimulated oxygen consumption rates in isolated mitochondria from lard based HFD fed older mice regardless of the substrates used (Figure 24). ATP synthesis rates were measured to determine whether the comparable oxygen consumption rates represented coupled or uncoupled respiration in the high fat diet fed groups. ATP synthesis rates were not significantly different in isolated mitochondria from control and high fat diet fed groups using pyruvate-malate as substrates (Figure 26). We further tested for differences in ATP synthesis

rates in isolated mitochondria from lard-based diet using PC-malate as substrates and observed no significant differences (Figure 27). This lack of differences in ATP synthesis rates using PC-malate as substrates was also true with isolated mitochondria from older mice fed saturated fat rich diet (Figure 26, top panel). We further assessed whether the isolated mitochondria from these different groups showed differences in generation of reactive oxygen species, particularly hydrogen peroxide ($H_2O_2$). We did not detect any significant differences in rate of generation of $H_2O_2$ in isolated mitochondria (Figure 28 and 29). Taken together, these results suggest that regardless of the degree of saturation of fatty acids, 20 weeks of high fat diet feeding in 10-week and 20-week old mice did not induce mitochondrial dysfunction.

**4.3.7. HFD feeding caused modest increases in extracellular matrix remodeling related genes:**

Given HFD reproducibly caused hypertrophy across multiple cohorts without cardiac dysfunction, we asked whether this form of compensated hypertrophy was associated with expression of pathological hypertrophic markers (Figure 30 and 31). We therefore assessed gene expression changes in markers related to pathological hypertrophy, remodeling and fibrosis. Regardless of the type of diet, there were no significant differences in natriuretic peptides including ANP and BNP. A modest increase in Myh7 was apparent within older mice subject to 20 weeks of high fat feeding using lard-based diet compared to the control diet. Modest increases in collagen transcripts and matrix metalloproteinases (MMP2 and MMP9) was evident in high fat diet groups regardless of saturated fat content or age of the mice. Taken together, high fat feeding regardless of the fatty acid saturation did not induce large differences in genes that are characteristic of pathological hypertrophy.

### 4.3.8. HFD feeding altered gene expression of fatty acid metabolism related genes:

Since HFD feeding increased triglyceride accumulation in the heart independent of impaired systolic function, we wondered if metabolic adaptations in the heart could have prevented free fatty acid accumulation and limited lipotoxicity. We therefore probed for changes in expression of genes involved in triglyceride formation and fatty acid oxidation (FAO) and fatty acid uptake (Figure 32 and 33). The expression of fatty acid importer CD36 was significantly induced. Expression levels of Dgat1, the enzyme catalyzing the rate limiting step in TAG synthesis, was not altered. A significant increase in expression of Plin5, a protein that regulates lipid droplet formation and breakdown was observed. Expression of Cpt1b and Acs1L were increased. AcadL expression was increased in both younger and older mice fed a lard-based diet. In both younger and older mice, Hadh expression levels were increased when fed a lard-based diet but decreased when fed a saturated fat-rich diet. Similarly, Pnpla2 expression levels were increased in both younger and older mice fed lard-based high fat diet but decreased when fed saturated fat based diet. Expression levels of Pdk4 exhibited the most robust increase. Taken together, these data are consistent with an increased fatty acid uptake and oxidation in response to HFD. Increased FAO has been linked to increased ROS generation in mitochondria that may activate increased uncoupling. Consistent with prior observations by our group and others (S. Boudina et al., 2012; Cole et al., 2011) uncoupling protein 3 expression (Ucp3) was induced following high fat feeding regardless of degree of fatty acid saturation or age at the time HFD initiation. As no differences in mitochondrial ROS synthesis rates were observed in isolated mitochondria between control and high fat fed mice (Figure 28 and 29), we examined other antioxidant defense mechanisms. Catalase expression was significantly increased by HFD

independent of the type of diet and age at time of initiation with HFD. However, expression levels of SOD-2 and HO-1 were unchanged (Figure 34 and 35).

**4.3.9. Concomitant hypertension induced by L-NAME does not cause cardiac dysfunction in C57BL/6J mice fed lard-based high fat diet:**

Since hypertension is a common co-morbidity in diabetic and obese individuals (G. Chen, McAlister, Walker, Hemmelgarn, & Campbell, 2011; "Treatment of Hypertension in Adults With Diabetes," 2003), we tested whether HFD feeding in conjunction with hypertension would induce cardiac dysfunction. We modeled this by feeding mice HFD and by adding L-NAME to the drinking water (1mg/mL). Mean Arterial Pressure (MAP) and systolic pressure were increased as early as 8 weeks after exposure to L-NAME (Supplementary Table 2). Animals were maintained on this protocol for 20 weeks with periodical assessment of cardiac function by TTE every 5 weeks. By 16 weeks, HFD fed mice had elevated fat mass compared to mice on control diet but L-NAME group on HFD had significantly lower fat mass and weight gain relative to HFD alone (Supplementary Figure 2C). L-NAME supplementation in HFD fed mice also attenuated insulin resistance (lower fasting insulin levels and more responsive to i.p. insulin during ITT) although glucose intolerance was comparable to that of water-fed HFD (Supplementary Figure 3D). LV function as measured by echocardiography was unaltered at all the time points tested regardless of the diet or by L-NAME supplementation (Figure 36). At the end of 20 weeks, we performed LV catheterization to determine changes in first derivatives of LV pressures. Neither HFD alone nor HFD along with L-NAME significantly altered these parameters of cardiac contractility (Figure 37). Since cardiac function measured at any time point did not show significant differences compared to control groups or HFD alone group, the

increased mortality in this group might be related to non-cardiac reasons. To determine whether the combination of metabolic stress and hypertension resulted in exaggerated cardiac hypertrophy (Figure 38) or pulmonary congestion (Figure 38), we measured heart weights and lung weights at the time of sacrifice but did not observe significant differences between mice fed a high fat diet alone or in combination with L-NAME.

**4.4 Discussion:**

A cardiomyopathy associated with diabetes and obesity has been implicated as a precursor to heart failure but the mechanisms that contribute to development of cardiomyopathy in diabetes and obesity are not completely understood. A number of potential mechanisms have been raised including lipotoxicity, mitochondrial dysfunction and oxidative stress (S. Boudina & Abel, 2010; Z. Y. Fang, Prins, & Marwick, 2004). While many models of cardiac dysfunction in diabetes and obesity have been reported in the literature, whether mitochondrial dysfunction and lipotoxicity are sufficient to induce cardiac dysfunction in these dysmetabolic states are less certain. In the present study, we initially sought to understand the molecular pathways that contribute to cardiac dysfunction in diet-induced obese mice. However, failure to uncover a cardiac phenotype by echocardiography despite using a similar dietary regimen and mouse strain motivated us to thoroughly characterize the reproducibility and utility of this diet-induced obesity model for diabetic cardiomyopathy. To this end, we utilized the C57BL/6J mouse strain for characterizing the cardiac phenotype upon long term high fat feeding. We focused on this strain given its susceptibility to diet-induced obesity and also the widespread use of this strain for metabolic studies (Collins, Martin, Surwit, & Robidoux, 2004). While additional environmental factors could potentially contribute to development of a cardiac phenotype with fat feeding, our

results suggest that chronic fat feeding in mice might not be a reproducible model with which to understand the cardiomyopathy related to diabetes and obesity.

Since a lard-based high fat diet did not result in cardiac phenotype as assessed by echocardiography and LV catheterization, we wondered if the fatty acid composition could influence the development of cardiac dysfunction in the diet-induced obesity model of cardiomyopathy. Lard based diets have a saturated fat content that approximates to 20% total calories and the remaining 40% calories in a lard based high fat diet are contributed by unsaturated fatty acids. Previous studies have shown in cell culture models that unsaturated fatty acids such as oleate can rescue the apoptotic effect of excess saturated fatty acids such as palmitate (Listenberger et al., 2003; Miller et al., 2005). To address the possibility that high amounts of unsaturated fatty acids could potentially protect cardiomyocytes from detrimental effects of lower amounts of saturated fatty acids, we developed a formulation that was enriched with saturated fat (up to 95% of total fatty acids). Using this formulation which was also enriched with sucrose (19% of total calories), mice developed insulin resistance and were glucose intolerant by 16-18 weeks. Periodic assessment of cardiac function by echocardiography failed to reveal systolic dysfunction.

To test the possibility that age at the time of induction with high fat diet may alter the cardiac function of high fat fed mice, we also fed an older cohort of mice (20 weeks old) alongside the younger cohort (10 weeks old) for 20 weeks. Cardiac function as assessed by echocardiography was not significantly different between the control and high fat groups. LV catheterization of the younger cohort at the time of sacrifice suggested normal cardiac function that was comparable to that of the control groups. Other studies in mice and rats have utilized a

cocoa-butter based diet, which is rich in saturated fatty acids such as palmitate and stearate. These studies were conducted for 8-16 weeks and the diet used did not cause obesity or insulin resistance. These studies also reported a lack of cardiac dysfunction by feeding the diet alone (Chess, Lei, Hoit, Azimzadeh, & Stanley, 2008; Isidore C. Okere, Chandler, et al., 2006; I. C. Okere et al., 2005; Isidore C. Okere, Young, et al., 2006). Under conditions of additional stress such as pressure overload or myocardial infarction, the cocoa-butter rich diet produced beneficial effects or no effects on cardiac function (Berthiaume et al., 2010; Berthiaume et al., 2012; Chess et al., 2008; Christopher et al., 2010; Morgan et al., 2006; Rennison et al., 2009). With regards to our study, the saturated fat rich diet used is a mixture of medium chain and long chain fatty acids (Table 3) and feeding mice with the customized diet resulted in insulin resistance and obesity. Despite the metabolic perturbations, there was no apparent cardiac dysfunction as assessed by echocardiography or LV catheterization emphasizing the resilience of these mouse hearts to development of cardiac dysfunction despite persistent metabolic stress.

We assessed cardiac steatosis, mitochondrial function and reactive oxygen species production rates to determine whether changes in any of these parameters as previously reported in literature were induced by high fat feeding in our cohorts. We observed modest but significant increases in cardiac triglyceride content in younger groups fed with - both saturated fat rich diet and lard-based HFDs. Cardiac mitochondrial dysfunction has been suggested to ensue in rodent models of obesity- both genetic and dietary (Anderson et al., 2009; S. Boudina et al., 2012; Sihem Boudina et al., 2007; D. Chen et al., 2018; Sverdlov et al., 2016). Our analysis of cardiac mitochondria using seahorse bioanalyzer did not indicate mitochondrial oxidative defects, uncoupling or ROS overproduction.

It is also possible that the lack of cardiac functional phenotype in the several high fat fed groups reveal robust compensatory responses that preserved cardiac function. While we failed to uncover a cardiac functional phenotype using the techniques at our disposal, we reproducibly observed cardiac hypertrophy in high fat fed mice with the hypertrophic phenotype being more pronounced in younger mice fed a high fat diet than older mice. However, when we probed for markers of pathological cardiac hypertrophy, we did not observe significant increases in natriuretic peptides- ANP and BNP that are induced by increased wall stress, nor did we note elevations in Myh7- the isoform that is increased in expressed under pathological conditions. We also investigated whether the high fat fed mice exhibited increased fibrosis in their hearts and found no significant differences between control and high fat groups. Modest, but significant increases in expression levels of collagen transcripts were found in hearts of high fat fed groups but these increases were not associated with collagen accumulation or cardiac fibrosis. Taken together, our data suggests that HFD feeding up to 20 weeks, regardless of the degree of fatty acid saturation and age at the time of initiation with HFD is insufficient to cause cardiac dysfunction as measured by transthoracic echocardiography and LV catheterization. In addition, our data also reveals that cardiac mitochondria could be resistant to dysfunction upon HFD feeding and that HFD might not invariably cause oxidative stress or lipotoxicity in the myocardium. Gross morphological changes suggest that cardiac hypertrophy without an increase in interstitial fibrosis ensues with HFD feeding for 20 weeks and is not accompanied by altered cardiac function.

Since hypertension is a common comorbidity in diabetic and obese individuals and the risk of heart disease is amplified under these conditions, we tested whether mice fed HFD and

concomitantly exposed to L-NAME developed cardiac dysfunction. We did not observe significant changes in cardiac function or increased pulmonary congestion as assessed by lung weights. A more recent study reported the high fat diet and L-NAME combination as a model of Heart Failure with Preserved Ejection Fraction (HFpEF) (Schiattarella et al., 2019). Diastolic function as measured by Pressure-Volume loops in this study indicated an increased end-diastolic pressure and ventricular compliance was compromised as demonstrated by an increased slope of end-diastolic pressure with increasing volume (Schiattarella et al., 2019). However, this phenotype was observed in the C57BL/6N strain (Schiattarella et al., 2019) and the dependence on mouse strain of cardiac outcomes following metabolic stress and hypertension may be particularly important. Supporting this argument is the recent finding that the functional Nicotinamide nucleotide transhydrogenase (Nnt) protein which is expressed in the C57BL/6N mice but not in the C57BL/6J mice can operate in reverse mode to generate NADH and thereby deplete NADPH levels under conditions of pathological workload such as pressure overload (Nickel et al., 2015). An increased oxidative stress in the cardiomyocytes of C57BL/6N mice was correlated with increased nuclear oxidative damage and increased fibrotic gene expression and compromised function. C57BL/6J mice which express a truncated non-functional Nnt protein have preserved cardiac function and relatively small increases in fibrosis following pressure overload (Nickel et al., 2015).

In conclusion, high fat diet feeding regardless of saturation content of the fat and concomitant hypertension might not be sufficient to induce cardiac dysfunction in C57BL/6J mice. In the present study, mitochondrial function is not impaired by fat feeding, lipid accumulation following high fat feeding is minimal possibly resulting from increased fatty acid

oxidation and utilization in the myocardium of fat fed mice. These results highlight the remarkable resilience of the mouse heart to cardiac dysfunction following metabolic stress.

**4.5 Limitations:**

Our findings suggest that high fat feeding in C57BL/6J mice may not represent a reliable model to study mechanisms of diabetic cardiomyopathy. The use of a single strain of mice in this study however does not inform of whether other strains may be sensitive to cardiac dysfunction induced by metabolic stress. We utilized 2D-echocardiography and LV catheterization to measure cardiac function and both these techniques measure cardiac function in a load-dependent manner. Use of a gold-standard technique such as PV-loops may inform us of subtle cardiac functional deficits that we were unable to uncover with the conventional techniques. We assessed mitochondrial function using whole mitochondrial fraction. Sophisticated isolation procedures may allow for assessment of whether there may be differences in sensitivity of the subpopulations (subsarcolemmal and interfibrillar) to metabolic stress.

# REFERENCES


Anderson, E. J., Kypson, A. P., Rodriguez, E., Anderson, C. A., Lehr, E. J., & Neufer, D. P. (2009). Substrate-Specific Derangements in Mitochondrial Metabolism and Redox Balance in the Atrium of the Type 2 Diabetic Human Heart. *Journal of the American College of Cardiology, 54*(20), 1891-1898. doi:10.1016/j.jacc.2009.07.031

Battiprolu, P. K., Hojayev, B., Jiang, N., Wang, Z. V., Luo, X., Iglewski, M., . . . Hill, J. A. (2012). Metabolic stress-induced activation of FoxO1 triggers diabetic cardiomyopathy in mice. *J Clin Invest, 122*(3), 1109-1118. doi:10.1172/jci60329

Belke, D. D., Larsen, T. S., Gibbs, E. M., & Severson, D. L. (2000). Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (db/db) mice. *Am J Physiol Endocrinol Metab, 279*(5), E1104-1113. doi:10.1152/ajpendo.2000.279.5.E1104

Berthiaume, J. M., Bray, M. S., McElfresh, T. A., Chen, X., Azam, S., Young, M. E., . . . Chandler, M. P. (2010). The myocardial contractile response to physiological stress improves with high saturated fat feeding in heart failure. *American journal of physiology. Heart and circulatory physiology, 299*(2), 21. doi:10.1152/ajpheart.00270.2010

Berthiaume, J. M., Young, M. E., Chen, X., McElfresh, T. A., Yu, X., & Chandler, M. P. (2012). Normalizing the metabolic phenotype after myocardial infarction: Impact of subchronic high fat feeding. *Journal of Molecular and Cellular Cardiology, 53*(1), 125-133. doi:10.1016/j.yjmcc.2012.04.005

Boudina, S., & Abel, E. D. (2010). Diabetic cardiomyopathy, causes and effects. *Rev Endocr Metab Disord, 11*(1), 31-39. doi:10.1007/s11154-010-9131-7

Boudina, S., Han, Y. H., Pei, S., Tidwell, T. J., Henrie, B., Tuinei, J., . . . Abel, E. D. (2012). UCP3 regulates cardiac efficiency and mitochondrial coupling in high fat-fed mice but not in leptin-deficient mice. *Diabetes, 61*(12), 3260-3269. doi:10.2337/db12-0063

Boudina, S., Sena, S., Theobald, H., Sheng, X., Wright, J. J., Hu, X., . . . Abel, D. E. (2007). Mitochondrial Energetics in the Heart in Obesity-Related Diabetes Direct Evidence for Increased Uncoupled Respiration and Activation of Uncoupling Proteins. *Diabetes, 56*(10), 2457-2466. doi:10.2337/db07-0481

Brainard, R. E., Watson, L. J., DeMartino, A. M., Brittian, K. R., Readnower, R. D., Boakye, A., . . . Jones, S. P. (2013). High Fat Feeding in Mice Is Insufficient to Induce Cardiac Dysfunction and Does Not Exacerbate Heart Failure. *PLOS ONE*(12). doi:10.1371/journal.pone.0083174

Buchanan, J., Mazumder, P. K., Hu, P., Chakrabarti, G., Roberts, M. W., Yun, U., . . . Abel, D. E. (2005). Reduced cardiac efficiency and altered substrate metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity. *Endocrinology, 146*(12), 5341-5349. doi:10.1210/en.2005-0938

Bugger, H., & Abel, E. D. (2014). Molecular mechanisms of diabetic cardiomyopathy. *Diabetologia, 57*(4), 660-671. doi:10.1007/s00125-014-3171-6

Chen, D., Li, X., Zhang, L., Zhu, M., & Gao, L. (2018). A high□fat diet impairs mitochondrial biogenesis, mitochondrial dynamics, and the respiratory chain complex in rat myocardial tissues. *Journal of Cellular Biochemistry*. doi:10.1002/jcb.27068

Chen, G., McAlister, F. A., Walker, R. L., Hemmelgarn, B. R., & Campbell, N. R. (2011). Cardiovascular outcomes in framingham participants with diabetes: the importance of blood pressure. *Hypertension, 57*(5), 891-897. doi:10.1161/hypertensionaha.110.162446

Chess, D. J., Lei, B., Hoit, B. D., Azimzadeh, A. M., & Stanley, W. C. (2008). Effects of a High Saturated Fat Diet on Cardiac Hypertrophy and Dysfunction in Response to Pressure Overload. *Journal of Cardiac Failure, 14*(1), 82-88. doi:10.1016/j.cardfail.2007.09.004

Chiu, H.-C., Kovacs, A., Ford, D. A., Hsu, F.-F., Garcia, R., Herrero, P., . . . Schaffer, J. E. (2001). A novel mouse model of lipotoxic cardiomyopathy. *Journal of Clinical Investigation, 107*(7), 813-822. doi:10.1172/JCI10947

Christoffersen, C., Bollano, E., Lindegaard, M. L., Bartels, E. D., Goetze, J. P., Andersen, C. B., & Nielsen, L. B. (2003). Cardiac lipid accumulation associated with diastolic dysfunction in obese mice. *Endocrinology, 144*(8), 3483-3490. doi:10.1210/en.2003-0242

Christopher, B. A., Huang, H.-M., Berthiaume, J. M., McElfresh, T. A., Chen, X., Croniger, C. M., . . . Chandler, M. P. (2010). Myocardial insulin resistance induced by high fat feeding in heart failure is associated with preserved contractile function. *American Journal of Physiology-Heart and Circulatory Physiology, 299*(6). doi:10.1152/ajpheart.00687.2010

Cole, M. A., Murray, A. J., Cochlin, L. E., Heather, L. C., McAleese, S., Knight, N. S., . . . Clarke, K. (2011). A high fat diet increases mitochondrial fatty acid oxidation and uncoupling to decrease efficiency in rat heart. *Basic Res Cardiol, 106*(3), 447-457. doi:10.1007/s00395-011-0156-1

Collins, S., Martin, T. L., Surwit, R. S., & Robidoux, J. (2004). Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. *Physiol Behav, 81*(2), 243-248. doi:10.1016/j.physbeh.2004.02.006

Dirkx, E., Schwenk, R. W., Glatz, J., Luiken, J., & van Eys, G. (2011). High fat diet induced diabetic cardiomyopathy. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA), 85*(5), 219-225. doi:10.1016/j.plefa.2011.04.018

Dong, F., Zhang, X., Yang, X., Esberg, L. B., Yang, H., Zhang, Z., . . . Ren, J. (2006). Impaired cardiac contractile function in ventricular myocytes from leptin-deficient ob/ob obese mice. *J Endocrinol, 188*(1), 25-36. doi:10.1677/joe.1.06241

Fang, C. X., Dong, F., Thomas, P. D., Ma, H., He, L., & Ren, J. (2008). Hypertrophic cardiomyopathy in high-fat diet-induced obesity: role of suppression of forkhead transcription factor and atrophy gene transcription. *American journal of physiology. Heart and circulatory physiology, 295*(3). doi:10.1152/ajpheart.00319.2008

Fang, Z. Y., Prins, J. B., & Marwick, T. H. (2004). Diabetic cardiomyopathy: evidence, mechanisms, and therapeutic implications. *Endocr Rev, 25*(4), 543-567. doi:10.1210/er.2003-0012

Galderisi, M., Anderson, K. M., Wilson, P. W., & Levy, D. (1991). Echocardiographic evidence for the existence of a distinct diabetic cardiomyopathy (the Framingham Heart Study). *Am J Cardiol, 68*(1), 85-89.

Gupte, A. A., Minze, L. J., Reyes, M., Ren, Y., Wang, X., Brunner, G., . . . Hsueh, W. A. (2013). High-fat feeding-induced hyperinsulinemia increases cardiac glucose uptake and mitochondrial function despite peripheral insulin resistance. *Endocrinology, 154*(8), 2650-2662. doi:10.1210/en.2012-2272

Kannel, W. B., Hjortland, M., & Castelli, W. P. (1974). Role of diabetes in congestive heart failure: the Framingham study. *Am J Cardiol, 34*(1), 29-34.

Kannel, W. B., & McGee, D. L. (1979). Diabetes and cardiovascular disease. The Framingham study. *Jama, 241*(19), 2035-2038.

Kenchaiah, S., Evans, J. C., Levy, D., Wilson, P. W., Benjamin, E. J., Larson, M. G., . . . Vasan, R. S. (2002). Obesity and the risk of heart failure. *N Engl J Med, 347*(5), 305-313. doi:10.1056/NEJMoa020245

Lark, D. S., Torres, M. J., Lin, C. T., Ryan, T. E., Anderson, E. J., & Neufer, P. D. (2016). Direct real-time quantification of mitochondrial oxidative phosphorylation efficiency in permeabilized skeletal muscle myofibers. *Am J Physiol Cell Physiol, 311*(2), C239-245. doi:10.1152/ajpcell.00124.2016

Lauer, M. S., Anderson, K. M., Kannel, W. B., & Levy, D. (1991). The impact of obesity on left ventricular mass and geometry. The Framingham Heart Study. *Jama, 266*(2), 231-236.

Listenberger, L. L., Han, X., Lewis, S. E., Cases, S., Farese, R. V., Jr., Ory, D. S., & Schaffer, J. E. (2003). Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Natl Acad Sci U S A, 100*(6), 3077-3082. doi:10.1073/pnas.0630588100

Liu, L., Shi, X., Bharadwaj, K. G., Ikeda, S., Yamashita, H., Yagyu, H., . . . Goldberg, I. J. (2009). DGAT1 Expression Increases Heart Triglyceride Content but Ameliorates Lipotoxicity. *Journal of Biological Chemistry, 284*(52), 36312-36323. doi:10.1074/jbc.M109.049817

Makrecka-Kuka, M., Krumschnabel, G., & Gnaiger, E. (2015). High-Resolution Respirometry for Simultaneous Measurement of Oxygen and Hydrogen Peroxide Fluxes in Permeabilized Cells, Tissue Homogenate and Isolated Mitochondria. *Biomolecules, 5*(3), 1319-1338. doi:10.3390/biom5031319

Marciniak, C., Marechal, X., Montaigne, D., Neviere, R., & Lancel, S. (2014). Cardiac contractile function and mitochondrial respiration in diabetes-related mouse models. *Cardiovascular Diabetology, 13*(1), 118. doi:10.1186/s12933-014-0118-7

Mazumder, P. K., O'Neill, B. T., Roberts, M. W., Buchanan, J., Yun, U., Cooksey, R. C., . . . Abel, D. E. (2004). Impaired Cardiac Efficiency and Increased Fatty Acid Oxidation in Insulin-Resistant ob/ob Mouse Hearts. *Diabetes, 53*(9), 2366-2374. doi:10.2337/diabetes.53.9.2366

Miller, T. A., LeBrasseur, N. K., Cote, G. M., Trucillo, M. P., Pimentel, D. R., Ido, Y., . . . Sawyer, D. B. (2005). Oleate prevents palmitate-induced cytotoxic stress in cardiac myocytes. *Biochem Biophys Res Commun, 336*(1), 309-315. doi:10.1016/j.bbrc.2005.08.088

Morgan, E. E., Rennison, J. H., Young, M. E., McElfresh, T. A., Kung, T. A., Tserng, K.-Y., . . . Chandler, M. P. (2006). Effects of chronic activation of peroxisome proliferator-activated receptor-α or high-fat feeding in a rat infarct model of heart failure. *American Journal of Physiology-Heart and Circulatory Physiology, 290*(5). doi:10.1152/ajpheart.01014.2005

Nickel, A. G., von Hardenberg, A., Hohl, M., Loffler, J. R., Kohlhaas, M., Becker, J., . . . Maack, C. (2015). Reversal of Mitochondrial Transhydrogenase Causes Oxidative Stress in Heart Failure. *Cell Metab, 22*(3), 472-484. doi:10.1016/j.cmet.2015.07.008

Okere, I. C., Chandler, M. P., McElfresh, T. A., Rennison, J. H., Sharov, V., Sabbah, H. N., . . . Stanley, W. C. (2006). Differential effects of saturated and unsaturated fatty acid diets on cardiomyocyte apoptosis, adipose distribution, and serum leptin. *American journal of physiology. Heart and circulatory physiology, 291*(1), 44. doi:10.1152/ajpheart.01295.2005

Okere, I. C., Chess, D. J., McElfresh, T. A., Johnson, J., Rennison, J., Ernsberger, P., . . . Stanley, W. C. (2005). High-fat diet prevents cardiac hypertrophy and improves contractile function in the hypertensive dahl salt-sensitive rat. *Clin Exp Pharmacol Physiol, 32*(10), 825-831. doi:10.1111/j.1440-1681.2005.04272.x

Okere, I. C., Young, M. E., McElfresh, T. A., Chess, D. J., Sharov, V. G., Sabbah, H. N., . . . Stanley, W. C. (2006). Low carbohydrate/high-fat diet attenuates cardiac hypertrophy, remodeling, and altered gene expression in hypertension. *Hypertension (Dallas, Tex. : 1979), 48*(6), 1116-1123. doi:10.1161/01.HYP.0000248430.26229.0f

Ouwens, D. M., Boer, C., Fodor, M., de Galan, P., Heine, R. J., Maassen, J. A., & Diamant, M. (2005). Cardiac dysfunction induced by high-fat diet is associated with altered myocardial insulin signalling in rats. *Diabetologia, 48*(6), 1229-1237. doi:10.1007/s00125-005-1755-x

Ouwens, D. M., Diamant, M., Fodor, M., Habets, D. D. J., Pelsers, M., El Hasnaoui, M., . . . Luiken, J. (2007). Cardiac contractile dysfunction in insulin-resistant rats fed a high-fat diet is associated with elevated CD36-mediated fatty acid uptake and esterification. *Diabetologia, 50*(9), 1938-1948. doi:10.1007/s00125-007-0735-8

Park, S.-Y., Cho, Y.-R., Kim, H.-J., Higashimori, T., Danton, C., Lee, M.-K., . . . Kim, J. K. (2005). Unraveling the Temporal Pattern of Diet-Induced Insulin Resistance in Individual Organs and Cardiac Dysfunction in c57bl/6 Mice. *Diabetes, 54*(12), 3530-3540. doi:10.2337/diabetes.54.12.3530

Park, S. Y., Cho, Y. R., Kim, H. J., Higashimori, T., Danton, C., Lee, M. K., . . . Kim, J. K. (2005). Unraveling the temporal pattern of diet-induced insulin resistance in individual organs and cardiac dysfunction in C57BL/6 mice. *Diabetes, 54*(12), 3530-3540.

Rennison, J. H., McElfresh, T. A., Chen, X., Anand, V. R., Hoit, B. D., Hoppel, C. L., & Chandler, M. P. (2009). Prolonged exposure to high dietary lipids is not associated with lipotoxicity in heart failure. *Journal of Molecular and Cellular Cardiology, 46*(6), 883-890. doi:10.1016/j.yjmcc.2009.02.019

Schiattarella, G. G., Altamirano, F., Tong, D., French, K. M., Villalobos, E., Kim, S. Y., . . . Hill, J. A. (2019). Nitrosative stress drives heart failure with preserved ejection fraction. *Nature*. doi:10.1038/s41586-019-1100-z

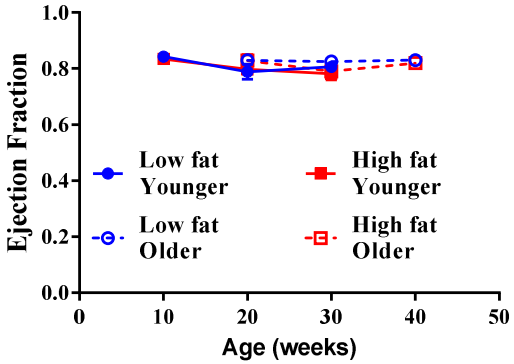
Semeniuk, L. M., Kryski, A. J., & Severson, D. L. (2002). Echocardiographic assessment of cardiac function in diabetic db/db and transgenic db/db-hGLUT4 mice. *Am J Physiol Heart Circ Physiol, 283*(3), H976-982. doi:10.1152/ajpheart.00088.2002

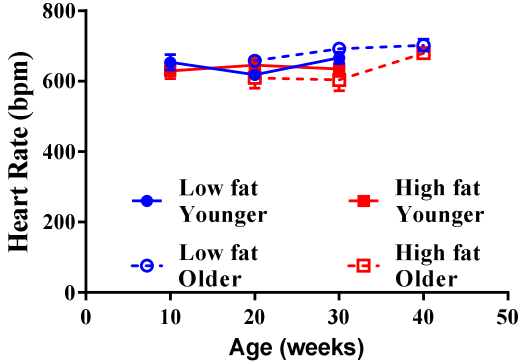
Sverdlov, A. L., Elezaby, A., Behring, J. B., Bachschmid, M. M., Luptak, I., Tu, V. H., . . . Colucci, W. S. (2015). High fat, high sucrose diet causes cardiac mitochondrial dysfunction due in part to oxidative post-translational modification of mitochondrial complex II. *Journal of Molecular and Cellular Cardiology, 78*, 165-173. doi:10.1016/j.yjmcc.2014.07.018

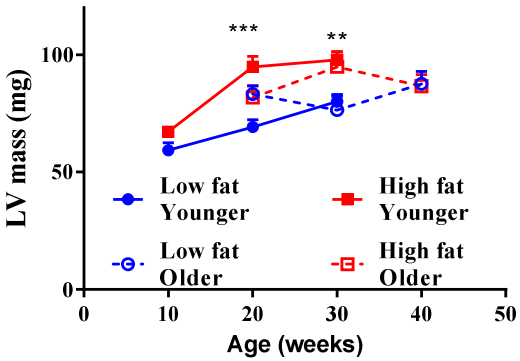
Sverdlov, A. L., Elezaby, A., Qin, F., Behring, J. B., Luptak, I., Calamaras, T. D., . . . Colucci, W. S. (2016). Mitochondrial Reactive Oxygen Species Mediate Cardiac Structural, Functional, and Mitochondrial Consequences of Diet-Induced Metabolic Heart Disease. *Journal of the American Heart Association, 5*(1). doi:10.1161/JAHA.115.002555

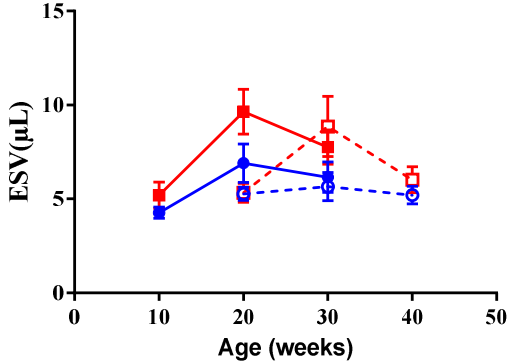
Treatment of Hypertension in Adults With Diabetes. (2003). *26*(suppl 1), s80-s82. doi:10.2337/diacare.26.2007.S80 %J Diabetes Care

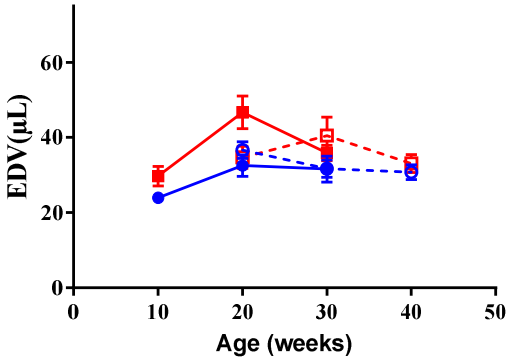
Wende, A. R., & Abel, E. D. (2010). Lipotoxicity in the heart. *Biochim Biophys Acta, 1801*(3), 311-319. doi:10.1016/j.bbalip.2009.09.023


Ejection Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Age (weeks)
Low fat Younger
High fat Younger
Low fat Older
High fat Older


Heart Rate (bpm)
800
600
400
200
0
0
10
20
30
40
50
Age (weeks)
Low fat Younger
High fat Younger
Low fat Older
High fat Older


LV mass (mg)
***
**
100
50
0
0
10
20
30
40
50
Age (weeks)
Low fat Younger
High fat Younger
Low fat Older
High fat Older


ESV(µL)
15
10
5
0
0
10
20
30
40
50
Age (weeks)


EDV(µL)
60
40
20
0
0
10
20
30
40
50
Age (weeks)

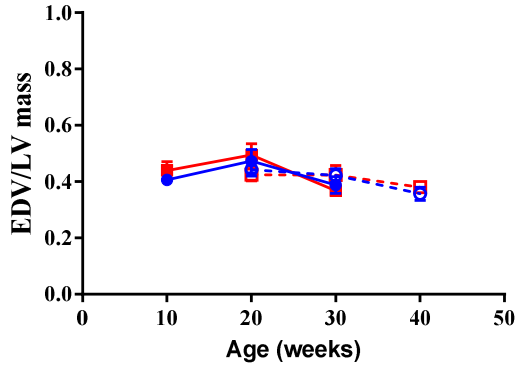
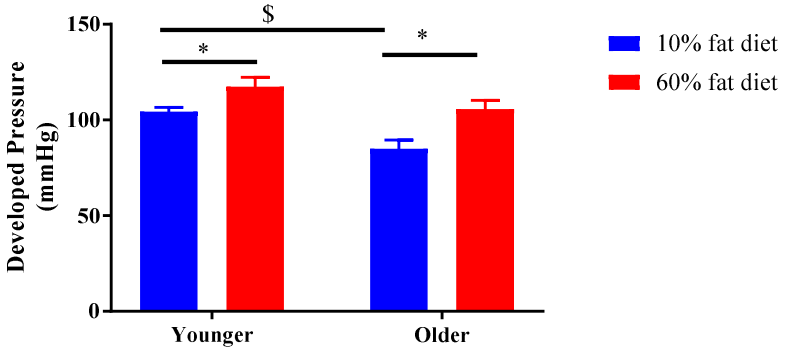
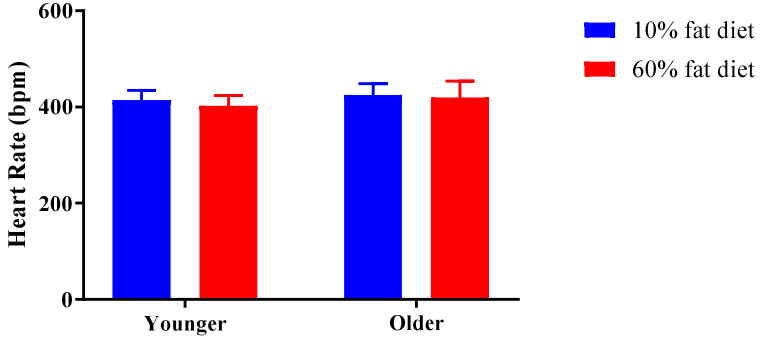
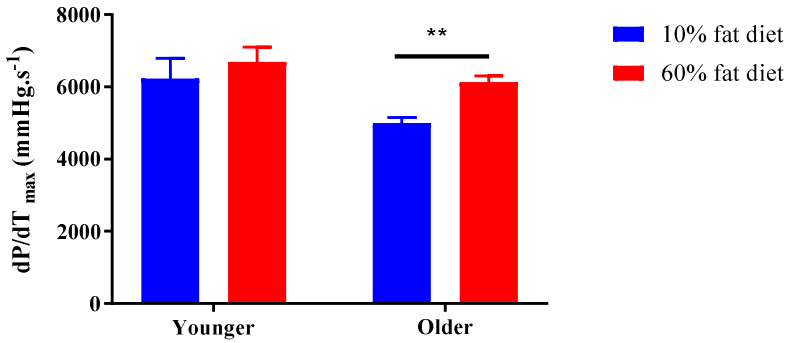

EDV/LV mass
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Age (weeks)

**Figure 18. Cardiac function measured by echocardiography in mice that were fed a lard-based high fat diet.**

Mice were 10 weeks of age (younger) or 20 weeks of age (older) at the time of dietary intervention. Data are presented as Mean±SEM and were analyzed by repeated measures two-way ANOVA. Statistical significance was set at $p<0.05$ and no significant interaction was observed between age and diet (N>9/group). Within age group data were analyzed by student's t-test (**-$p<0.01$, ***-$p<0.001$ for comparisons within younger mice).


Developed Pressure (mmHg)
150
100
50
0
$
*
*
Younger
Older
10% fat diet
60% fat diet


Heart Rate (bpm)
600
400
200
0
Younger
Older
10% fat diet
60% fat diet


dP/dT max (mmHg.s-1)
8000
6000
4000
2000
0
**
Younger
Older
10% fat diet
60% fat diet

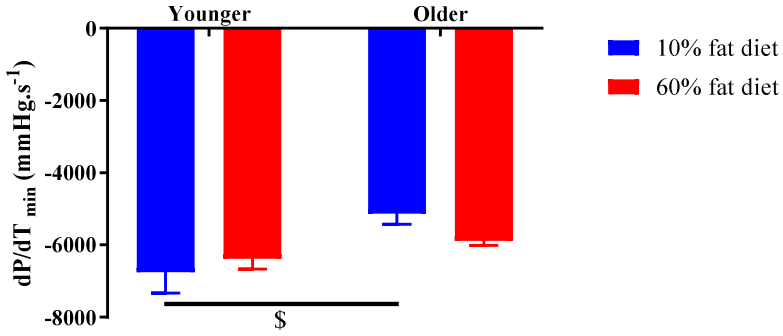
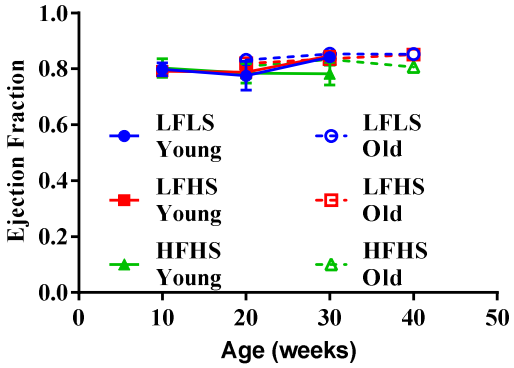
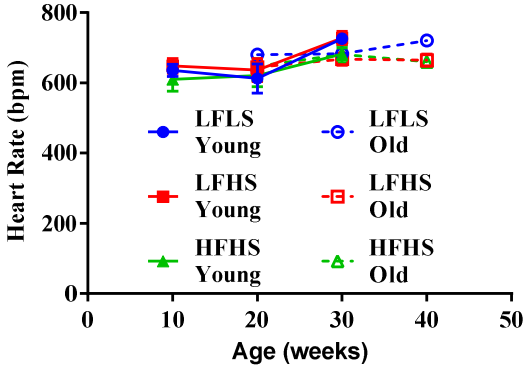
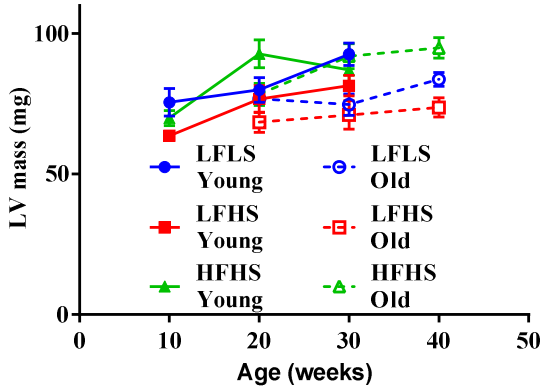

dP/dT min (mmHg.s-1)
0
-2000
-4000
-6000
-8000
Younger
Older
$
10% fat diet
60% fat diet

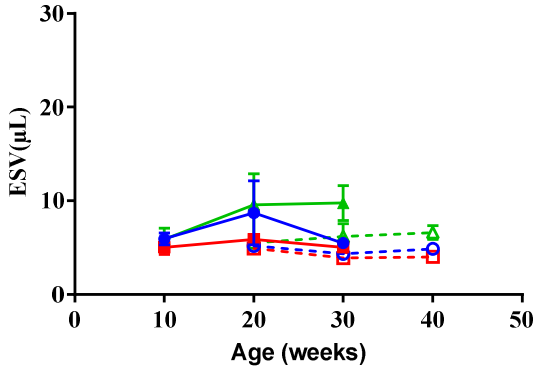
**Figure 19. Cardiac function as assessed by invasive hemodynamics in mice fed lard-based high fat diet.**

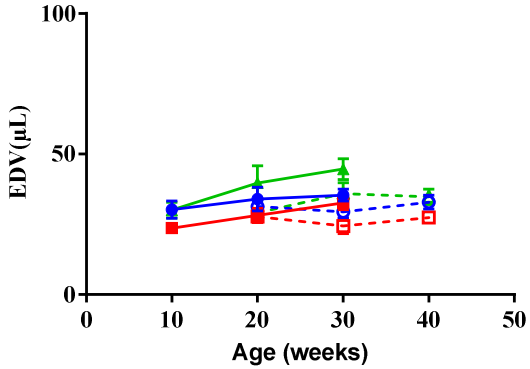
Data were analyzed by two-way ANOVA followed by post-hoc analysis using Tukey's test. Statistical significance was set at $p<0.05$ and data are presented as Mean±SEM($-$p<0.05, N=4 per group). Within group differences were assessed by student's t-test (*-p<0.05, **-p<0.01). Younger and Older refer to mice that were 10 weeks of age or 20 weeks of age at the time of beginning the dietary intervention.


Ejection Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Age (weeks)
LFLS Young
LFLS Old
LFHS Young
LFHS Old
HFHS Young
HFHS Old


Heart Rate (bpm)
800
600
400
200
0
0
10
20
30
40
50
Age (weeks)
LFLS Young
LFLS Old
LFHS Young
LFHS Old
HFHS Young
HFHS Old


LV mass (mg)
100
50
0
0
10
20
30
40
50
Age (weeks)
LFLS Young
LFLS Old
LFHS Young
LFHS Old
HFHS Young
HFHS Old


ESV(µL)
30
20
10
0
0
10
20
30
40
50
Age (weeks)


EDV(µL)
100
50
0
0
10
20
30
40
50
Age (weeks)

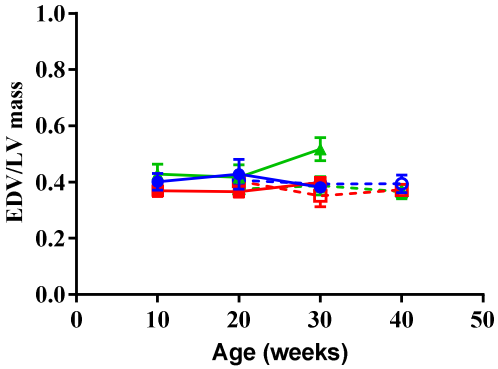
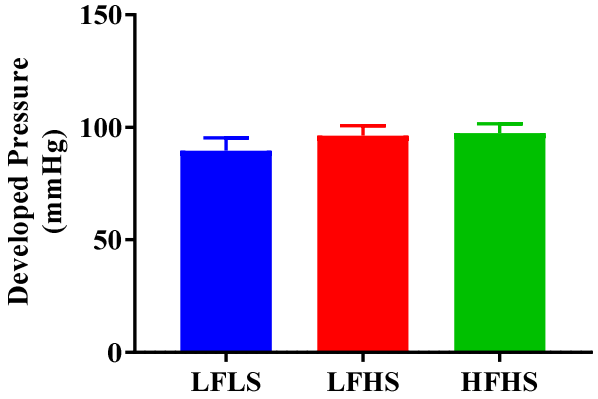
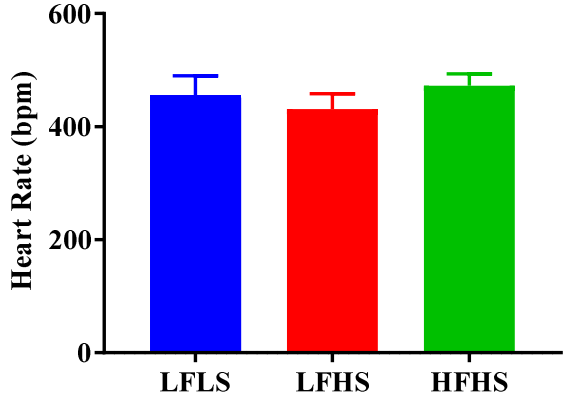
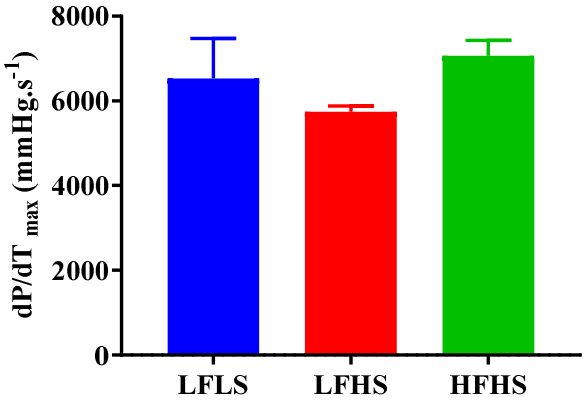

EDV/LV mass
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Age (weeks)

**Figure 20. Cardiac function measured by echocardiography in mice that were fed a saturated fat rich diet**.

Mice were 10 weeks of age (younger) or 20 weeks of age (older) at the time of dietary intervention. Data are presented as Mean±SEM and were analyzed by repeated measures two-way ANOVA. Statistical significance was set at $p<0.05$ and no significant interaction was observed between age and diet (N>6/group).


Developed Pressure (mmHg)
150
100
50
0
LFLS
LFHS
HFHS


Heart Rate (bpm)
600
400
200
0
LFLS
LFHS
HFHS


dP/dT max (mmHg.s-1)
8000
6000
4000
2000
0
LFLS
LFHS
HFHS

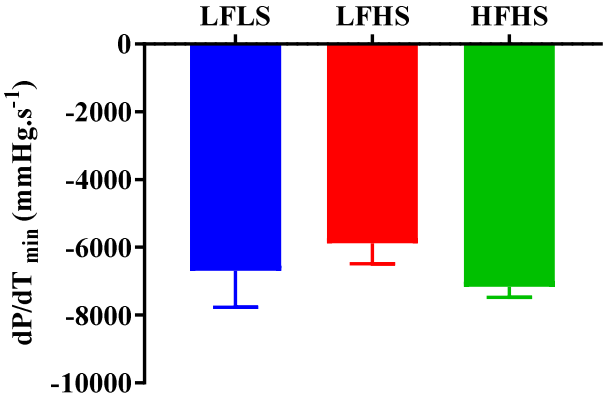
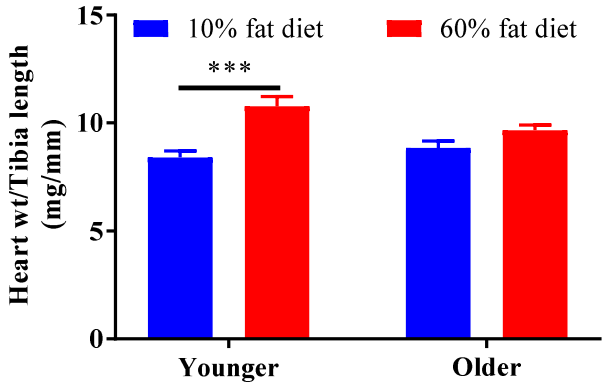

dP/dT min (mmHg.s-1)
LFLS
LFHS
HFHS
0
-2000
-4000
-6000
-8000
-10000

**Figure 21. Cardiac function as assessed by LV catheterization in younger mice fed saturated fat rich diet.**

Data are presented as Mean+SEM and were analyzed by one-way ANOVA and are represented as Mean+SEM. Statistical significance was set at $p<0.05$ (N=4/group).

A)




B)

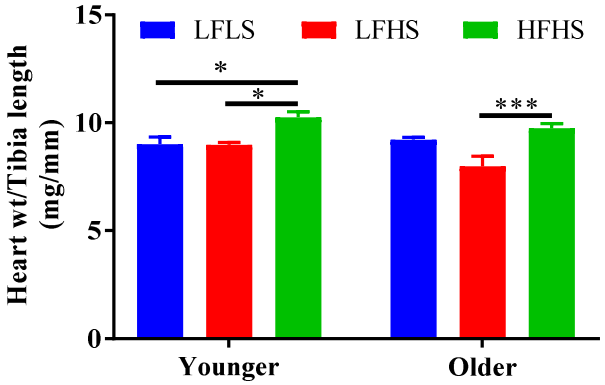



**Figure 22. Cardiac hypertrophy induced by HFD feeding in younger and older mice.**

A) lard based diet for 20 weeks or B) saturated fat rich diet for 20 weeks. Data are presented as Mean+SEM and were analyzed by two-way ANOVA followed by post-hoc analysis using Tukey's test. Statistical significance was set at $p<0.05$ (*- $p<0.05$, ***- $p<0.001$, $N>6$/group). Younger and older refer to mice that that were 10 weeks of age or 20 weeks of age respectively at the time of dietary intervention.

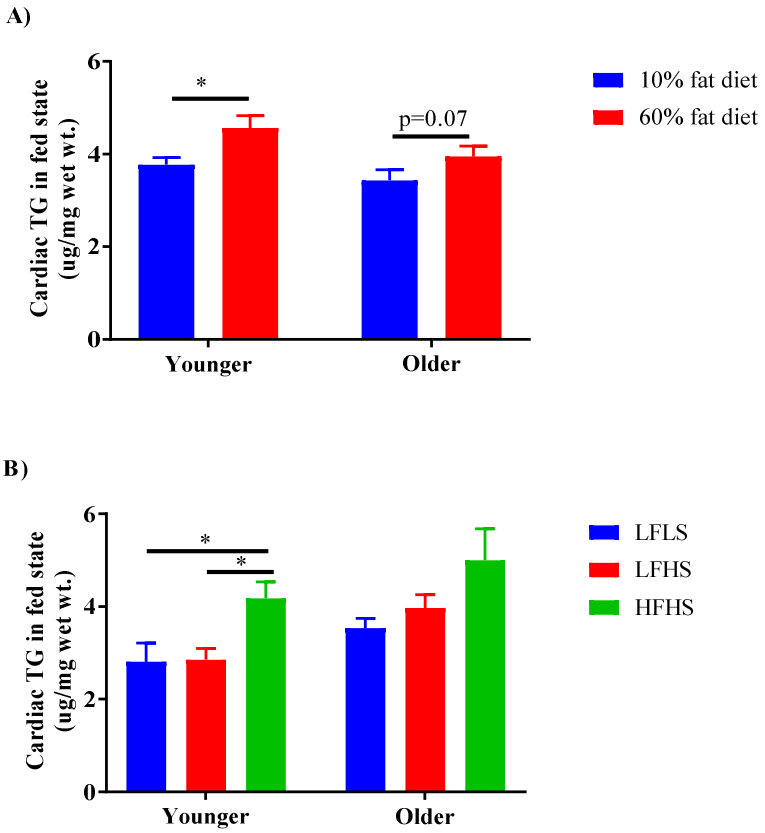
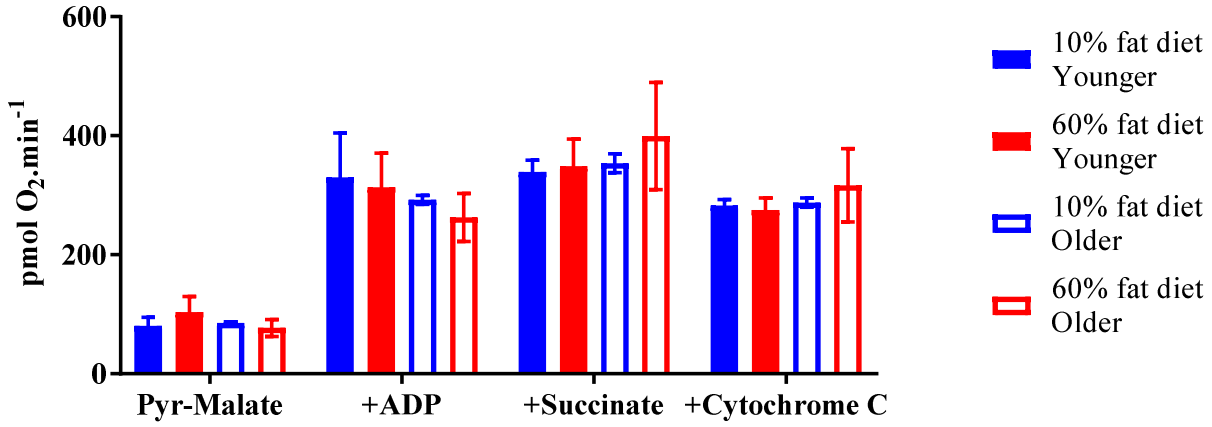



**Figure 23. Cardiac triglyceride content in mice fed a (A) lard-based high fat diet or (B) saturated fat rich diet for 20 weeks.**

Data are presented as Mean+SEM and were analyzed by one-tail student's t-test for determining statistical differences within age group in (A) and statistical significance is reported at $p<0.05$. ($N>9$/group, *-$p<0.05$). No interaction between age and diet was found upon analysis using two-way ANOVA. In (B) data are presented as Mean+SEM and were analyzed by one-way ANOVA to determine statistical differences followed by Tukey's post-hoc analysis and statistical significance was set at $p<0.05$ ($N>6$/group, *-$p<0.05$). Two-way ANOVA revealed no significant interaction between diet and age for data presented in (B).


600
400
200
0
pmol O2.min-1
Pyr-Malate
+ADP
+Succinate
+Cytochrome C
10% fat diet Younger
60% fat diet Younger
10% fat diet Older
60% fat diet Older

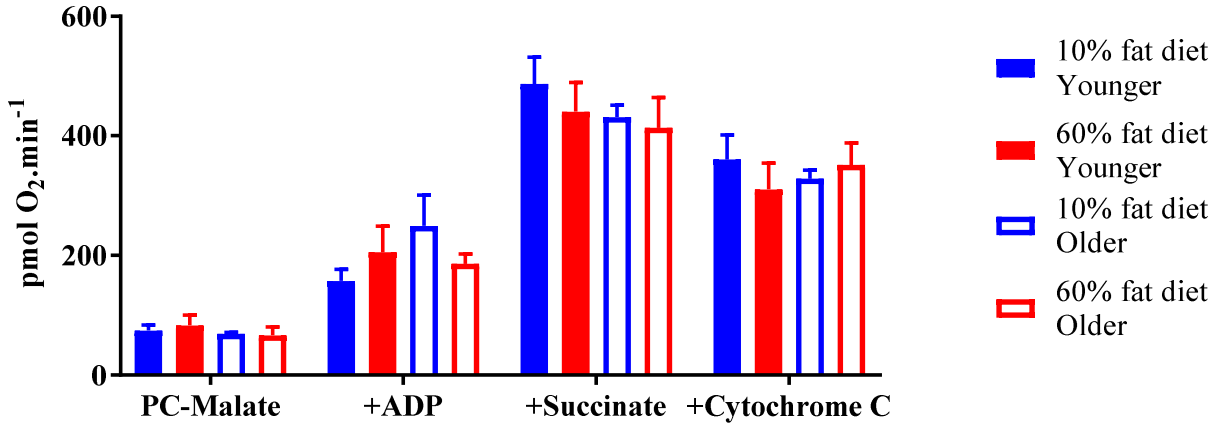
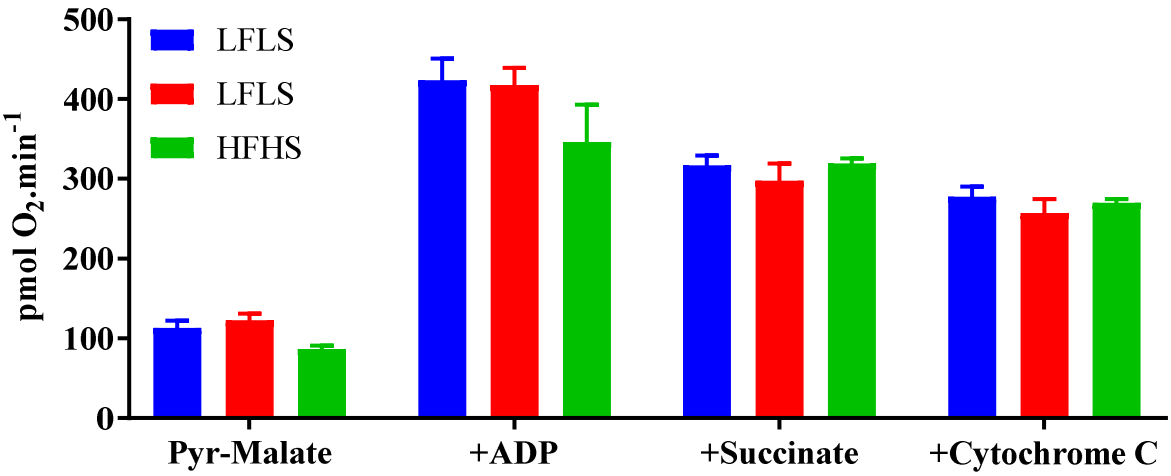

600
400
200
0
pmol O2.min-1
PC-Malate
+ADP
+Succinate
+Cytochrome C
10% fat diet Younger
60% fat diet Younger
10% fat diet Older
60% fat diet Older

**Figure 24. Oxygen consumption in isolated mitochondria from the hearts of mice fed lard-based high fat diet using different substrates.**

Pyruvate-Malate (top) or Palmitoylcarnitine (PC)- malate (bottom) were used as substrates. Data are presented as Mean+SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's test to determine interaction between diet and age. Multiple t-tests were performed for determining effect of diet on mitochondrial function within the same age groups. Statistical significance was set at $p<0.05$ ($N>3$/group). Younger and older refer to mice that that were 10 weeks of age or 20 weeks of age respectively at the time of dietary intervention. No statistical significance was found.


500
400
300
200
100
0
pmol O2.min-1
LFLS
LFLS
HFHS
Pyr-Malate
+ADP
+Succinate
+Cytochrome C

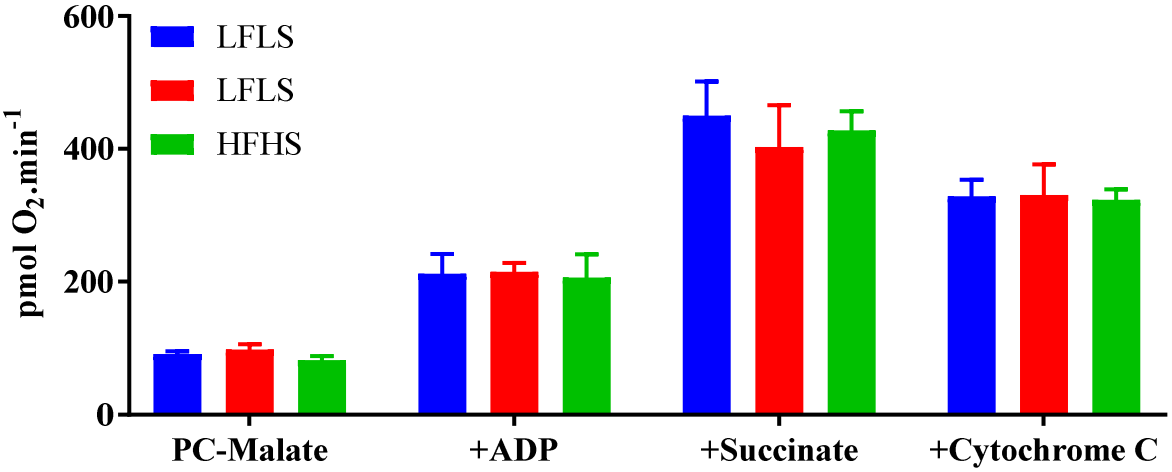

600
400
200
0
pmol O2.min-1
LFLS
LFLS
HFHS
PC-Malate
+ADP
+Succinate
+Cytochrome C

**Figure 25. Oxygen consumption in isolated mitochondria from the hearts of mice fed saturated fat rich diet using different substrates.**

Pyruvate-Malate (top) or Palmitoylcarnitine (PC)- malate (bottom) were used as substrates. Data are presented as Mean+SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's test to determine interaction between diet and age. Statistical significance was set at $p<0.05$ (N=4/group). Only younger mice that that were 10 weeks of age at the time of dietary intervention were used for the experiment. No statistical significance was found.

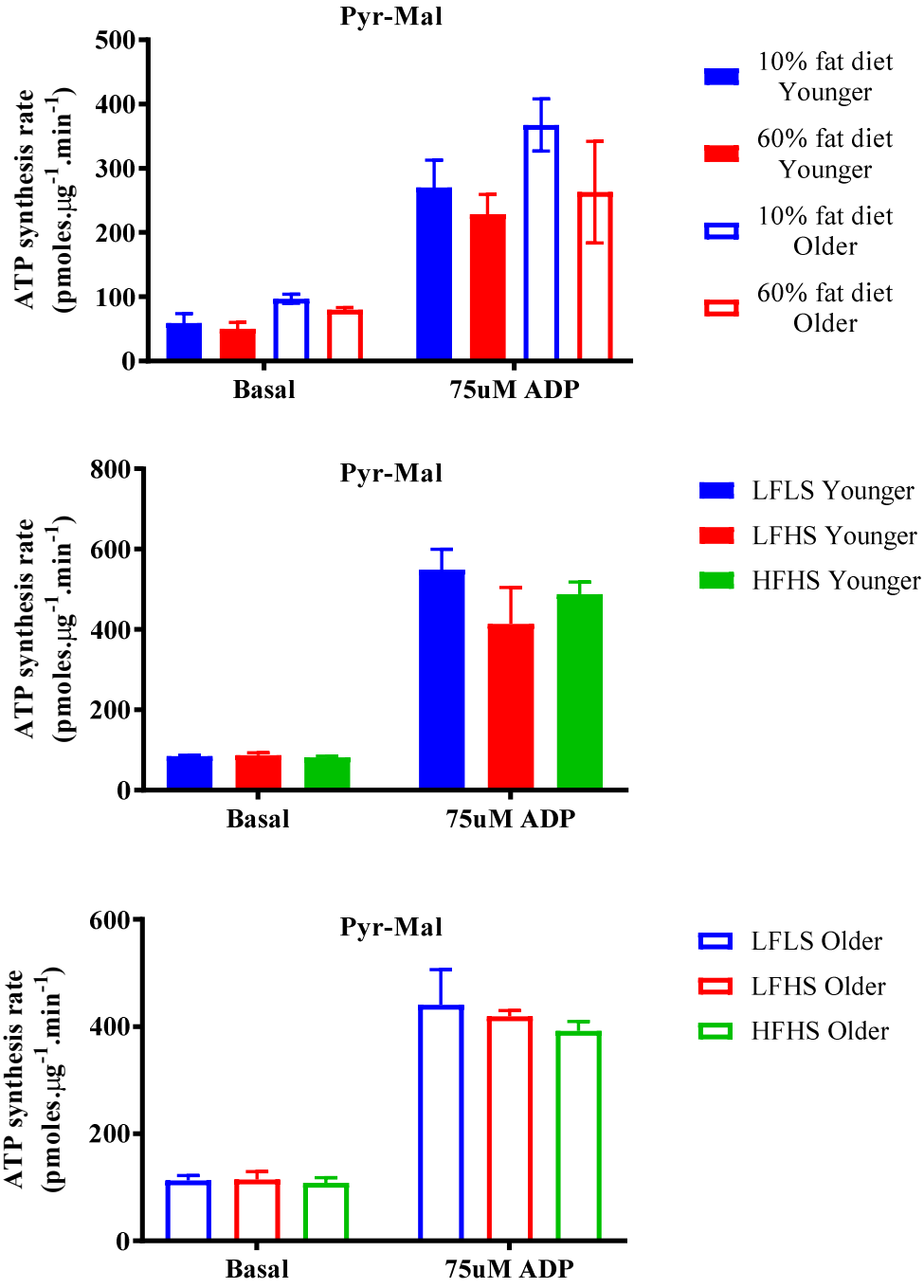
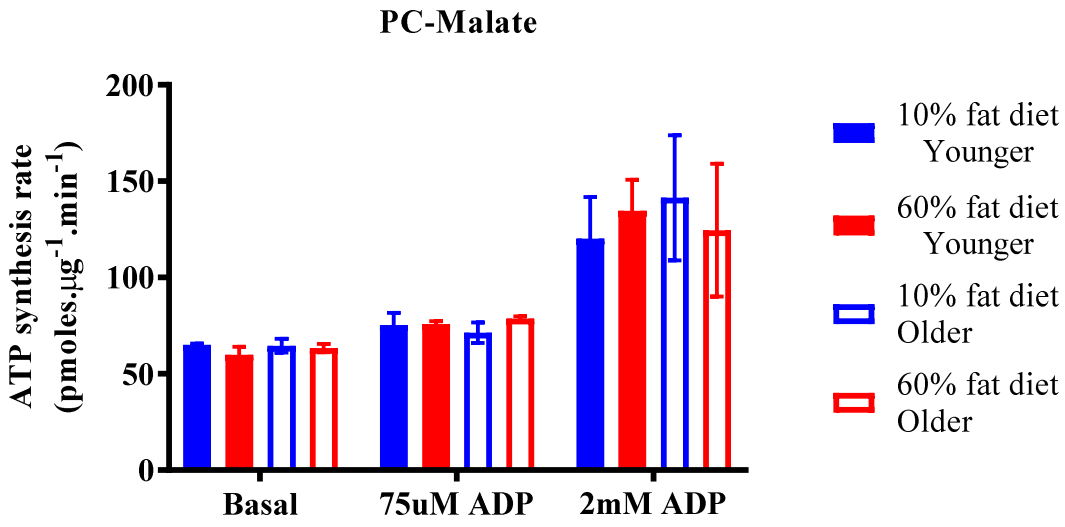

Pyr-Mal
ATP synthesis rate (pmoles.µg-1.min-1)
500
400
300
200
100
0
Basal
75uM ADP
10% fat diet Younger
60% fat diet Younger
10% fat diet Older
60% fat diet Older
Pyr-Mal
ATP synthesis rate (pmoles.µg-1.min-1)
800
600
400
200
0
Basal
75uM ADP
LFLS Younger
LFHS Younger
HFHS Younger
Pyr-Mal
ATP synthesis rate (pmoles.µg-1.min-1)
600
400
200
0
Basal
75uM ADP
LFLS Older
LFHS Older
HFHS Older

**Figure 26. Pyruvate-Malate driven ATP synthesis rates measured in isolated mitochondria from hearts of mice fed different fat enriched diets.**

Lard based high fat diet (top) or saturated fat rich diet (bottom). Data are presented as Mean+SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's test. Additionally, for analysis on samples from lard based high fat study, multiple t-tests were performed to determine effect of diet on ATP synthesis rates within each age group. Statistical significance was set at $p<0.05$ ($N>4$/group). Younger and older groups refer to mice that that were 10 weeks of age or 2 weeks of age respectively at the time of dietary intervention were used for the experiment. No statistical significance was found.


PC-Malate
ATP synthesis rate (pmoles.µg-1.min-1)
200
150
100
50
0
Basal
75uM ADP
2mM ADP
10% fat diet Younger
60% fat diet Younger
10% fat diet Older
60% fat diet Older

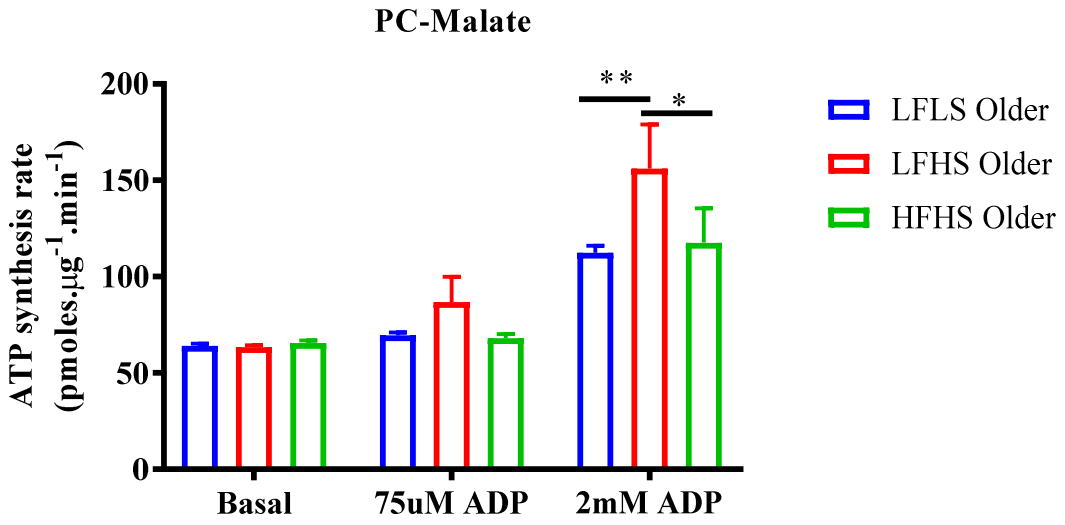
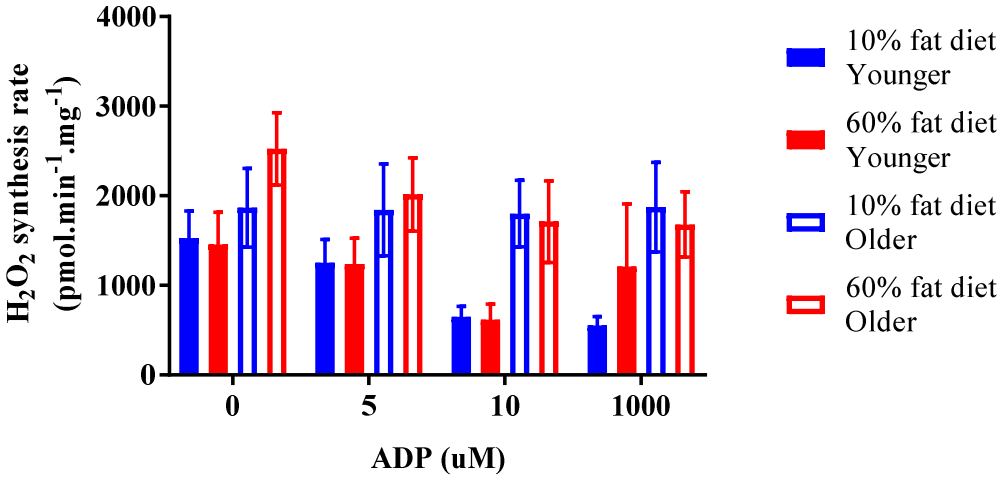

PC-Malate
ATP synthesis rate (pmoles.µg-1.min-1)
200
150
100
50
0
**
*
Basal
75uM ADP
2mM ADP
LFLS Older
LFHS Older
HFHS Older

**Figure 27. Palmitoylcarnitine-Malate driven ATP synthesis rates measured in isolated mitochondria from hearts of mice fed different fat enriched diets.**

Lard based high fat diet (top) or saturated fat rich diet (bottom). Data are presented as Mean+SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's test. Additionally, for analysis on samples from lard based high fat study, multiple t-tests were performed to determine effect of diet on ATP synthesis rates within each age group. Statistical significance was set at $p<0.05$ ($N>3$/group). Only younger mice that that were 10 weeks of age at the time of dietary intervention were used for the experiment. No statistical significance was found.


4000
3000
2000
1000
0
$H_2O_2$ synthesis rate
(pmol.min$^{-1}$.mg$^{-1}$)
0
5
10
1000
ADP (uM)
10% fat diet Younger
60% fat diet Younger
10% fat diet Older
60% fat diet Older

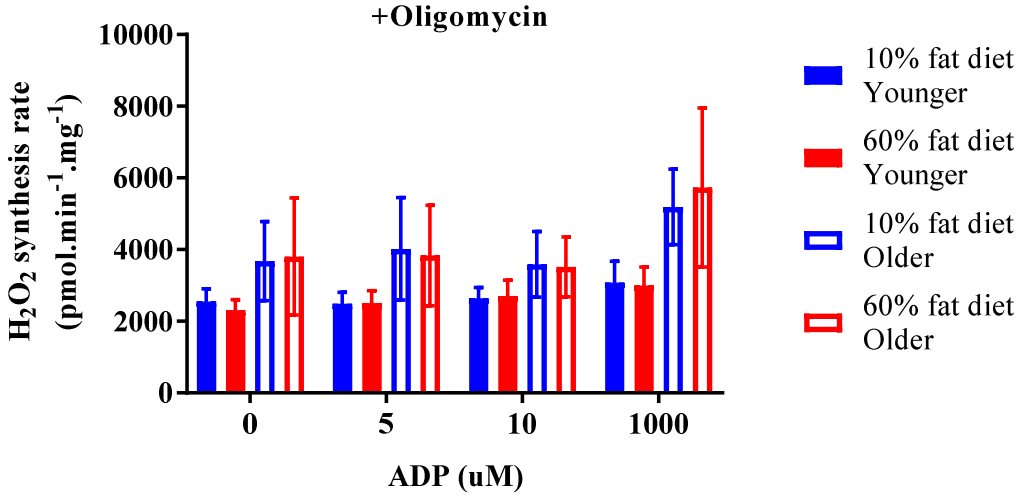
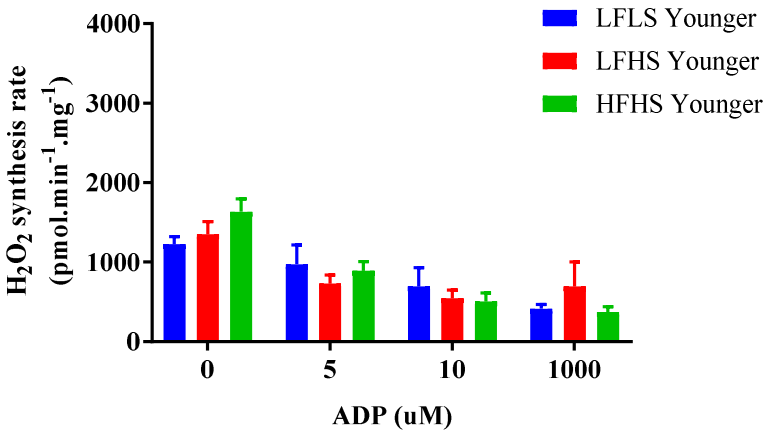
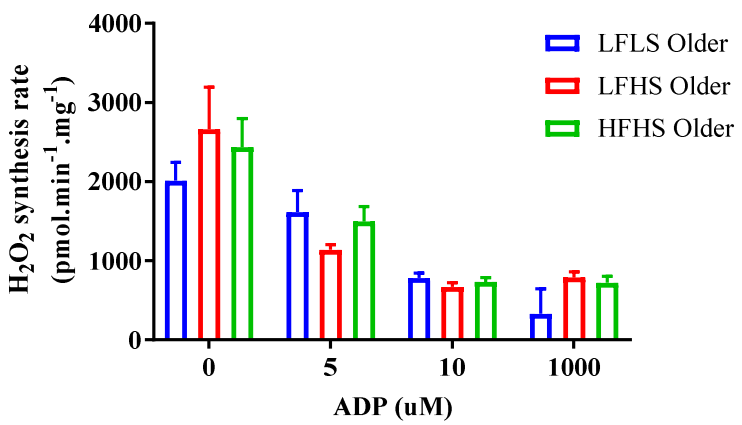
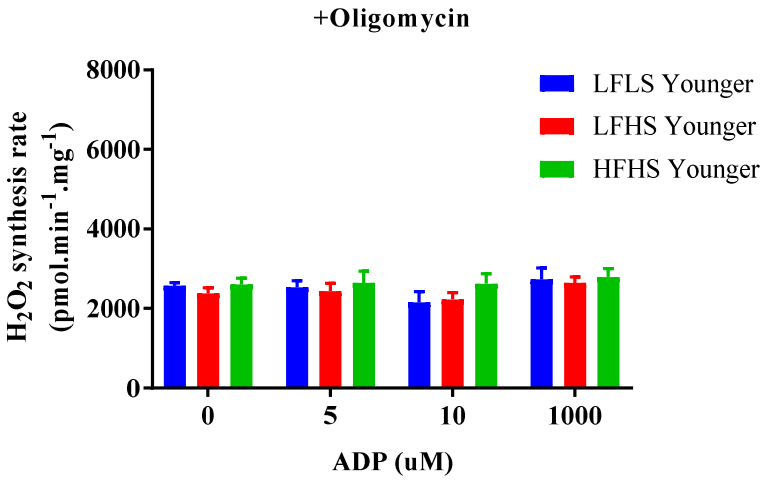

+Oligomycin
10000
8000
6000
4000
2000
0
$H_2O_2$ synthesis rate
(pmol.min$^{-1}$.mg$^{-1}$)
0
5
10
1000
ADP (uM)
10% fat diet Younger
60% fat diet Younger
10% fat diet Older
60% fat diet Older

**Figure 28. ROS synthesis rates in isolated mitochondria from hearts of mice fed a lard-based high fat diet**.

Succinate/glutamate/malate were used as substrate in the absence (top) or presence (bottom) of oligomycin. Data are presented as Mean+SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's test. Multiple t-tests were performed for determining effect of diet on mitochondrial function within the same age groups. Statistical significance was set at $p<0.05$ ($N>3$/group). Younger and older refer to mice that that were 10 weeks of age or 20 weeks of age respectively at the time of dietary intervention. No statistical significance was found.


LFLS Younger
LFHS Younger
HFHS Younger
$H_2O_2$ synthesis rate (pmol.min$^{-1}$.mg$^{-1}$)
4000
3000
2000
1000
0
0
5
10
1000
ADP (uM)


LFLS Older
LFHS Older
HFHS Older
$H_2O_2$ synthesis rate (pmol.min$^{-1}$.mg$^{-1}$)
4000
3000
2000
1000
0
0
5
10
1000
ADP (uM)


+Oligomycin
LFLS Younger
LFHS Younger
HFHS Younger
$H_2O_2$ synthesis rate (pmol.min$^{-1}$.mg$^{-1}$)
8000
6000
4000
2000
0
0
5
10
1000
ADP (uM)

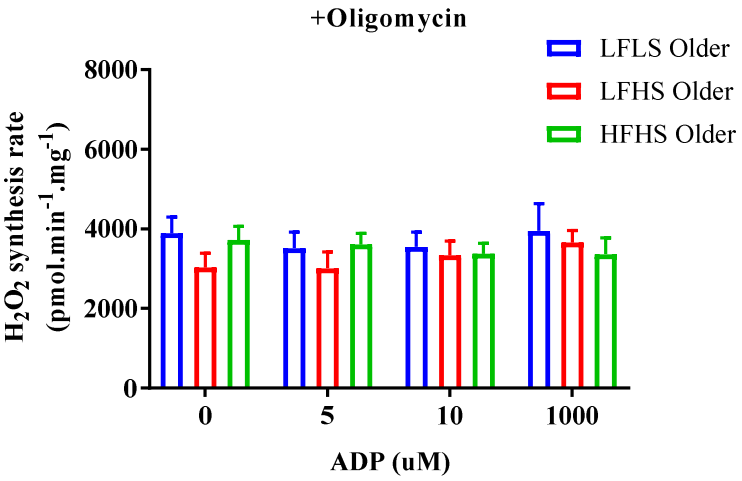

+Oligomycin
LFLS Older
LFHS Older
HFHS Older
$H_2O_2$ synthesis rate (pmol.min$^{-1}$.mg$^{-1}$)
8000
6000
4000
2000
0
0
5
10
1000
ADP (uM)

**Figure 29. ROS synthesis rates in isolated mitochondria from hearts of mice fed a saturated fat rich diet**.

Succinate/glutamate/malate were used as substrate in the absence (top) or presence (bottom) of oligomycin. Data are presented as Mean+SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's test. Statistical significance was set at $p<0.05$ ($N>3$/group).

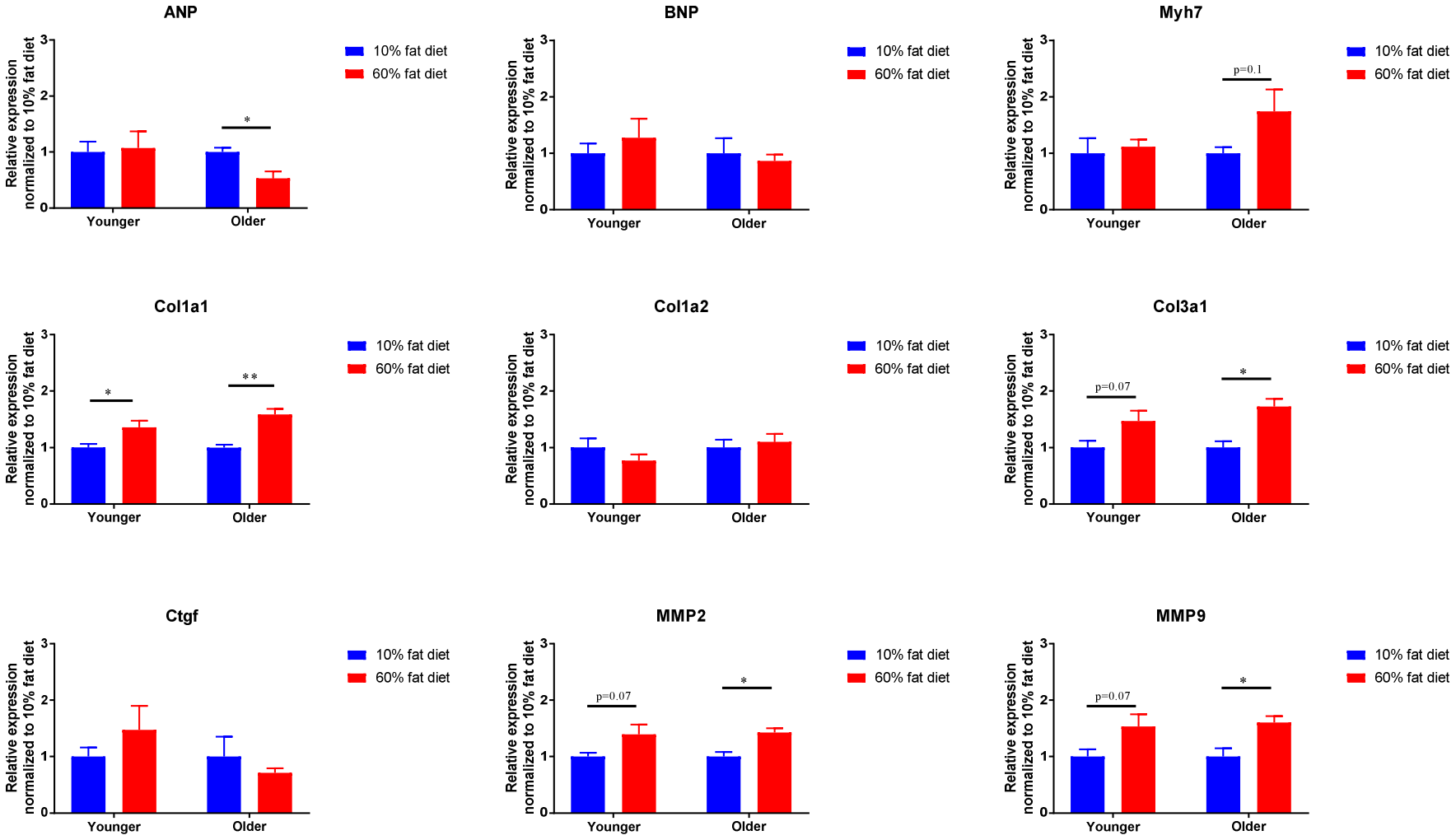

ANP
Relative expression normalized to 10% fat diet
10% fat diet
60% fat diet
*
Younger
Older
BNP
Relative expression normalized to 10% fat diet
10% fat diet
60% fat diet
Younger
Older
Myh7
Relative expression normalized to 10% fat diet
10% fat diet
60% fat diet
p=0.1
Younger
Older
Col1a1
Relative expression normalized to 10% fat diet
10% fat diet
60% fat diet
*
**
Younger
Older
Col1a2
Relative expression normalized to 10% fat diet
10% fat diet
60% fat diet
Younger
Older
Col3a1
Relative expression normalized to 10% fat diet
10% fat diet
60% fat diet
p=0.07
*
Younger
Older
Ctgf
Relative expression normalized to 10% fat diet
10% fat diet
60% fat diet
Younger
Older
MMP2
Relative expression normalized to 10% fat diet
10% fat diet
60% fat diet
p=0.07
*
Younger
Older
MMP9
Relative expression normalized to 10% fat diet
10% fat diet
60% fat diet
p=0.07
*
Younger
Older

**Figure 30**.**Gene expression of cardiac hypertrophy related genes (ANP,BNP, Myh7) and ECM remodeling related genes (Col1a1, Col1a2, Col3a1, Ctgf, MMP2, MMP9) in the hearts of mice fed a lard-based high fat diet for 20 weeks.**

Data are presented as Mean±SEM and were analyzed by student's t-test. Statistical significance was set at $p<0.05$ (N=5/group). Younger and older refer to mice that that were 10 weeks of age or 20 weeks of age respectively at the time of dietary intervention were used for the experiment.

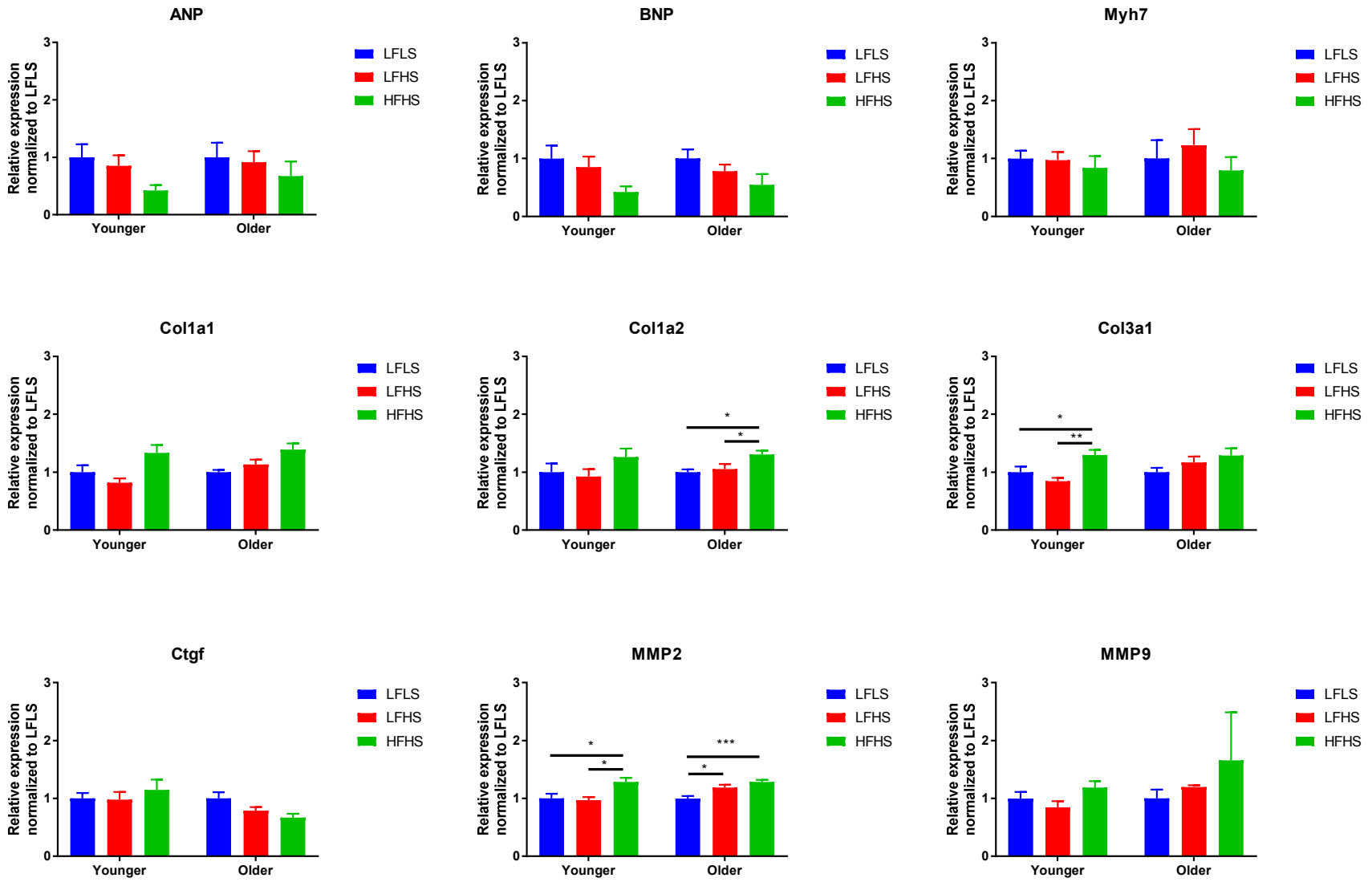
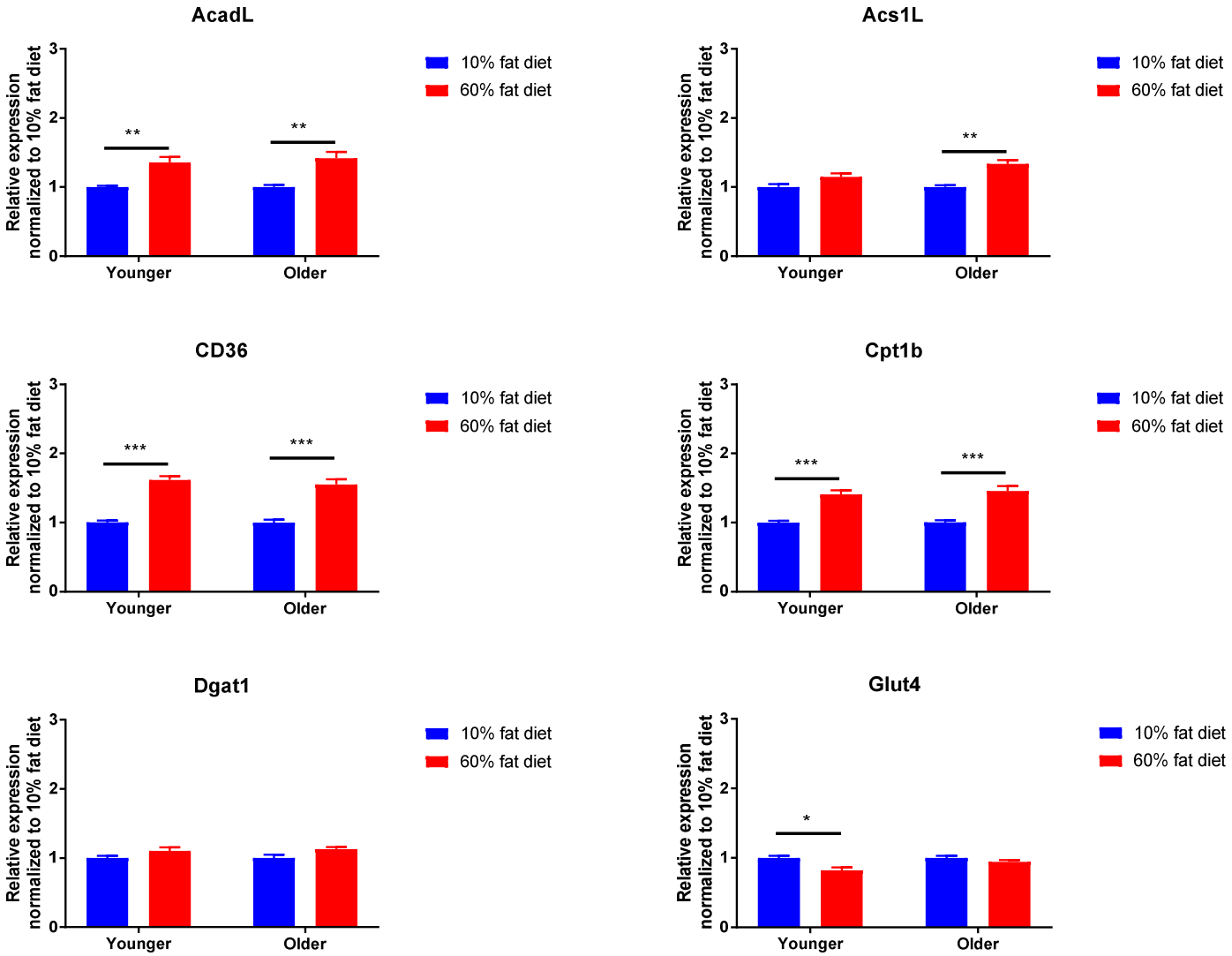

ANP
BNP
Myh7
Col1a1
Col1a2
Col3a1
Ctgf
MMP2
MMP9
Relative expression normalized to LFLS
0
1
2
3
Younger
Older
LFLS
LFHS
HFHS
*
**
***

**Figure 31. Gene expression of cardiac hypertrophy related genes (ANP,BNP, Myh7) and ECM remodeling related genes (Col1a1, Col1a2, Col3a1, Ctgf, MMP2, MMP9) in the hearts of mice fed a saturated fat rich diet for 20 weeks.**

Data are presented as Mean±SEM and were analyzed by one-way ANOVA followed by post-hoc analysis using Tukey's test to determine within group differences. Statistical significance was set at $p<0.05$ ($N>6$/group). Younger and older refer to mice that that were 10 weeks of age or 20 weeks of age respectively at the time of dietary intervention were used for the experiment.


AcadL
Relative expression normalized to 10% fat diet
0
1
2
3
**
**
Younger
Older
10% fat diet
60% fat diet
Acs1L
Relative expression normalized to 10% fat diet
0
1
2
3
**
Younger
Older
10% fat diet
60% fat diet
CD36
Relative expression normalized to 10% fat diet
0
1
2
3
***
***
Younger
Older
10% fat diet
60% fat diet
Cpt1b
Relative expression normalized to 10% fat diet
0
1
2
3
***
***
Younger
Older
10% fat diet
60% fat diet
Dgat1
Relative expression normalized to 10% fat diet
0
1
2
3
Younger
Older
10% fat diet
60% fat diet
Glut4
Relative expression normalized to 10% fat diet
0
1
2
3
*
Younger
Older
10% fat diet
60% fat diet

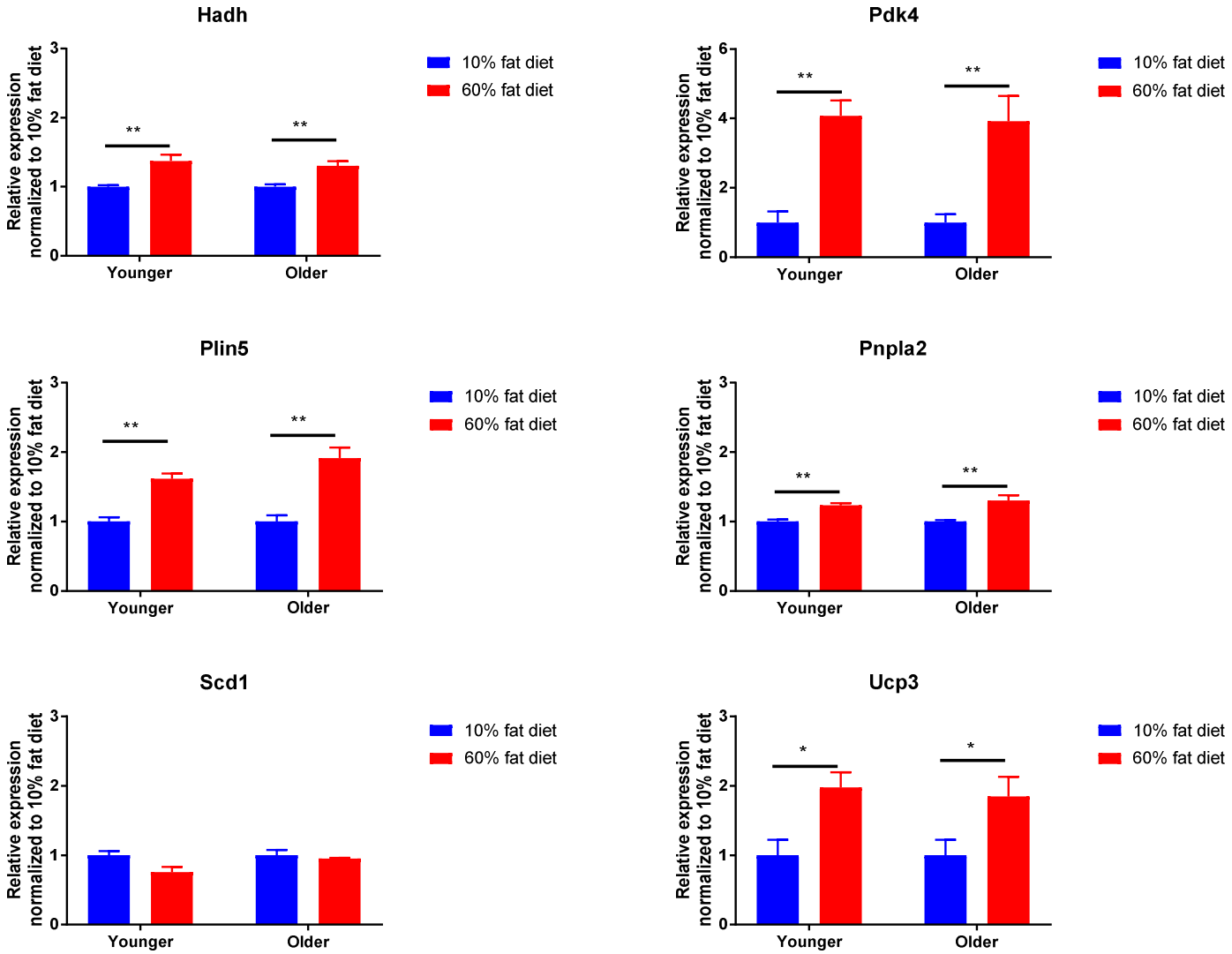
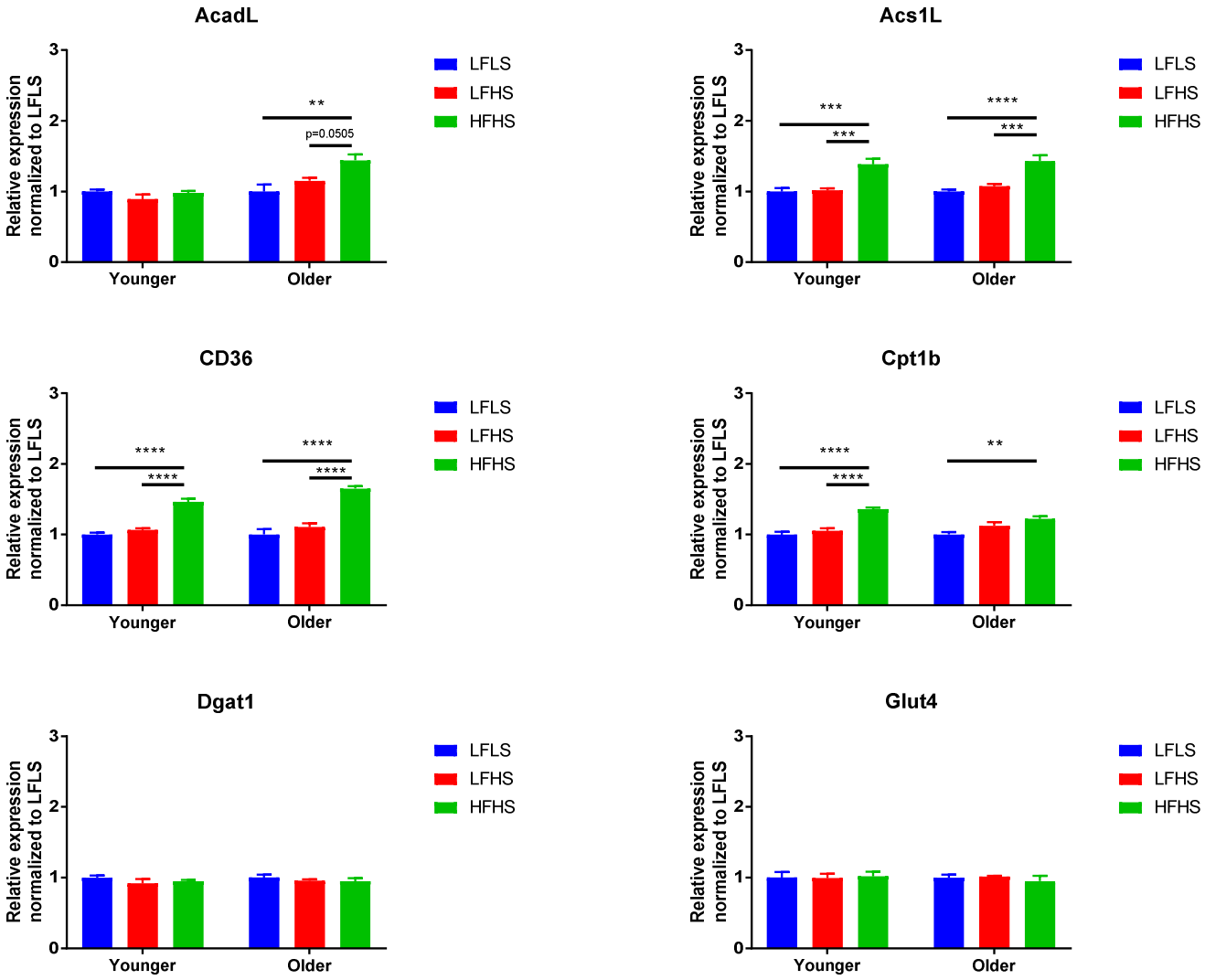

Hadh
Relative expression normalized to 10% fat diet
0
1
2
3
**
**
Younger
Older
10% fat diet
60% fat diet
Pdk4
Relative expression normalized to 10% fat diet
0
2
4
6
**
**
Younger
Older
10% fat diet
60% fat diet
Plin5
Relative expression normalized to 10% fat diet
0
1
2
3
**
**
Younger
Older
10% fat diet
60% fat diet
Pnpla2
Relative expression normalized to 10% fat diet
0
1
2
3
**
**
Younger
Older
10% fat diet
60% fat diet
Scd1
Relative expression normalized to 10% fat diet
0
1
2
3
Younger
Older
10% fat diet
60% fat diet
Ucp3
Relative expression normalized to 10% fat diet
0
1
2
3
*
*
Younger
Older
10% fat diet
60% fat diet

**Figure 32. Gene expression of metabolism related genes in the hearts of mice fed a lard-based high fat diet for 20 weeks.** Data are presented as Mean±SEM and were analyzed by unpaired student's t-test to determine fat feeding induced differences within each age group. Statistical significance was set at $p<0.05$ (N=5/group, *-$p<0.05$, **- $p<0.01$, ***-$p<0.001$, ****-$p<0.0001$). Younger and older refer to mice that that were 10 weeks of age or 20 weeks of age respectively at the time of dietary intervention were used for the experiment.


AcadL
Relative expression normalized to LFLS
3
2
1
0
**
p=0.0505
Younger
Older
LFLS
LFHS
HFHS
Acs1L
***
***
****
***
CD36
****
****
****
****
Cpt1b
****
****
**
Dgat1
Glut4

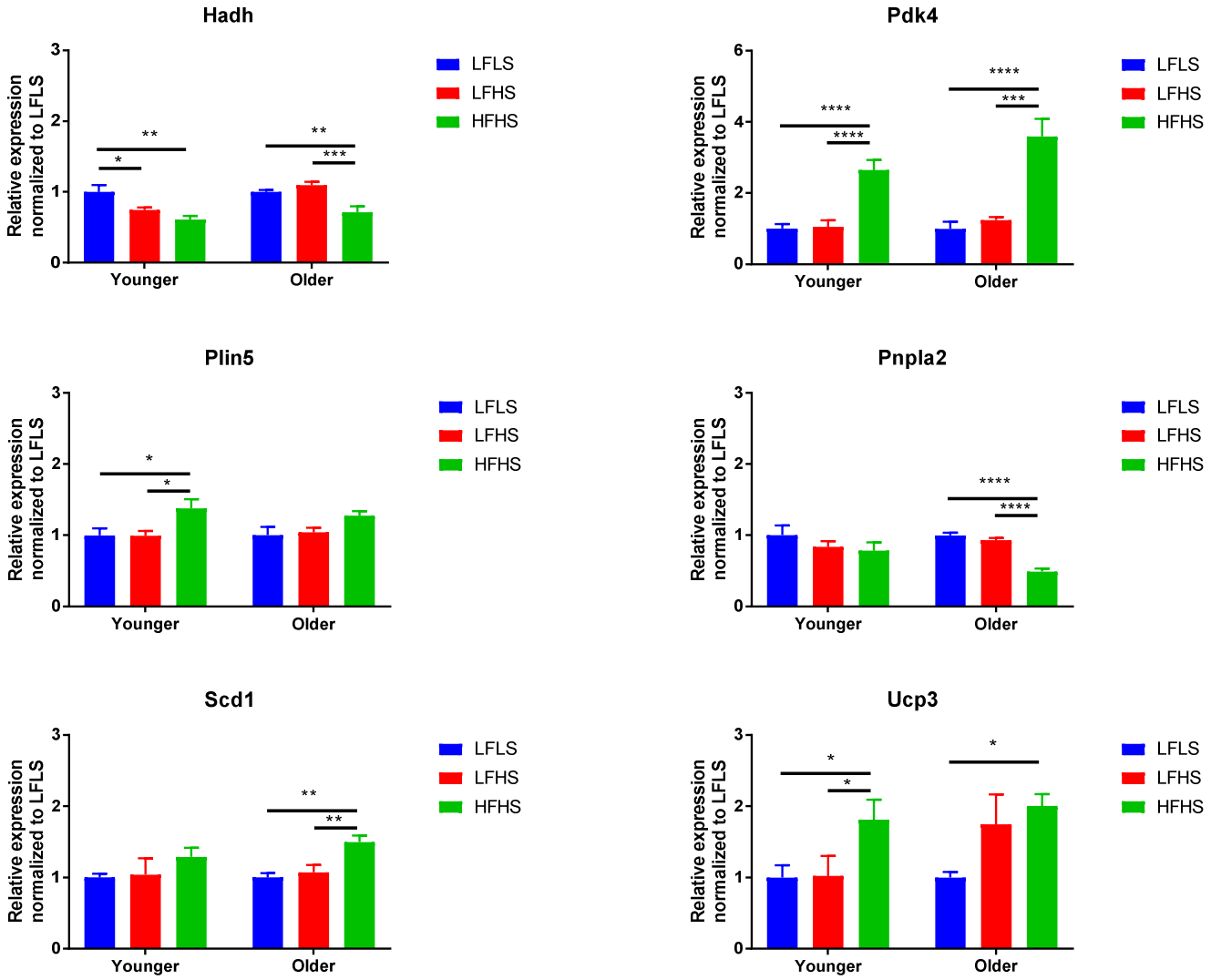
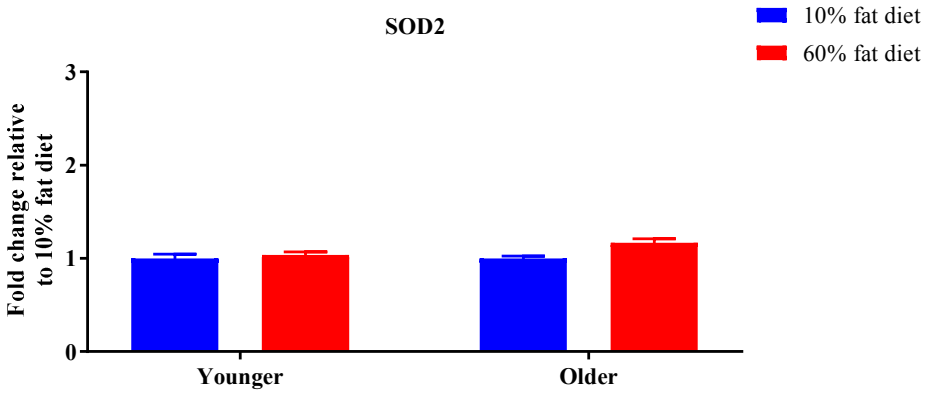
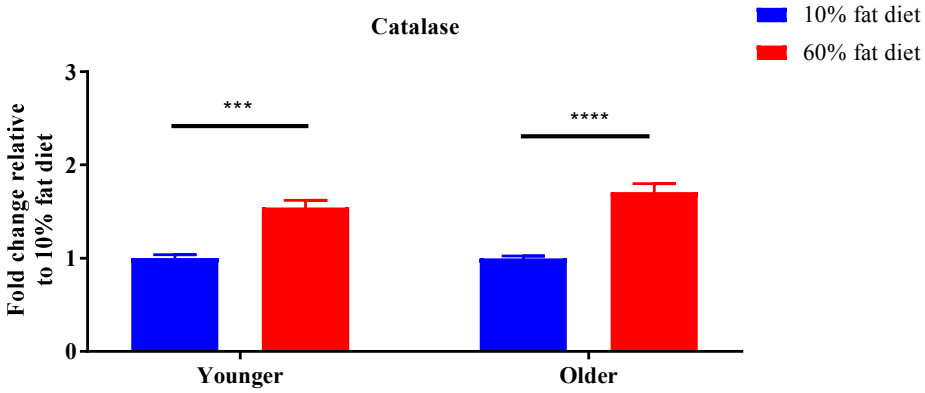

Hadh
Relative expression normalized to LFLS
LFLS
LFHS
HFHS
Younger
Older
Pdk4
Plin5
Pnpla2
Scd1
Ucp3

**Figure 33. Gene expression of metabolism related genes in the hearts of mice fed a saturated fat rich diet for 20 weeks.**

Data are presented as Mean±SEM and were analyzed by one-way ANOVA followed by post-hoc analysis using Tukey's test to determine fat feeding induced changes within each age group. Statistical significance was set at $p<0.05$ ($N>6$/group). Younger and older refer to mice that that were 10 weeks of age or 20 weeks of age respectively at the time of dietary intervention were used for the experiment


SOD2
10% fat diet
60% fat diet
Fold change relative to 10% fat diet
3
2
1
0
Younger
Older


Catalase
10% fat diet
60% fat diet
Fold change relative to 10% fat diet
***
****
3
2
1
0
Younger
Older

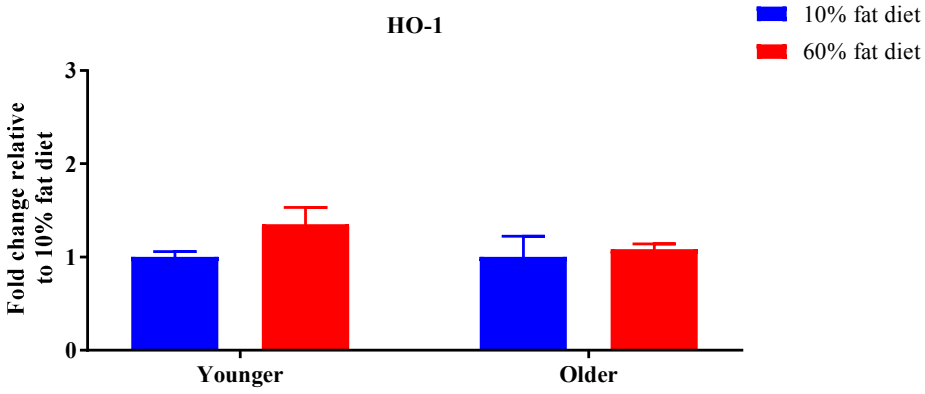
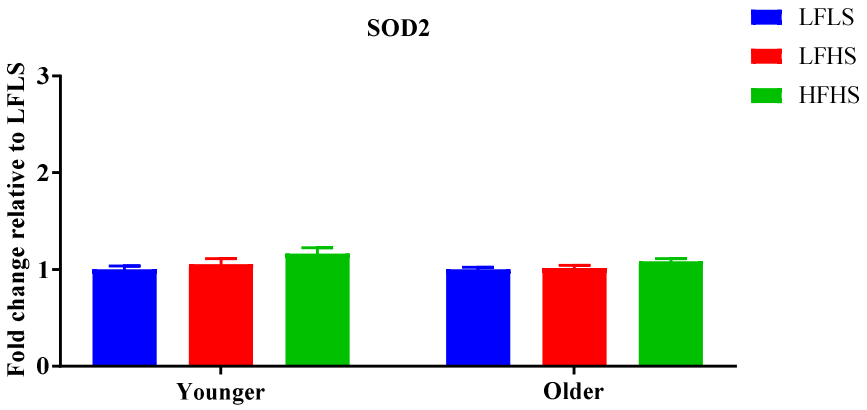
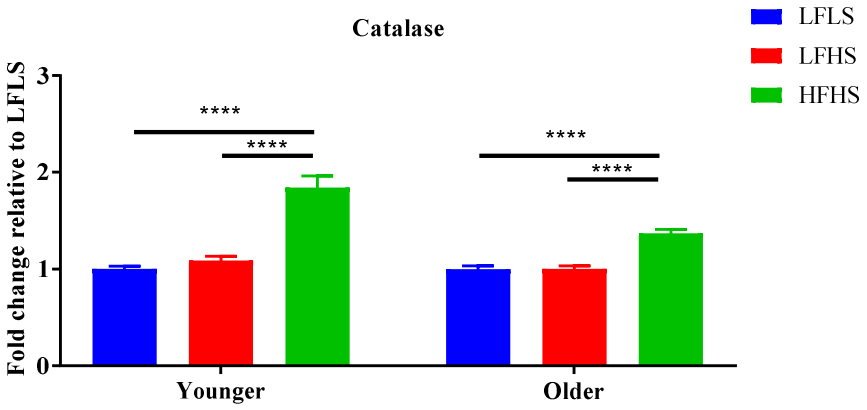

HO-1
10% fat diet
60% fat diet
Fold change relative to 10% fat diet
3
2
1
0
Younger
Older

**Figure 34. Gene expression of enzymes important for neutralizing ROS in the hearts of mice fed a lard-based diet for 20 weeks.**

Data are presented as Mean±SEM and were analyzed by student's t-test to determine fat feeding induced differences within each age group. Statistical significance was set at $p<0.05$ (N=5/group). Younger and older refer to mice that that were 10 weeks of age or 20 weeks of age respectively at the time of dietary intervention were used for the experiment.


SOD2
LFLS
LFHS
HFHS
Fold change relative to LFLS
0
1
2
3
Younger
Older


Catalase
LFLS
LFHS
HFHS
Fold change relative to LFLS
****
****
****
****
0
1
2
3
Younger
Older

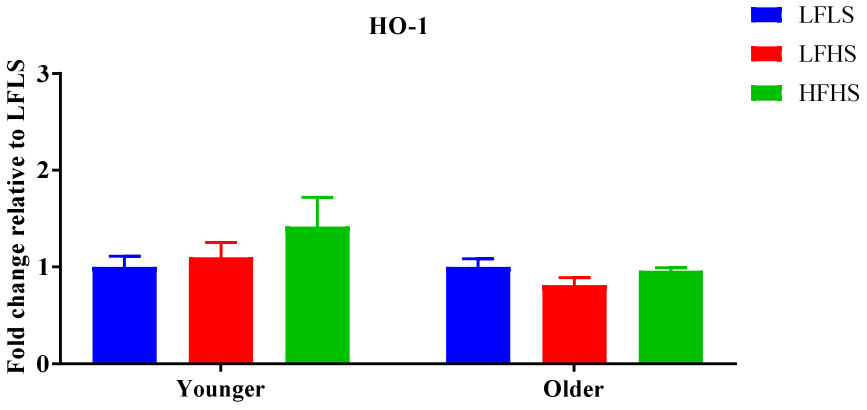
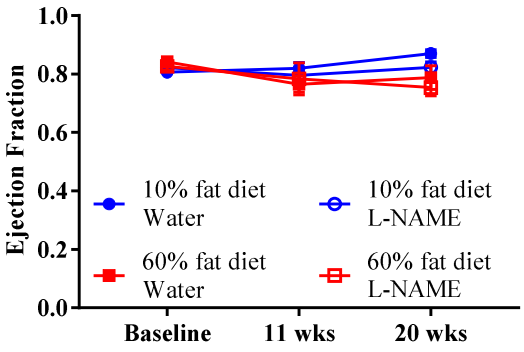
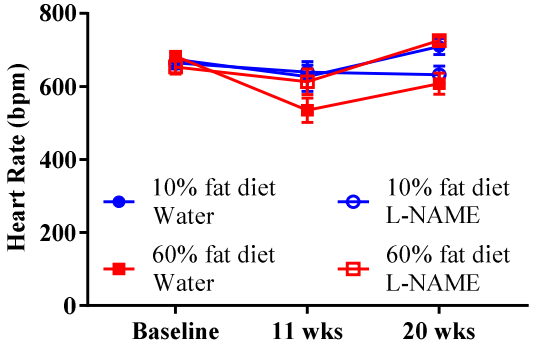
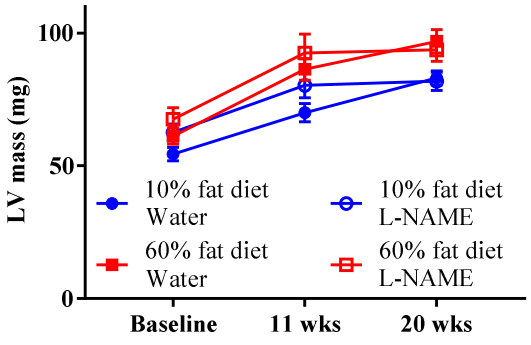

HO-1
LFLS
LFHS
HFHS
Fold change relative to LFLS
0
1
2
3
Younger
Older

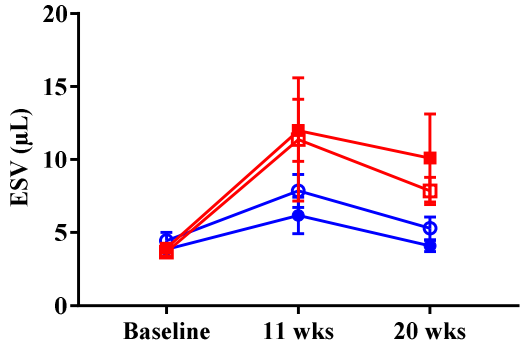
**Figure 35. Gene expression of enzymes important for neutralizing ROS in the hearts of mice fed a saturated fat rich diet for 20 weeks.**

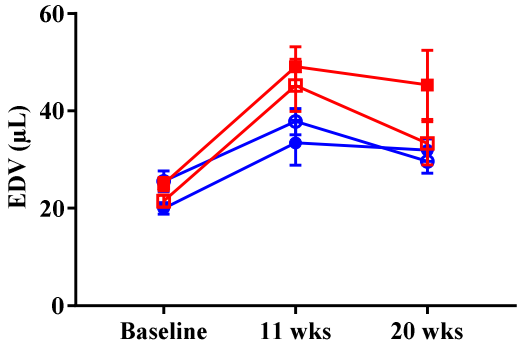
Data are presented as Mean±SEM and were analyzed by one-way ANOVA followed by post-hoc analysis using Tukey's test to determine fat feeding induced differences within each age group. Statistical significance was set at $p<0.05$ ($N>6$/group). Younger and older refer to mice that that were 10 weeks of age or 20 weeks of age respectively at the time of dietary intervention were used for the experiment.


Ejection Fraction
1.0
0.8
0.6
0.4
0.2
0.0
10% fat diet Water
10% fat diet L-NAME
60% fat diet Water
60% fat diet L-NAME
Baseline
11 wks
20 wks


Heart Rate (bpm)
800
600
400
200
0
10% fat diet Water
10% fat diet L-NAME
60% fat diet Water
60% fat diet L-NAME
Baseline
11 wks
20 wks


LV mass (mg)
100
50
0
10% fat diet Water
10% fat diet L-NAME
60% fat diet Water
60% fat diet L-NAME
Baseline
11 wks
20 wks


ESV (µL)
20
15
10
5
0
Baseline
11 wks
20 wks


EDV (µL)
60
40
20
0
Baseline
11 wks
20 wks

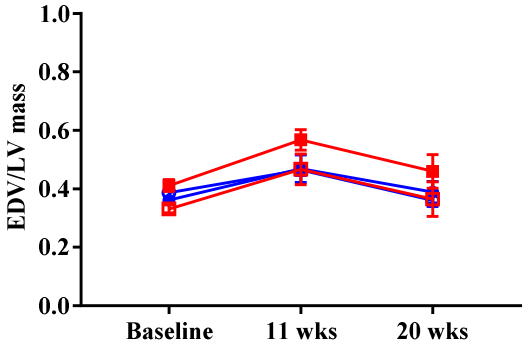
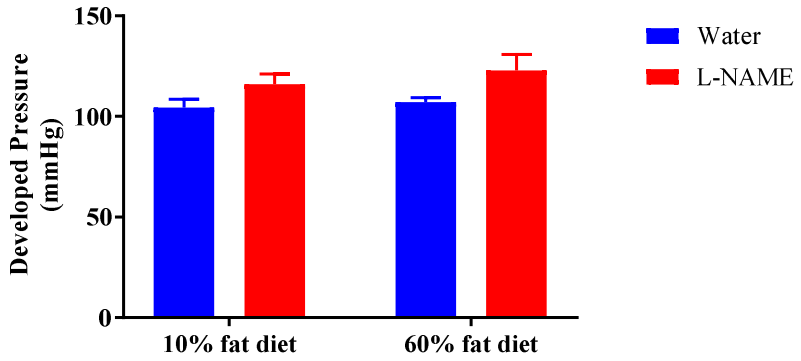
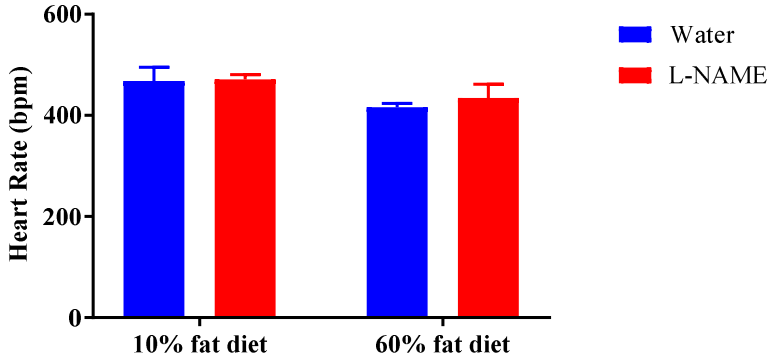
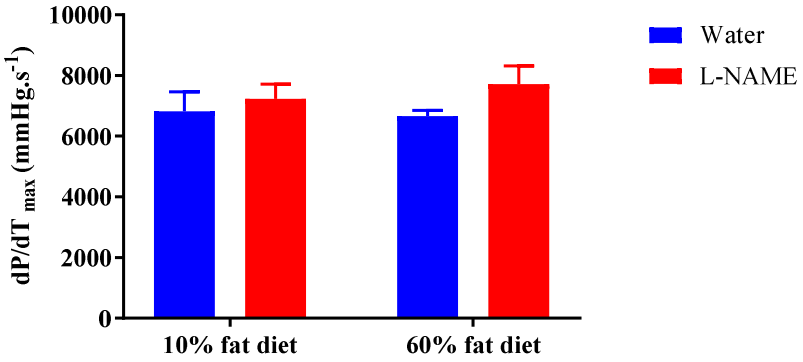

EDV/LV mass
1.0
0.8
0.6
0.4
0.2
0.0
Baseline
11 wks
20 wks

**Figure 36. Cardiac function measured by echocardiography in mice that were fed a lard-based high fat diet and concomitantly exposed to L-NAME (1mg/mL).**

Data are presented as Mean±SEM and were analyzed by repeated measures two-way ANOVA and statistical significance was set at $p<0.05$. No significant changes in cardiac function were observed ($N>5$/group).


Developed Pressure (mmHg)
150
100
50
0
10% fat diet
60% fat diet
Water
L-NAME


Heart Rate (bpm)
600
400
200
0
10% fat diet
60% fat diet
Water
L-NAME


dP/dT max (mmHg.s-1)
10000
8000
6000
4000
2000
0
10% fat diet
60% fat diet
Water
L-NAME

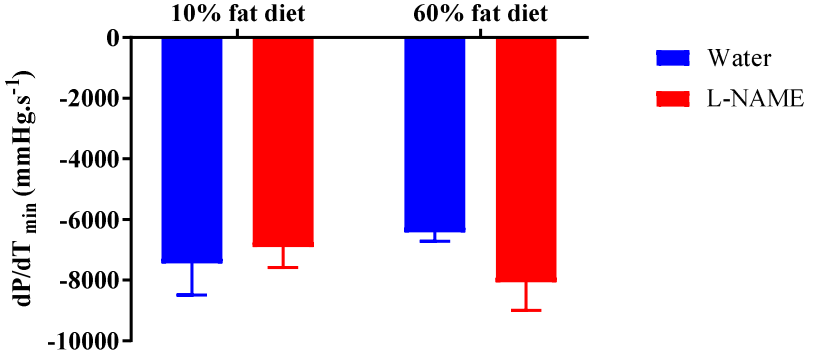

dP/dT min (mmHg.s-1)
10% fat diet
60% fat diet
0
-2000
-4000
-6000
-8000
-10000
Water
L-NAME

**Figure 37. Cardiac function as assessed by invasive hemodynamics in mice concomitantly exposed to lard-based HFD and L-NAME.**

Data are presented as Mean+SEM and were analyzed by two-way ANOVA followed by post-hoc analysis using Tukey's test.

Statistical significance was set at $p<0.05$ ($N\geq5$/group)

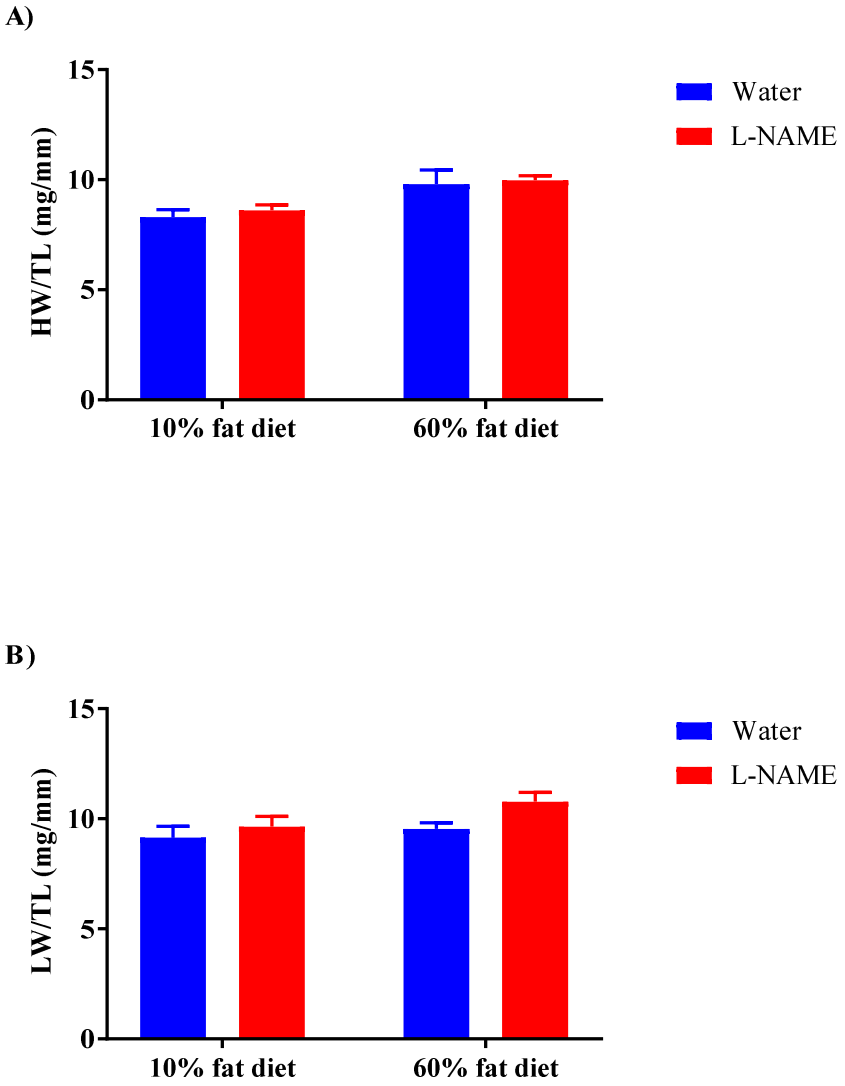



**Figure 38. Indices of cardiac hypertrophy (A) and pulmonary congestion (B) in mice concomitantly fed lard-based HFD and L-NAME (1mg/mL) for 20 weeks.**

Data in (A) and (B) were analyzed by two-way ANOVA followed by post-hoc analysis using Tukey's multiple comparisons test (N>4/group). Statistical significance is reported at $p<0.05$.

| Parameter | 10% fat diet (N=6) | | 60% fat diet (N=6) | |
|---|---|---|---|---|
| | Baseline | 41 weeks | Baseline | 41 weeks |
| Ejection Fraction | 0.816±0.0156 | 0.819±0.01 | 0.776±0.03 | 0.834±0.03 |
| Heart Rate (bpm) | 705±6.99 | 625.8±27.9 | 687.8±22.9 | 543.8±32.8* |
| ESV (µL) | 5.29±0.49 | 5.51±0.46 | 7.87±1.47 | 8.61±2.14 |
| EDV (µL) | 28.89±2.04 | 30.88±1.95 | 35.52±4.96 | 50.73±5.23* |
| EDV/LV mass | 0.439±0.019 | 0.483±0.031 | 0.43±0.037 | 0.49±0.049 |
| LV mass (mg) | 65.83±3.57 | 82.56±8.83 | 64.36±3.92 | 102.17±5.51* |

**Supplementary Table 1. Cardiac function measured by transthoracic echocardiography in mice fed a lard-based high fat diet for 41 weeks beginning at 9 weeks of age.**

Data were analyzed by repeated measures two-way ANOVA and are represented as Mean±SEM followed by post-hoc analysis using Sidak's test. Significance is reported at $p<0.05$ (*- $p<0.05$ compared to 10% fat diet at the same time point).

| Parameter | 10% fat diet (N=5) | 10% fat diet + L-NAME (N=5) | 60% fat diet (N=5) | 60% fat diet + L-NAME (N=5) |
|---|---|---|---|---|
| **Systolic Blood pressure (mmHg)** | 127.16±2.4 | 140.4±1.7 | 118±7.2 | 155.4±3.2 *** |
| **Diastolic Blood pressure (mmHg)** | 78.1±1.6 | 86.6±4.3 | 73.3±5.4 | 99±2.32 *** |
| **Mean Arterial pressure (mmHg)** | 94.4±1.6 | 104.5±4.4 | 88.25±5.97 | 117.8±2.4 * |
| **Pulse (bpm)** | 627.7±9.4 | 499.5±31.15 ** | 669±16.12 | 535.14±22.2 ** |

**Supplementary Table 2. Tail-cuff plethysmography derived blood pressure measurements indicating altered hemodynamics in L-NAME groups.**

Data are presented as Mean±SEM and were analyzed by two-way ANOVA followed by Tukey's post-hoc analysis. Statistical significance was set at $p<0.05$. *- $p<0.05$, **- $p<0.01$, ***-$p<0.001$ compared to water group fed with the same diet.

| | | Lard based diets | | Saturated fat based diets | | |
|---|---|---|---|---|---|---|
| | | 10% fat diet | 60% fat diet | LFLS | LFHS | HFHS |
| Carbohydrate | Sucrose | 7.1 | 7.1 | 7.1 | 19.5 | 19.5 |
| | Maltodextrin | 12.3 | 12.3 | 12.3 | 12.3 | 0 |
| | Corn Starch | 50 | 0 | 50 | 37.5 | 0 |
| Protein | | 20 | 20 | 20 | 20 | 20 |
| Fat | Saturated | 2.26 | 19.1 | 5.24 | 5.24 | 54.7 |
| | MUFA | 2.98 | 21.5 | 1.33 | 1.33 | 1.7 |
| | PUFA | 4.7 | 19.1 | 3.3 | 3.3 | 3.3 |

**Supplementary Table 3. Composition of different diets.**

(values presented as % calories of total)

| Ingredient | 10% fat diet | 60% fat diet | LFLS | LFHS | HFHS |
|---|---|---|---|---|---|
| Coconut Oil, 101 | 0 | 0 | 20 | 20 | 245 |
| Lard | 20 | 245 | 0 | 0 | 0 |
| Soybean Oil | 25 | 25 | 25 | 25 | 25 |
| **Total (g)** | 45 | 270 | 45 | 45 | 270 |
| **COMPOSITION OF FAT (in grams)** | | | | | |
| | **10% fat diet** | **60% fat diet** | **LFLS** | **LFHS** | **HFHS** |
| C2, Acetic | 0 | 0 | 0 | 0 | 0 |
| C4, Butyric | 0 | 0 | 0 | 0 | 0 |
| C6, Caproic | 0 | 0 | 0.1 | 0.1 | 1.47 |
| C8, Caprylic | 0 | 0 | 1.5 | 1.5 | 18.865 |
| C10, Capric | 0 | 0.1 | 1.18 | 1.2 | 14.455 |
| C12, Lauric | 0 | 0.2 | 9.5 | 9.5 | 116.62 |

**Supplementary Table 4. Fat composition of different diets.** (according to the manufacturer)

| | | | | | |
|---|---|---|---|---|---|
| C14, Myristic | 0.3 | 2.8 | 3.6 | 3.6 | 44.125 |
| C14:1, Myristoleic, n-9 | 0 | 0 | 0 | 0 | 0 |
| C15 | 0 | 0.2 | 0 | 0 | 0 |
| C16, Palmitic | 6.4 | 49.9 | 4.3 | 4.3 | 23.9025 |
| C16:1, Palmitoleic, n-9 | 0.3 | 3.4 | 0 | 0 | 0.025 |
| C16:2, n-4 | 0 | 0 | 0 | 0 | 0 |
| C16:3, n-9 | 0 | 0 | 0 | 0 | 0 |
| C16:4, n-4 | 0 | 0 | 0 | 0 | 0 |
| C17 | 0.1 | 0.9 | 0 | 0 | 0.013 |
| C17:1 | 0 | 0 | 0 | 0 | 0 |
| C18, Stearic | 3.1 | 26.9 | 3.1 | 3.1 | 26.933 |
| C18:1, Oleic, n-9 | 12.3 | 86.3 | 5.9 | 5.9 | 7.71 |
| C18:2, Linoleic | 17.8 | 72.7 | 12.9 | 12.9 | 13.011 |
| C18:3, Linolenic | 2.1 | 5.1 | 1.9 | 1.9 | 1.85 |

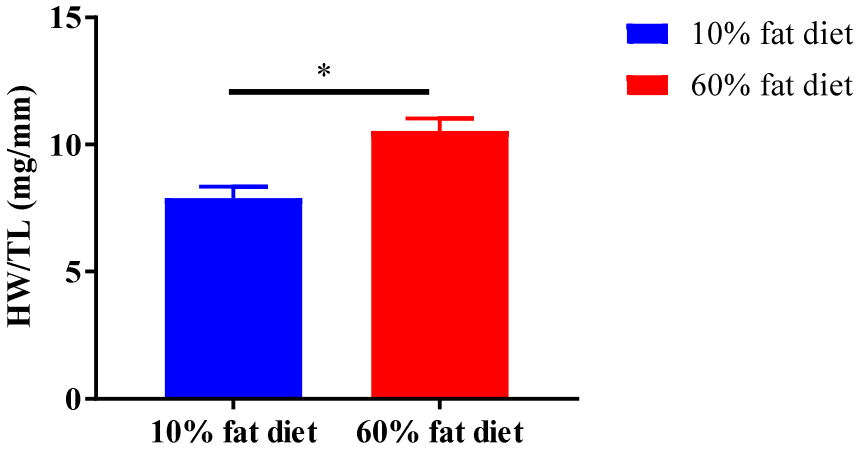
**Supplementary Table 4 continued**

| | | | | | |
|---|---|---|---|---|---|
| C18:4, Stearidonic | 0 | 0 | 0 | 0 | 0 |
| C20, Arachidic | 0.1 | 0.5 | 0.1 | 0.1 | 0.088 |
| C20:1 | 0.2 | 1.6 | 0.1 | 0.1 | 0.063 |
| C20:2 | 0.2 | 2 | 0 | 0 | 0 |
| C20:3, n-6 | 0 | 0.3 | 0 | 0 | 0 |
| C20:3, n-3 | 0 | 0 | 0 | 0 | 0 |
| C20:4, Arachidonic, n-6 | 0.1 | 0.7 | 0 | 0 | 0 |
| C20:4, n-3 | 0 | 0 | 0 | 0 | 0 |
| C20:5, Eicosapentaenoic, n-3 | 0 | 0 | 0 | 0 | 0 |
| C21:5, n-3 | 0 | 0 | 0 | 0 | 0 |
| C22, Behenic | 0.1 | 0.1 | 0.1 | 0.1 | 0.063 |
| C22:1, Erucic | 0 | 0 | 0 | 0 | 0 |
| C22:4, Clupanodonic, n-6 | 0 | 0 | 0 | 0 | 0 |
| C22:5, Docosapentaenoic, n-3 | 0 | 0.2 | 0 | 0 | 0 |

**Supplementary Table 4 continued**

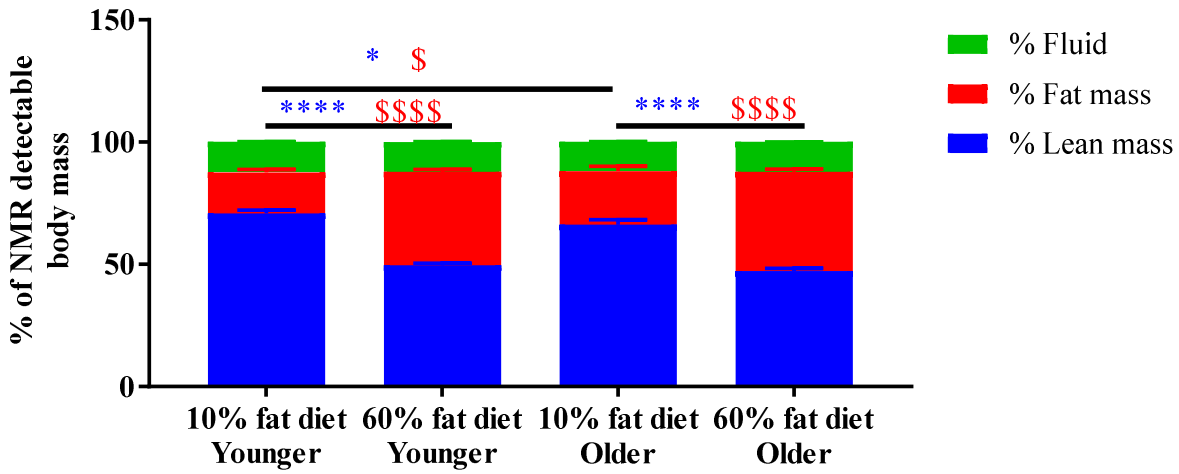
| | | | | | |
|---|---|---|---|---|---|
| C22:6, Docosahexaenoic, n-3 | 0 | 0 | 0 | 0 | 0 |
| C24, Lignoceric | 0 | 0 | 0 | 0 | 0.038 |
| C24:1 | 0 | 0 | 0 | 0 | 0 |
| **Total** | **43.1** | **254** | **44.4** | **44.4** | **269.229** |
| Saturated (g) | 10.1 | 81.7 | 23.6 | 23.6 | 246.57 |
| Monounsaturated (g) | 12.8 | 91.2 | 6 | 6 | 7.798 |
| Polyunsaturated (g) | 20.2 | 81 | 14.8 | 14.8 | 14.861 |
| Saturated (%) | 23.5 | 32.2 | 53.2 | 53.2 | 91.584 |
| Monounsaturated (%) | 29.7 | 35.9 | 13.5 | 13.5 | 2.896 |
| Polyunsaturated (%) | 46.8 | 31.9 | 33.3 | 33.3 | 5.52 |
| n6 | 17.9 | 73.7 | 12.9 | 12.9 | 13.011 |
| n3 | 2.1 | 5.3 | 1.9 | 1.9 | 1.85 |
| n6:n3 ratio | 8.4 | 13.9 | 7 | 7 | 7.033 |
| trans fat (gm) | | | 0 | 0 | 0 |

**Supplementary Table 4 continued**




**Supplementary Figure 1. Chronic high fat diet feeding (lard-based) for 45 weeks caused cardiac hypertrophy in C57BL/6J mice.**

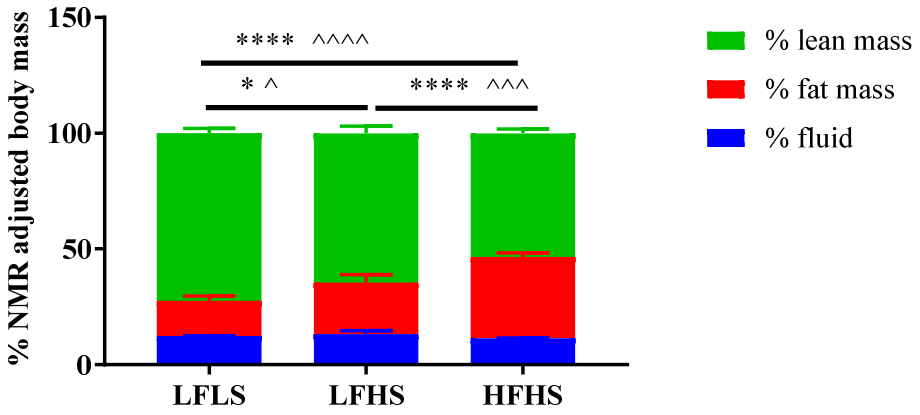
Data were analyzed by student's t-test and represented as Mean+SEM. (*- $p<0.05$, $N>3$/group)



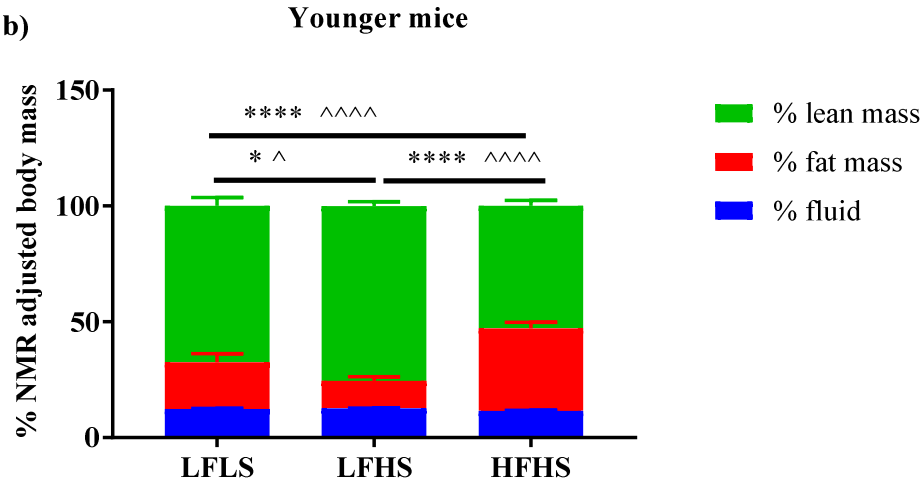

**Supplementary Figure 2. Body mass composition in mice fed lard-based high fat diet for 16 weeks.**

Data were analyzed by two-way ANOVA followed by post-hoc analysis using Tukey's test. Statistical significance was set at $p<0.05$ (N>6/group). Data are represented as Mean+SEM. Younger and older refer to mice that that were 10 weeks of age or 20 weeks of age respectively at the time of dietary intervention. (*,$- $p<0.05$, ****,$$$$ -$p<0.0001$, *- significance for lean mass, $- significance for fat mass)

a)


Older mice
% NMR adjusted body mass
150
100
50
0
**** ^^^^
* ^
**** ^^^
LFLS
LFHS
HFHS
% lean mass
% fat mass
% fluid

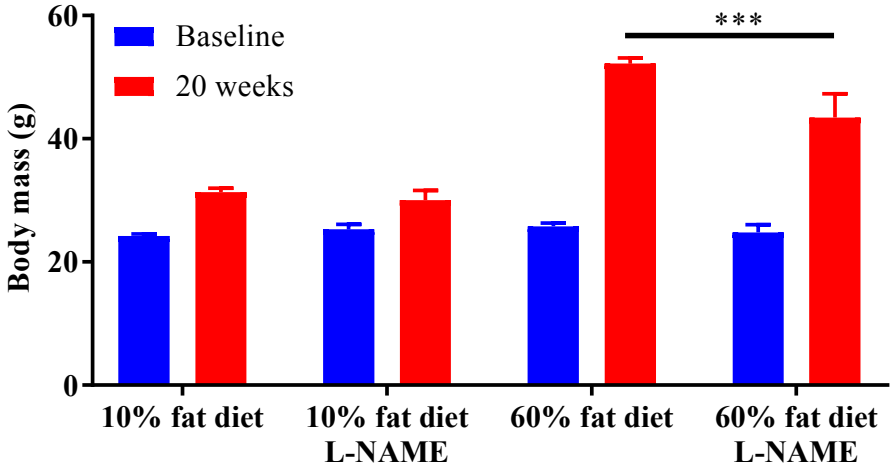
b)


Younger mice
% NMR adjusted body mass
150
100
50
0
**** ^^^^
* ^
**** ^^^^
LFLS
LFHS
HFHS
% lean mass
% fat mass
% fluid

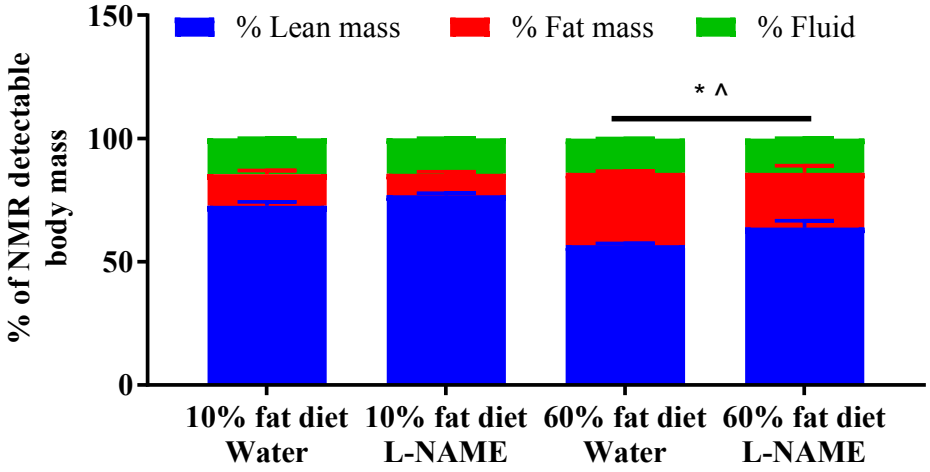
**Supplementary Figure 3. Body mass composition in mice fed saturated fat rich diet for 16 weeks in (a) Older mice and (b) Younger mice.**

Data were analyzed by two-way ANOVA followed by post-hoc analysis using Tukey's test. Statistical significance was set at $p<0.05$ (N>6/group). Data are presented as Mean+SEM. Older and Younger refer to mice that that were 10 weeks of age or 20 weeks of age respectively at the time of dietary intervention. *,^- $p<0.05$; ****,^^^^-$p<0.0001$; *- comparisons of fat mass, ^- comparisons of lean mass.

A)

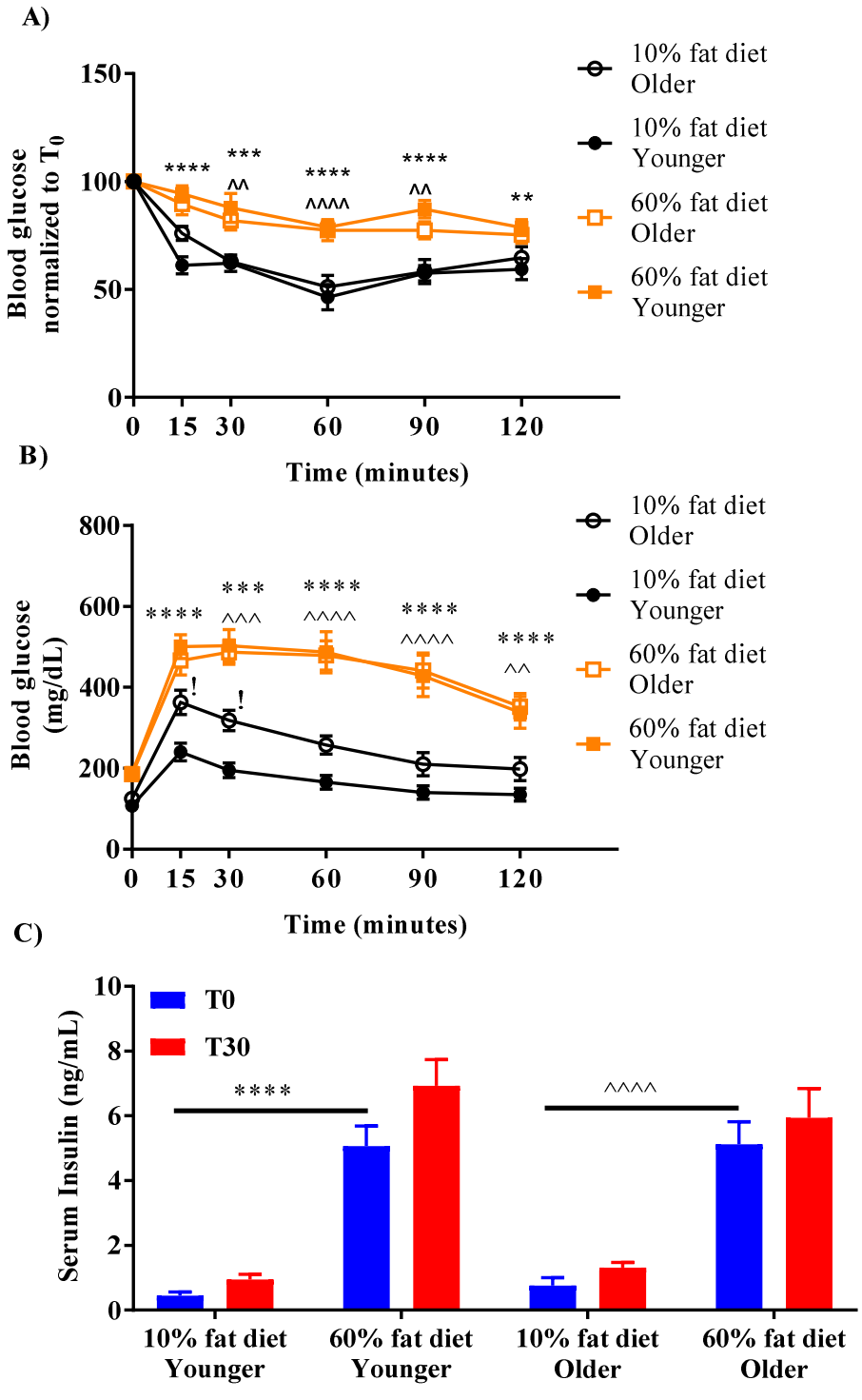

60
40
20
0
Body mass (g)
Baseline
20 weeks
***
10% fat diet
10% fat diet L-NAME
60% fat diet
60% fat diet L-NAME

B)


150
100
50
0
% of NMR detectable body mass
% Lean mass
% Fat mass
% Fluid
* ^
10% fat diet Water
10% fat diet L-NAME
60% fat diet Water
60% fat diet L-NAME

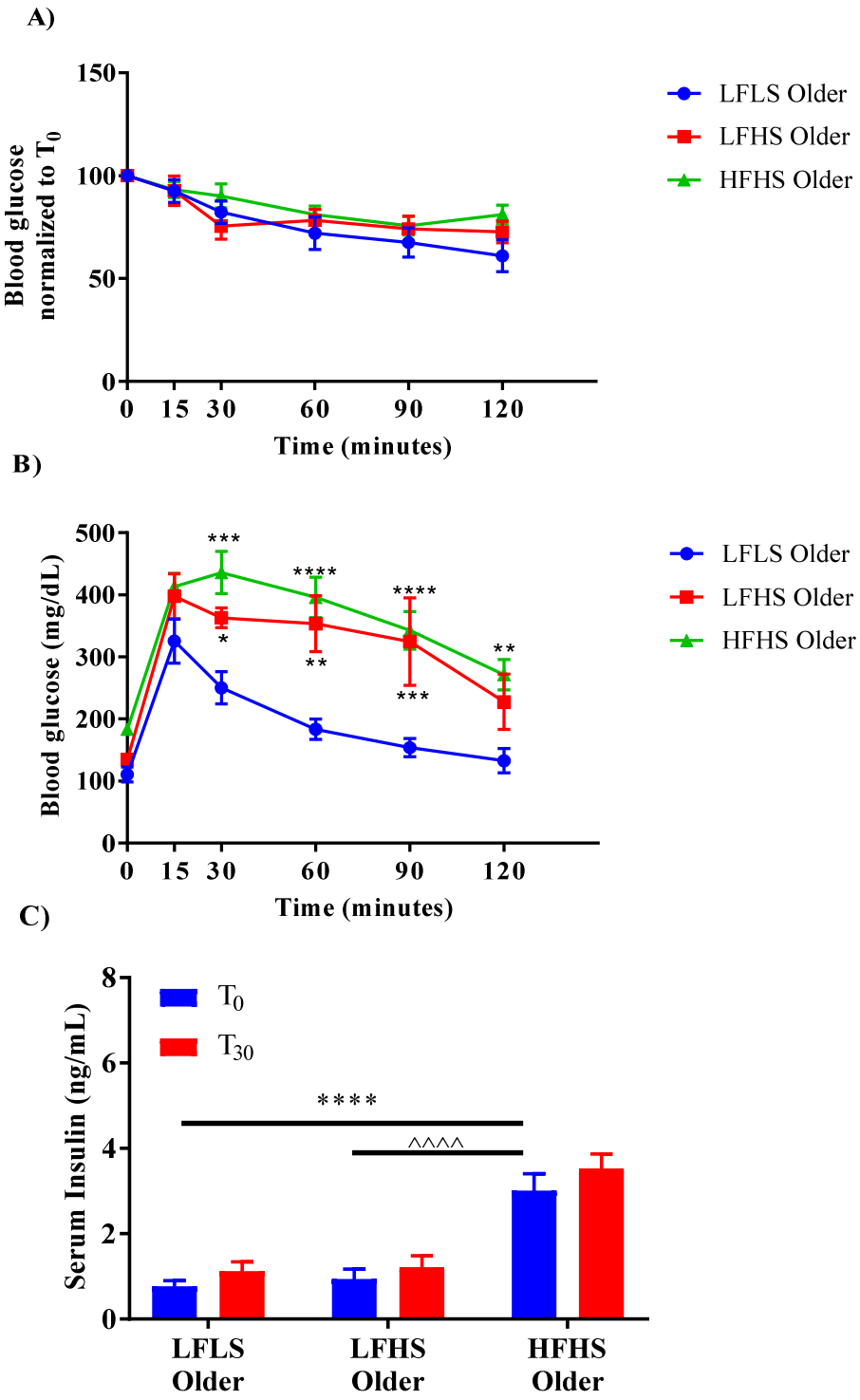
**Supplementary Figure 4. Concomitant L-NAME exposure decreased body weight gain and fat mass expansion in high fat fed mice.**

A) Gravimetric measures. Data are presented as Mean+SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's multiple comparisons test. Statistical significance is reported at $p<0.05$ ($N>5$ per group, ***-$p<0.001$). B) NMR derived body composition measurements. Data are presented as Mean+SEM and were analyzed by two-way ANOVA followed by post-hoc analysis using Tukey's multiple comparisons test. Statistical significance is reported at $p<0.05$ ($N>5$ per group, *-$p<0.05$ for comparison of lean mass, ^- $p<0.05$ for comparison of fat mass).


A)
Blood glucose normalized to T0
150
100
50
0
0 15 30 60 90 120
Time (minutes)
10% fat diet Older
10% fat diet Younger
60% fat diet Older
60% fat diet Younger
B)
Blood glucose (mg/dL)
800
600
400
200
0
0 15 30 60 90 120
Time (minutes)
C)
Serum Insulin (ng/mL)
10
8
6
4
2
0
T0
T30
10% fat diet Younger
60% fat diet Younger
10% fat diet Older
60% fat diet Older

**Supplementary Figure 5. Measures of insulin resistance in mice fed a lard-based diet for 18 weeks.**

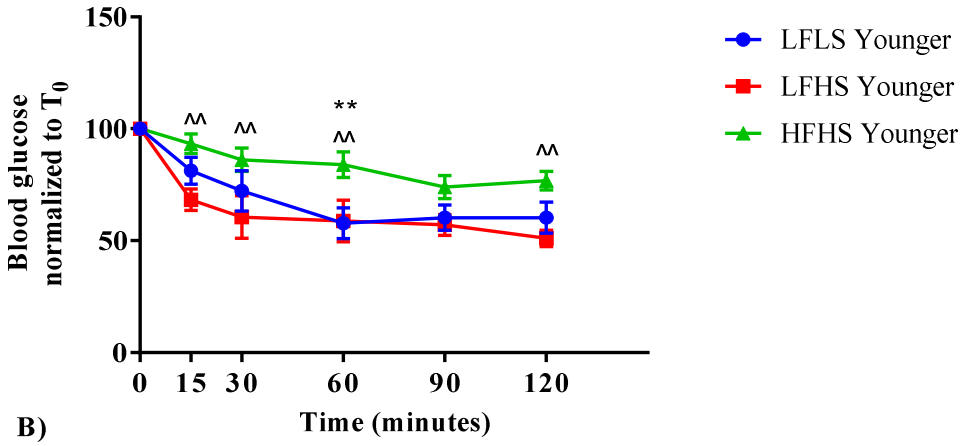
(A) Insulin sensitivity as measured by ITT (B) Glucose tolerance as measured by i.p. GTT. (C) Fasting serum insulin (T0) and serum insulin after i.p. glucose load (T30) in mice as measured by insulin ELISA. Data are represented as Mean+SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's test. Statistical significance was set at $p<0.05$ ($N>6$/group). Younger and older refer to mice that were 10 weeks of age or 20 weeks of age respectively at the time of dietary intervention. (**,^^-$p<0.01$, ***-$p<0.001$. ****,^^^^-$p<0.0001$; *- 10% diet young vs 60% diet young; ^- 10% diet old vs. 60% diet old, !- 10% diet young vs. 10% diet old)


A)
Blood glucose normalized to T0
150
100
50
0
0 15 30 60 90 120
Time (minutes)
LFLS Older
LFHS Older
HFHS Older
B)
Blood glucose (mg/dL)
500
400
300
200
100
0
***
****
*
**
****
***
**
0 15 30 60 90 120
Time (minutes)
LFLS Older
LFHS Older
HFHS Older
C)
Serum Insulin (ng/mL)
8
6
4
2
0
T0
T30
****
^^^^
LFLS Older
LFHS Older
HFHS Older

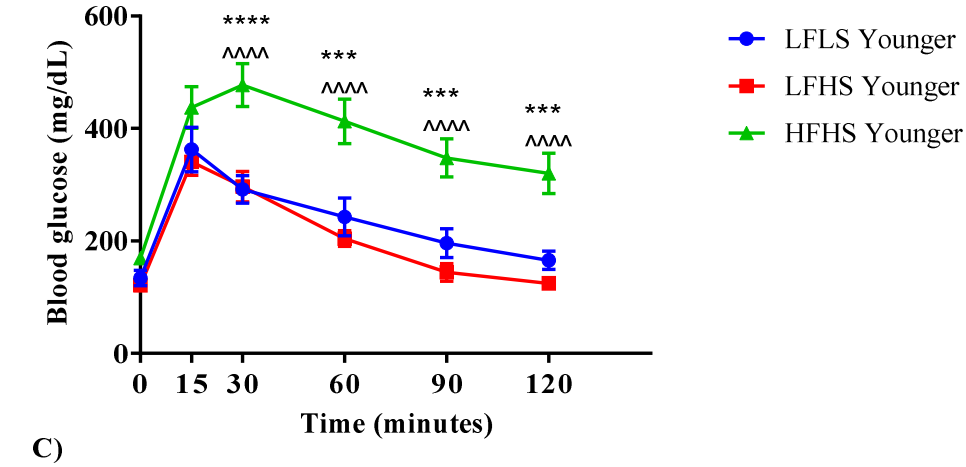
**Supplementary Figure 6. Measures of insulin resistance in mice fed a saturated fat rich diet for 18 weeks.**

(A) Insulin sensitivity as measured by ITT (B) Glucose tolerance as measured by i.p. GTT. (C) Fasting serum insulin (T0) and serum insulin after i.p. glucose load (T30) in mice as measured by insulin ELISA. Data are represented as Mean+SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's test. Statistical significance was set at $p<0.05$ (N>6/group, *- $p<0.05$, **- $p<0.01$, ***-$p<0.001$, ****-$p< 0.0001$ vs LFLS Older, ^^^^-$p< 0.0001$ vs LFHS Older). Older refers to mice that were 20 weeks of age at the time of dietary intervention.

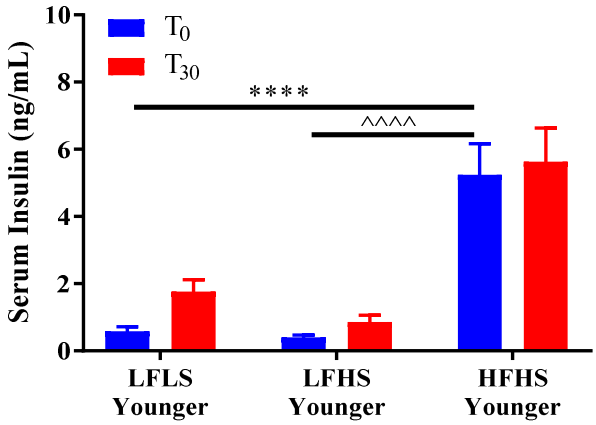
A)


Blood glucose normalized to T0
150
100
50
0
0 15 30 60 90 120
Time (minutes)
^^
^^
**
^^
^^
LFLS Younger
LFHS Younger
HFHS Younger

B)


Blood glucose (mg/dL)
600
400
200
0
0 15 30 60 90 120
Time (minutes)
****
^^^^
***
^^^^
***
^^^^
***
^^^^
LFLS Younger
LFHS Younger
HFHS Younger

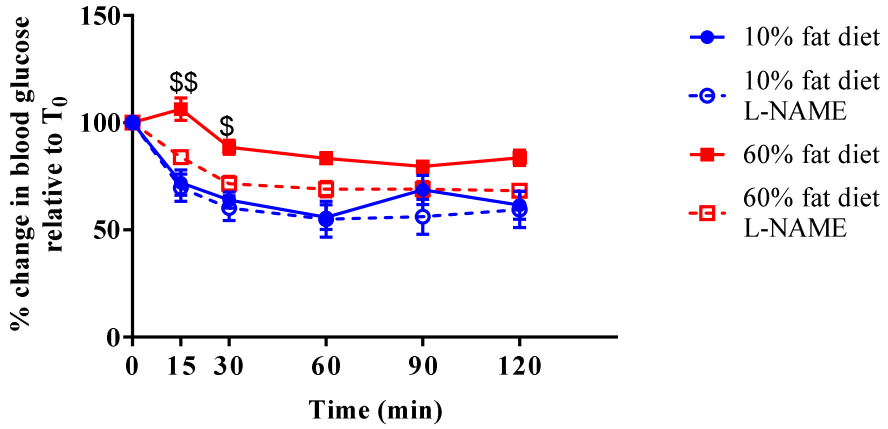
C)


Serum Insulin (ng/mL)
10
8
6
4
2
0
T0
T30
****
^^^^
LFLS Younger
LFHS Younger
HFHS Younger

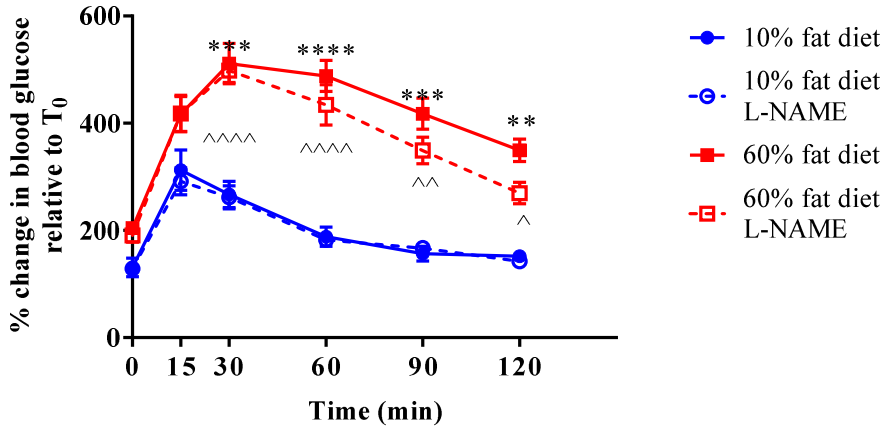
**Supplementary Figure 7. Measures of insulin resistance in mice fed a saturated fat rich diet for 18 weeks.**

(A) Insulin sensitivity as measured by ITT (B) Glucose tolerance as measured by i.p. GTT. (C) Fasting serum insulin (T0) and serum insulin after i.p. glucose load (T30) in mice as measured by insulin ELISA. Data are represented as Mean+SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's test. Statistical significance was set at $p<0.05$ (N>6/group, *- $p<0.05$, **- $p<0.01$, ***-$p<0.001$, ****-$p< 0.0001$ vs LFLS Younger; ^^- $p<0.01$, ^^^^-$p< 0.0001$ vs LFHS Younger). Younger refers to mice that were 10 weeks of age at the time of dietary intervention.

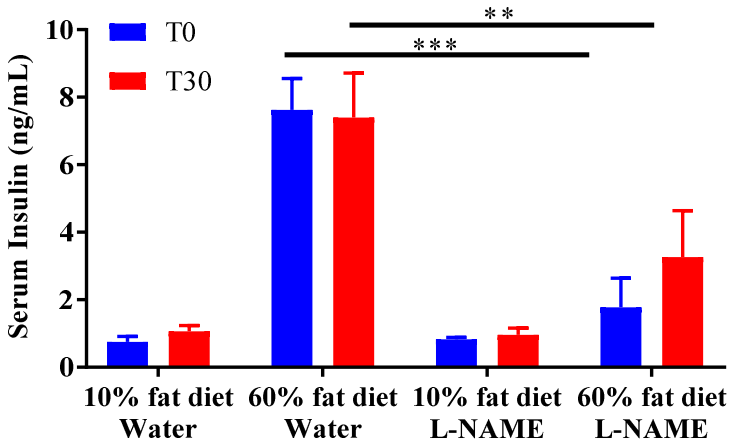
A)


% change in blood glucose relative to T0
150
100
50
0
$$
$
0 15 30 60 90 120
Time (min)
10% fat diet
10% fat diet L-NAME
60% fat diet
60% fat diet L-NAME

B)

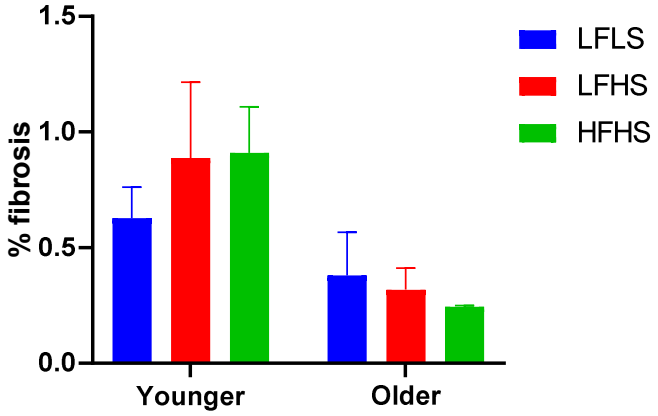

% change in blood glucose relative to T0
600
400
200
0
***
****
***
**
^^^^
^^^^
^^
^
0 15 30 60 90 120
Time (min)
10% fat diet
10% fat diet L-NAME
60% fat diet
60% fat diet L-NAME

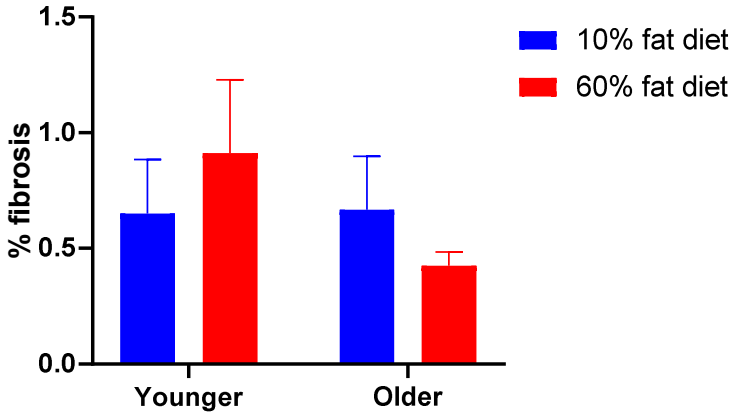
C)


Serum Insulin (ng/mL)
10
8
6
4
2
0
T0
T30
**
***
10% fat diet Water
60% fat diet Water
10% fat diet L-NAME
60% fat diet L-NAME

**Supplementary Figure 8. Concomitant L-NAME exposure decreased insulin resistance in high fat fed mice.**

A) Insulin tolerance test B) Glucose tolerance test. Data are presented as Mean+SEM and were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Tukey's multiple comparisons test . Statistical significance was set at $p<0.05$ (N>4/group, **,^^-$p<0.01$, ***-$p<0.001$, ****,^^^^-$p<0.0001$; *- between water fed 10% fat diet and 60% fat diet, ^- between L-NAME fed 10% fat diet and 60% fat diet; $- $p<0.05$, $$-$p<0.01$, $- between 60% fat diet and 60% fat diet+L-NAME ). C) Fasting serum insulin (T0) and serum insulin following i.p. glucose load (T30) in mice concotitantly exposed to L-NAMEand different diets. Data were analyzed by repeated measures two-way ANOVA followed by post-hoc analysis using Sidak's multiple comparisons test. Statistical significance is reported at $p<0.05$ (N>4/group).




**Supplementary Figure 9. Quantification of trichrome staining of heart sections from mice subject to 20 weeks of dietary intervention using saturated fat rich diet or a lard-based diet.** Younger mice refer to mice that were 10 weeks of age and Older mice refer to mice that were 20 weeks of age at the time of initiation of high fat diets. Data are presented as Mean+SEM and within age group comparisons were made using appropriate statistical tests. No significance was found between high fat and low fat groups (N>3 mice/group).

# CHAPTER 5: FUTURE DIRECTIONS

## 5.1 Effects of Acute Hyperinsulinemia on Cardiac Function and Myocardial β-Adrenergic Response

Acute hyperinsulinemia for durations of 5 min or 2h did not impair baseline contractile performance or myocardial β-adrenergic responsiveness in mice under isoflurane anesthesia and intact autonomic regulation of the heart. However, hyperinsulinemic dysmetabolic states such as type 2 diabetes and obesity are frequently associated with varying degrees of autonomic neuropathy and autonomic dysfunction (Vinik & Ziegler, 2007). An important future study to evaluate if insulin may have a direct cardio-depressant effect *in vivo*, would be to conduct experiments in animals in which the heart is devoid of autonomic innervation. This could be achieved by assessing myocardial contractility in mice subject to acute hyperinsulinemia and bilateral vagotomy. A dose response to isoproterenol following bilateral vagotomy would inform cardiac inotropic response in the face of acute hyperinsulinemia and under conditions of autonomic dysfunction or loss of autonomic regulation. By negating the influence of autonomic regulation on the heart that might compensate for changes in cardiac function following acute hyperinsulinemia and β-adrenergic stimulation, this approach could discern direct effects of insulin on beta adrenergic responsiveness *in vivo*.

## 5.2 High Fat Feeding induced Cardiac Dysfunction as a Model of Diabetic Cardiomyopathy

The lack of an animal model that closely reflects the etiology of cardiomyopathy associated with obesity and T2DM remains a limitation in our understanding of molecular mechanisms that underlie development and progression of diabetic cardiomyopathy. This may

further impede identification or development of therapeutic strategies to better manage and improve cardiac outcomes in T2DM and obesity. To address the existing problems with a reliable model that reflects the etiology of diabetic cardiomyopathy, we have considered age, saturated fat content in the diet as potential modifiers of response to high fat feeding induced cardiac dysfunction. We found no obvious contribution of age up to 20 weeks of age or of saturated fat content in the diet to development of cardiac dysfunction as measured by 2D echocardiography and by LV catheterization. An important shortcoming of both 2D-echocardiography and LV pressure tracings is that the functional assessment using these techniques is not load-independent. Utilizing more sophisticated techniques such as Pressure-Volume analysis allows for load-independent measurement of cardiac function that reflects the inherent contractile function of the cardiac muscle. Assessment of cardiac function using PV-loops allows us to thoroughly define the effects of metabolic stress on cardiac function.

### 5.2.1. Differential induction of PPARa targets- potential involvement in adaptation to metabolic stress:

One of the key observations in the high fat feeding protocols used in our studies is increased gene expression levels of the targets of PPARα. This increase is remarkable for Pdk4, the protein product of which is a key regulator of PDH activity. PDH activity regulates glucose oxidation and phosphorylation of the E1 subunit of PDH by Pdk4 in the heart decreases PDH activity thereby, decreasing glucose oxidation and indirectly increasing fatty acid oxidation in the heart (Chambers et al., 2011). Fatty acids are ligands of PPARα and the increased availability of free fatty acids in T2DM and obesity potentially increases the expression of PPARα target genes. The differential induction of the various PPARα genes in these mouse hearts is evidenced by variation in the fold increase in expression levels in high fat fed hearts

relative to control diet fed hearts. Some PPARα targets are induced more robustly than others. The mechanisms that contribute to the differential induction of these PPARα target genes are of interest because they may in part contribute to the overall adaptive phenotype that accompanies high fat feeding. This can be investigated by performing whole genome analysis of cardiac PPARα targets and determining the high affinity and low affinity binding sites for PPARα using high fat diet as the variable and a PPARα agonist treated group as a positive control. The enrichment scores of these sites and mapping of these sequences using bioinformatic approaches would permit the identification of differential binding of PPARα at its target genes under conditions of dietary fat overload. Using this information, we could then develop genetic models where some of these specific PPARα regulated targets (including Pdk4, but not limited to), based on their induction levels in the hearts of high fat fed animals could be overexpressed or deleted and their roles in myocardial adaptation to high fat feeding be investigated.

**5.2.2. Consideration of different mouse strains:**

One of the key constants throughout the high fat feeding studies was the strain of mouse used for determining the effect of metabolic stress on cardiac function. We chose to use the C57BL/6J mouse strain for these studies given its susceptibility to diet-induced obesity and development of insulin resistance (Collins et al., 2004). However; this strain of mouse appeared to be resistant to development of cardiac dysfunction induced by high fat feeding despite apparent development of metabolic perturbations. Whether other mouse strains may be susceptible to cardiac dysfunction induced by metabolic stress has not been thoroughly assessed. A recent study using the hybrid mouse diversity panel (HMDP) assessed sensitivity of different mouse strains to the development of cardiac hypertrophy, dysfunction and fibrosis induced by

isoproterenol (J. J. Wang et al., 2016). Of the 105 mouse strains tested, KK/HiJ strain was most susceptible to cardiac hypertrophy, dysfunction and fibrosis induced by isoproterenol. DBA/2J was moderately sensitive and C57BL/6J strain was markedly resistant to cardiac dysfunction and fibrosis induced by isoproterenol. This panel of 105 strains was also assessed for sensitivity to metabolic perturbations by high fat feeding (Parks et al., 2015). Of the strains tested, DBA/2J and KK/HiJ exhibited significant glucose intolerance and remarkably high fasting insulin levels when fed a high fat diet. Considering the myocardial sensitivity of the DBA/2J and KK/HiJ strains to morphological, histological and functional perturbations induced by isoproterenol and their sensitivity to metabolic perturbations induced by high fat feeding, it is possible that these strains may closely reflect cardiac abnormalities that accompany dysmetabolic states such as T2DM and obesity. To test whether these strains may indeed model diabetic cardiomyopathy, subjecting these strains to a chronic high fat feeding protocol with periodic assessment of cardiac function non-invasively using 2D-echocardiography may inform the contribution of genetic background as an under-appreciated variable in modeling diabetic cardiomyopathy in the mouse.

A more recent study reported the high fat diet and L-NAME combination as a model of Heart Failure with Preserved Ejection Fraction (HFpEF) (Schiattarella et al., 2019). Diastolic function as measured by Pressure-Volume loops in this study indicated an increased end-diastolic pressure and ventricular compliance was compromised as demonstrated by an increased slope of end-diastolic pressure with increasing volume (Schiattarella et al., 2019). However, this phenotype was observed in the C57BL/6N strain (Schiattarella et al., 2019) and the dependence on mouse strain of cardiac outcomes following metabolic stress and hypertension may be particularly important. Supporting this argument is the recent finding that the functional Nicotinamide nucleotide transhydrogenase (Nnt) protein which is expressed in the C57BL/6N

mice but not in the C57BL/6J mice. Under physiological conditions, Nnt replenishes and maintains the NADPH pool in mitochondria by utilizing NADH. But under conditions of pathological workload such as pressure overload, Nnt operates in reverse mode to increase NADH levels and thereby depleting the NADPH levels (Nickel et al., 2015). An increased oxidative stress in the cardiomyocytes of C57BL/6N mice was correlated with increased nuclear oxidative damage and increased fibrotic gene expression and compromised function (Nickel et al., 2015). C57BL/6J mice which express a truncated non-functional Nnt protein have preserved cardiac function and relatively small increases in fibrosis following pressure overload (Nickel et al., 2015). A future course of action would be to attempt to reproduce the phenotype in C57BL/6N strain using the combination of HFD and L-NAME to ensure that strain differences contributed to lack of cardiac phenotype in the C57BL/6J strain.

Depending on the outcome of pilot studies using these strains to determine whether high fat feeding in these strains causes cardiac dysfunction, a future course of action will be to generate GRK2 floxed mice on DBA/2J or KK/HiJ background depending on the sensitivity of these strains to metabolic stress. If a more sensitive strain is identified, then backcrossing the GRK2 floxed mice to an isogenic background that is more sensitive to metabolic-stress induced cardiac dysfunction would facilitate a deeper understanding of the role of GRK2 in diabetic cardiomyopathy. Knockout of GRK2 using α-MHC promoter driven inducible Cre recombinase in these mice could also determine whether GRK2 plays critical roles in the initiation or progression of diabetic cardiomyopathy. Further analysis of mitochondrial function and cardiac triglycerides in the GRK2 knockout line fed a high fat diet could also inform if GRK2 regulates cardiac triglyceride turnover and mitochondrial function under conditions of metabolic stress.

# REFERENCES


Chambers, K. T., Leone, T. C., Sambandam, N., Kovacs, A., Wagg, C. S., Lopaschuk, G. D., . . . Kelly, D. P. (2011). Chronic inhibition of pyruvate dehydrogenase in heart triggers an adaptive metabolic response. *J Biol Chem, 286*(13), 11155-11162. doi:10.1074/jbc.M110.217349

Collins, S., Martin, T. L., Surwit, R. S., & Robidoux, J. (2004). Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. *Physiol Behav, 81*(2), 243-248. doi:10.1016/j.physbeh.2004.02.006

Nickel, A. G., von Hardenberg, A., Hohl, M., Loffler, J. R., Kohlhaas, M., Becker, J., . . . Maack, C. (2015). Reversal of Mitochondrial Transhydrogenase Causes Oxidative Stress in Heart Failure. *Cell Metab, 22*(3), 472-484. doi:10.1016/j.cmet.2015.07.008

Parks, B. W., Sallam, T., Mehrabian, M., Psychogios, N., Hui, S. T., Norheim, F., . . . Lusis, A. J. (2015). Genetic architecture of insulin resistance in the mouse. *Cell Metab, 21*(2), 334-347. doi:10.1016/j.cmet.2015.01.002

Schiattarella, G. G., Altamirano, F., Tong, D., French, K. M., Villalobos, E., Kim, S. Y., . . . Hill, J. A. (2019). Nitrosative stress drives heart failure with preserved ejection fraction. *Nature*. doi:10.1038/s41586-019-1100-z

Vinik, A. I., & Ziegler, D. (2007). Diabetic cardiovascular autonomic neuropathy. *Circulation, 115*(3), 387-397. doi:10.1161/circulationaha.106.634949

Wang, J. J., Rau, C., Avetisyan, R., Ren, S., Romay, M. C., Stolin, G., . . . Lusis, A. J. (2016). Genetic Dissection of Cardiac Remodeling in an Isoproterenol-Induced Heart Failure Mouse Model. *PLoS Genet, 12*(7), e1006038. doi:10.1371/journal.pgen.1006038